# Purification and preliminary characterization of *Bothrops moojeni* venom components active on haemostasis

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Prof. Dr. Eberhard Parlow
Dekan

Wissenschaft ist der gegenwärtige Stand unseres Irrtums

Jakob Franz Kern (1897-1924)

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# Abstract

For almost every factor involved in haemostasis there exists a venom protein that can mimic, activate or deactivate it. Many of these components are insensitive to the physiological and therapeutically used coagulation inhibitors and, because of their unique features, are applied as molecular tools for diagnosis and therapy of haemostatic disorders. Batroxobin, purified from the venoms of *Bothrops moojeni* and *B. atrox* snakes, commonly known under its trade names Reptilase® and Defibrase® is still widely used in pharmaceutical and diagnostic applications. However, the venom material needed for its production is not further analyzed, although it still contains a large number of potentially active compounds. The goal of this *Botmo Thesis* was the investigation of the crude *B. moojeni* venom for the identification of new active ingredients affecting haemostasis. Various protein purification methods, mass spectrometry and biocomputing, as well as newly developed coagulation screening tools were applied.

*B. moojeni* crude venom and its fractions were used as model substances for the development of different screening tools applying mass spectrometry (MS) technology. The complexity of the venom provides a good selectivity assessment for the developed methods. By means of the novel MS approaches, 15 new sequences of Bradykinin-potentiating peptides (BPPs) were revealed, as well as the presence of acetylcholinesterase inhibiting venom component. To our knowledge, it is the first time acetylcholinesterase inhibiting properties were described in the venom of *B. moojeni* snake.

Further, two novel Lys49 phospholipases $A_2$ ($PLA_2$) have been purified and fully sequenced, showing a characteristic sequence in the *C*-terminal region known in the literature to interact with heparin. Both $PLA_2$s were able to interact *in vitro* with unfractionated heparin (UFH) and low molecular weight heparin (LMWH) neutralizing their anticoagulant properties. The revealed sequences, possessing the LMWH neutralization properties, may be of medical interest, because there is no LMWH antidote used in clinical applications.

Moreover, a procoagulant protein resembling the coagulation FVIIa could be identified in the *B. moojeni* venom. Such activity has never been reported for any snake venom until now and further investigations would be of medical interest.

In addition a new method was developed for the identification of active ingredients acting on blood platelets. It can be used for screening complex protein mixtures, such as *B. moojeni* venom, containing platelet activating prothrombin activators or thrombin like-enzymes. By means of this methodology, a platelet aggregation activator and platelet antagonist with broad inhibitory activity, which is most likely a disintegrin, could be identified.

Up to now, it could be shown that *B. moojeni* venom is a highly complex mixture of active ingredients and still remains a huge “source of undiscovered potential”. Further purifications and characterization steps are needed in order to get a complete activity profile of the venom, also in fields other than haemostasis. Nevertheless, in the framework of this *Botmo Thesis*, some active ingredients, also such of medical interest, were identified.

## Preface

The work described in this thesis (*Botmo Thesis*) was conducted in the framework of "*Bothrops moojeni* venom proteomics" project under the supervision of Prof. Dr. Beat Ernst, Institute of Molecular Pharmacy at the University of Basel. It was performed at Pentapharm Ltd. in Aesch under the guidance of Dr. Marianne Wilmer and Dr. Reto Schöni.

Parts of the *Botmo Thesis* have already been published in peer reviewed journals or will be submitted for publication. These manuscripts are presented in the corresponding paragraphs the way they were prepared for submission to the corresponding journals. The posters are included in Appendix.

**Manuscripts published or prepared:**

- Liesener A., Perchuc A.M., Schöni R., Wilmer M. and Karst U. "Screening for proteolytic activities in snake venom by means of a multiplexing electrospray ionization mass spectrometry assay scheme." *Rapid Commun Mass Spectrom.* **2005**; 19 (20): 2923-8.
- Perchuc A.M., Menin L., Stöcklin R., Bühler B. and Schöni R. "The Potential of *Bothrops moojeni* Venom in the Field of Hemostasis. Established Use and New Insights." *Pathophysiol Haemost Thromb*. **2005**; 34: 241-245
- Liesener A., Perchuc A.M., Schöni R., Schebb N.H., Wilmer M. and Karst U. "Screening of acetylcholinesterase inhibitors in snake venom by electrospray mass spectrometry." *Pure Appl. Chem*. **2007**; 79: 2339-2349
- Menin L., Perchuc A.M., Favreau P., Perret F., Schöni R. and Stöcklin R. "Precursor ion mass spectra for efficient screening of Pyroglutamate and Bradykinin potentiating peptide (BPP)-type substances in *Bothrops moojeni* snake venom using liquid chromatography/tandem mass spectrometry." *Toxicon* **2008** Jun 1; 51 (7): 1288-302
- Perchuc A.M., Menin L., Favreau P., Bühler B., Schöni R., Wilmer M. Ernst B. and Stöcklin R. "Isolation and characterization of two new Lys49 $PLA_2$s with heparin neutralizing properties from *Bothrops moojeni* snake venom." **In preparation** for *Toxicon*

- Ritz V., Bühler B., Zaborosch Ch., Wilmer M., Ernst B. and Perchuc A.M. "A protease possessing blood coagulation factor VIIa-like activity isolated from venom of *Bothrops moojeni* snake." **In preparation** for *Thrombosis Research*
- Demler C., Bühler B., Menin L., Stöcklin R., Wilmer M., Ernst B. and Perchuc A.M. "Platelet-active substances in the venom of *Bothrops moojeni* snake – a novel evaluation method using whole blood aggregometry." **In preparation** for *Platelets*
- Perchuc A.M. and Wilmer M. "Schlangengifte" in Hämostaseologie 2nd Edition **In press**

**Parts of this work were presented at conferences:**

**Oral presentations:**

- Perchuc A.M., Menin L., Stöcklin R., Bühler B. and Schöni R. "The Potential of *Bothrops moojeni* Venom in the Field of Hemostasis. Established Use and New Insights." *3rd International Conference on Exogenous Factors Affecting Thrombosis and Hemostasis (EFATH 2005)*; Sydney, Australia; August 13th-14th, **2005**
- Perchuc A.M., Menin L., Favreau P., Bühler B., Bulet P., Schöni R. and Stöcklin R. "Structural and heparin-binding properties of two *Bothrops moojeni* phospholipases $A_2$ and their derived C-terminal synthetic fragments." *NP2D - Natural Peptides to Drug*; Zermatt, Switzerland; April 18th-21st, **2006**
- Perchuc A.M., Menin L., Favreau P., Bühler B., Bulet P., Schöni R. and Stöcklin R. "Heparin-neutralizing properties of two novel Lys49 $PLA_2$ variants from the *Bothrops moojeni* venom." *15th World Congress on Animal, Plant & Microbial Toxins*; Glasgow, Scotland; July 23rd- 28th, **2006**

**Posters:**

- Perchuc A.M., Menin L., Stöcklin R., Bühler B. and Schöni R. "*Bothrops moojeni* Proteomics – A Second Look at the Venom." *15th European Symposium on Animal, Plant and Microbial Toxins*; Brdo Estate (Kranj), Slovenia; June 19th-23rd, **2004**

- Perchuc A.M., Menin L., Stöcklin R., Bühler B. and Schöni R. "*Bothrops moojeni* venom – a resource of undiscovered potential." *NP2D - Natural Peptides to Drug; Zermatt, Switzerland*; November 30th – December 3rd, **2004**
- Perchuc A.M., Menin L., Stöcklin R., Bühler B. and Schöni R. "The Potential of *Bothrops moojeni* Venom in the Field of Hemostasis. Established Use and New Insights." *50. Jahrestagung der Gesellschaft für Thrombose- und Hämostaseforschung (GTH)*; Basel, Switzerland; February 15th-18th, **2006**
- Perchuc A.M., Menin L., Heidl M., Favreau P., Bühler B., Bulet P., Schöni R. and Stöcklin R. "Two novel Lys49 $PLA_2$s from the venom of *Bothrops moojeni* and some synthetic peptides derived from their C-terminus are able to bind and neutralize anticoagulant activities of different heparins." *51. Jahrestagung der Gesellschaft für Thrombose- und Hämostaseforschung (GTH)*; Dresden, Germany; February 21st-24th, **2007**

# 1 Introduction

## 1.1 Snakes and their venoms

Venoms and venomous animals have always fascinated man. Very early in human history they were used as medicines, though it is difficult to date the first scientific studies [1].

Snakes are animals commonly known for their potential danger rather than for their benefits to mankind [2]. A negative image of the snake exists in most cultures due to the high frequency of snake bites and the seriousness of many cases of envenomation [3]. The poisonous effects of snake-bites have been known since antiquity. The most famously recorded account is the death of Cleopatra, which resulted from a self-inflicted bite on the 30$^{th}$ of August, 30 BC. It is believed that the snake that caused the death was the Egyptian cobra (*Naja haje*), because its neurotoxic venom triggers a quick and relatively painless death [4].

There are nearly 3000 snake species in the world, of which only 300 are known to be venomous [5]. The venomous species belong to the following families: *Elapidae*, which include the cobra and the mamba, and are probably the most venomous of all snakes, *Hydrophidae*, sea snakes, which cause rather rare incidents of bites, *Viperidae*, including the true vipers of the genus *Bitis* (the African giant snakes) and pit vipers such as the rattle snakes of North and South America; and the poisonous *Colubridae*, including the boomslang and the mangrove snake, nearly all of which are found on the Asian continent [5].

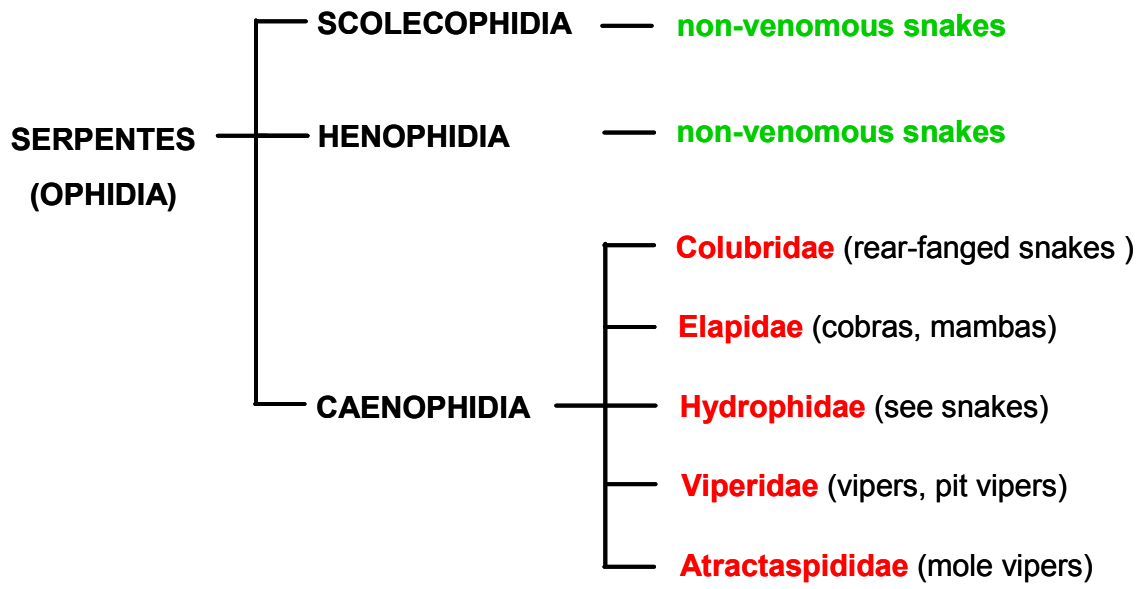


**Figure 1.** A general, simplified classification scheme of snakes [6].

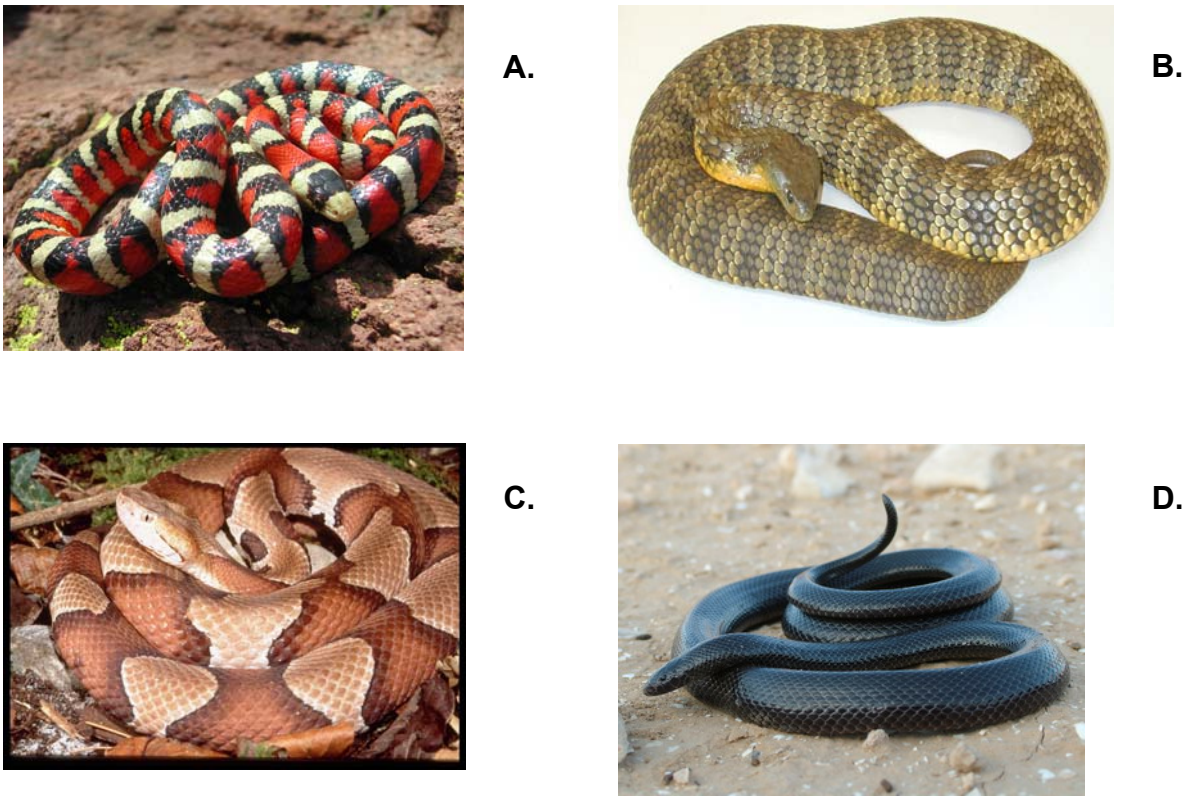


**Figure 2.** The venomous snakes: A. Colubridae, Kingsnake; B. Elapidae, *Notechis scutatus* (Tiger snake); C. Viperidae, pit viper, *Agkistrodon contortrix* (Copperhead); D. Atractaspididae, *Atractaspis engaddensis* (Black mole viper)

Snakes are usually called venomous when envenomations or human fatalities after their bites are reported. However, snake-bite does not necessarily lead to envenomation, for venomous snakes usually do not inject high amounts of venom when striking, which is quite reasonable when considering the primary biological significance of the venom in snake's life. The severity of snake venom poisoning not only depends on the venom amount injected but also on other aspects such as bite site, venom quality, age, weight and health of the victim, and, of course, the medical treatment given. As a consequence, the mortality rate following snake-bite is relatively low. Nevertheless, every snake bite needs medical care and attention [7, 8].

Snake venoms remain a great fascination to scientists, because they are probably the most complex of all the poisons. The compositions of snake venoms vary from species to species, are often age and sex dependent and differ according to geographical region within the same species [1, 7, 9-14]. The clinically important snake venoms are those of elapids and vipers, including the pit vipers [5].

Basically, venom is an adaptation intended for immobilizing or paralysing the prey hunted by certain animals for food. Its function is to affect vital biological systems leading to prey's death, as well as to contribute to digestion, as there is a strong evidence that the toxic (proteolytic) protein components present in the venom evolved from digestive enzymes. Similarly, it should be remembered that humans are not a natural prey species for any venomous snake and the effect of any venom component on the intended prey may be rather different to the effect in humans [7, 8].

Biochemically, snake venom can be described as a unique mixture of hundreds of different proteins. It is highly modified saliva, produced and stored by specialized salivary glands and injected by grooved teeth or the venom apparatus.

**Figure 3.** *Bothrops atrox*, solenoglyph type of venom apparatus. Solenoglyphs tend to have the most perfect of the venomous fangs, being entubed almost their entire length. The fang tends to be long, articulated and can be erected under the snake's control. It allows for deep injection of venom. It is characteristic of the vipers and adders.

The amount of venom injected during a strike varies from a few drops to about 1 ml depending on age or snake species [15, 16]. Fresh snake venom is a viscous, yellow to pale yellowish secretion. It can be dried in vacuum and then stored under refrigeration for years without significant loss of its potency and toxicity. Dry venoms are soluble in water, as the fresh crude venoms consist of about 70% of water. Proteins (enzymes and non-enzymatic polypeptides) constitute about 90% of the dry weight of crude venoms and are responsible for its toxicity. Other constituents are nucleotides, amino acids, sugars, mainly in the form of glycoproteins, lipids, some metal (especially zinc) and non-metal ions.

## 1.2 Toxic components of snake venoms

Snake venoms are regarded as the highest concentrated secretory fluids produced by vertebrate organisms. They contain several toxins that may act synergistically or individually [17]. Due to their structural and functional diversity, a classification is not simple, some components being multifunctional. Classically, the venoms are divided into four categories [1]:

1. neuromuscular toxins,
2. toxins that induce cytolysis,
3. factors acting on the blood coagulation, at different levels of the coagulation cascade and

4. haemorrhagins, that damage the vascular endothelium and are responsible for oedema and capillary haemorrhage.

Investigations have led to the general conclusion that elapid venoms contain large amounts of low molecular peptides and only few enzymes, whereas viperid venoms contain higher molecular proteins and are rich in enzymes [18].

The proteins and peptides present in snake venoms can be divided in two groups, enzymes and non-enzymatic components:

| **Enzymes** | **Non-enzymatic components** |
|---|---|
| • different hydrolases<br>• proteases such as serine proteases and metalloproteinases<br>• phospholipases<br>• phosphodiesterases<br>• nucleosidases<br>• ATPase<br>• cholinesterase<br>• L-amino acid oxidase, etc. | • neurotoxins<br>• cytotoxins<br>• cardiotoxins<br>• myotoxins<br>• protease inhibitors<br>• bradykinin-potentiating peptides (BPPs)<br>• choline esterase inhibitors<br>• phospholipase inhibitors<br>• nerve growth factors<br>• lectins<br>• disintegrins<br>• natriuretic peptides |

The non-proteinous snake venom components can be divided into organic and inorganic constituents. The organic components include: lipids, also in the form of phospholipids, carbohydrates (mucopolysaccharides), riboflavin as a part of L-amino acid oxidase and being responsible for the yellowish colour of the venom, nucleosides and nucleotides, amino acids and biogenic amines. The inorganic venom constituents comprise metall cations (mainly $K^+$, $Na^+$, $Ca^{2+}$, $Mg^{2+}$ and $Zn^{2+}$) contained in snake venom metalloproteinases (haemorrhagins). The presence of inorganic anions in snake venoms was described only in few studies [18].

## 1.3 Symptoms of snake bite, general overview

The most common reaction following the snake-bite is the fear, particularly of a rapid and unpleasant death. This fear may even dominate the clinical picture [6]. Nevertheless, every envenoming is a unique event with variables including: (i) venom composition, which can vary within species as well as amount of venom injected; (ii) mode and time-course of venom absorption, which depends on snake species, site of bite, patient size, patient activity and use of first aid; (iii) possible variations in patients susceptibility to various venom components such as neurotoxins and haemostatic factors; (iv) any clinical interventions such as use of antivenom [19].

Taking into account the protein nature of many venom toxins, envenomations, caused not just by snakes but also by any other venomous creature such as spiders, scorpions, bees or wasps, can be classified in three categories according to their responses to serotherapy [1]:

1. **Envenomations due to venom toxins acting on a defined cell targets such as ionic channels, synaptic receptors (neurotoxins and myotoxins):** The envenomation symptoms appear weakly or not at all, if the antivenom is administered promptly; the antibodies neutralize toxins before they reach their cell targets.
2. **Envenomations due to proteolytic enzymes triggering haemorrhagic syndromes and skin necrosis:** When the targets of the proteases are definite proteins such as fibrinogen, prothrombin or factor X, serotherapy will be efficient as in the previous case. When cell membranes undergo lysis, which may be digestion following the action of one or several proteases, the inflammatory reaction accompanying or following necrosis evolves *per se* and it is not sensitive to the neutralising action of antibody antiproteases. It is well known that the local effects of the venom and the skin damage are not greatly improved by serotherapy, which is, however, regularly active on coagulation disorders.
3. **Envenomations and allergic reactions:** Some venoms can be dangerous as they induce intense allergic reactions, rather than because of their neurotoxic or haemolytic activity. These venoms contain many proteins which are powerful allergens; sometimes the toxins, themselves, more often enzymes

(phospholipases, hyaluronidases).

Further, the most common clinical features of envenoming by elapidae snakes can be listed as follows: local swelling, abdominal pain, nausea, vomiting and headache, tender regional lymph nodes, early collapse, pre- or post-synaptic neurotoxicity, myotoxicity and coagulopathy [19]. The bites caused by viperidae snakes can be characterized by oedema (often gross and frequently painful and with erythema), dizziness and fainting, vomiting, convulsion, tachycardia, diarrhoea and lymphangitis. More severe cases have hypotension, shock and renal failure, dyspnoes, reduced blood coagulability even to incoagulability, melaena, haematemesis, haematuria, thrombocytopenia and, in some cases, local necrosis [2, 7, 9, 20, 21].

Although it may be stated in a simplified way that neurotoxic manifestations are predominant for elapid snakes and that cardiovascular symptoms are much more typical for vipers and pit vipers, there exist no clearly defined limits [6]. Usually, there is no key venom effect responsible for morbidity or mortality, yet different components may act synergistically with major venom effects causing a detrimental effect upon human health [8].

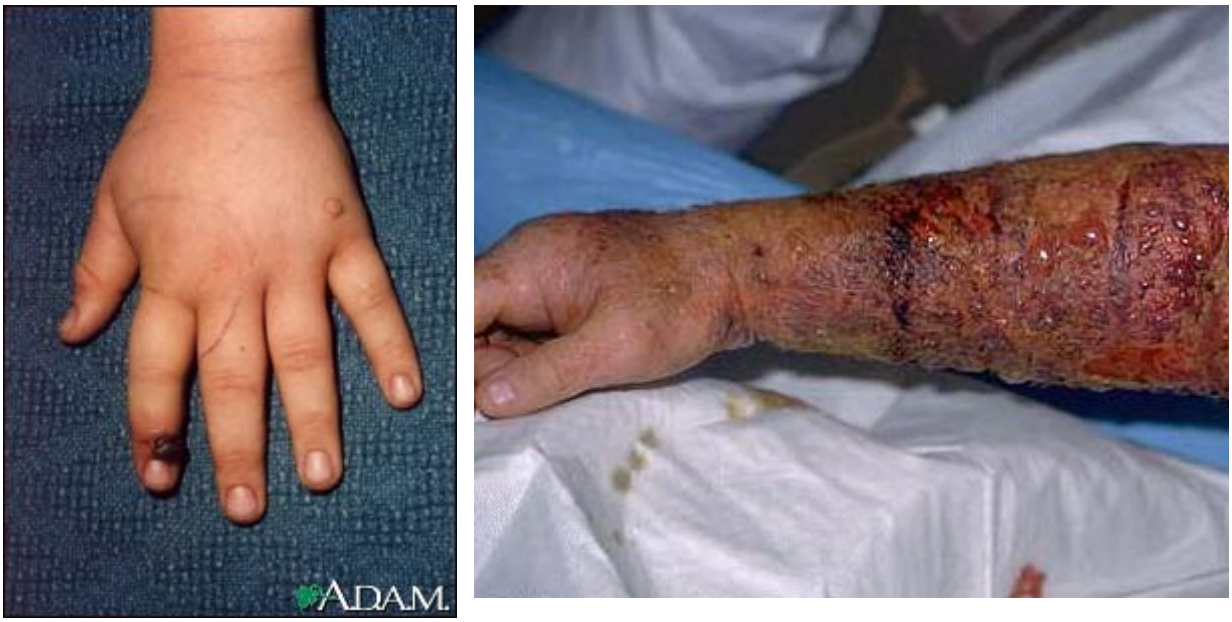


**Figure 4.** Symptoms of a snake bite: oedema and haemorrhage.

## 1.4 A short view of the history of snake venoms affecting haemostasis

The earliest reports of snake venoms affecting haemostasis occur in the XVIIth and the first half of the XVIIIth century with the observations of F. Redi, Nieremberius, van Helmont, R. Mead, and Geoffrey and Hunauld [1, 4]. F. Redi (1626-1696) lived in the times when the common belief was that the bad spirits formed by the snake were the reason for poisoning, including all of the symptoms, thus his experimental results were contrary to the common understanding of the day. So although his observations and assumptions were not easily accepted, he could draw the following conclusions from his experiments (described in *Observations on Vipers* (1664)) that; (i) all animals are affected by the venom, but smaller animals are killed more readily than the large ones; (ii) death occurs earlier or later depending on the place of the bite, whether the bite hits tissue only or veins or arteries; (iii) animals bitten by a viper which do not die, will suffer from the venom and recover without treatment; (iv) the venom is not poisonous if applied orally, which meant when swallowed in half a glass of wine; and (v) last but not least, the venom is a yellow liquid coming from the fangs of the viper [22, 23]. A hundred years later, the work of Redi was confirmed by F. Fontana, who correctly described the mechanism of envenomation, the venom gland, the duct and the canal within the fangs [22].

Independently from Redi's experiments, Nieremberius is said to have described in 1635 profuse bleeding associated with bites of "haemorrum" viper, while just a few years later in 1648 van Helmont considered that snake venoms were "so cold that they coagulated blood in the veins and arrested circulation" [4].

The first report of *in vitro* experiments on viper venom, *A Mechanical Account of Poisons in Several Essays,* was published in 1702 by R. Mead (1673-1754). His work is considered as the earliest description of the coagulant effects of snake venoms. The popularity of the book was such that it had to be reprinted "by booksellers twice or thrice, very improperly, in a *piratical* way!" Nevertheless, serious misconceptions as to the nature of snake venom and its effects were enclosed in Mead's report, such as considering a haemorrhage being caused by "pungent salts" and the venom being an acid [4, 24]. One of the "pirated versions" of the book was studied by F. Fontana,

considered nowadays as the father of modern toxinology, and influenced his early work.

F. Fontana (1730-1805) is usually credited with the first quantitative observations on viper venom, including the lethal effects of viper bite in animals [4]. In his *in vitro* experiments, he showed that venom components could not be coagulated in hot water but could be precipitated from solution by alcohol (*On the nature of the venom of the viper*). Further, for the *in vivo* experiments Fontana used 600 vipers to envenomate over 1000 animals in over 4000 experiments, demonstrating "something paradoxical", as by examination of the body of an animal which died because of an envenomation he observed not only blood coagulation but also fluidity of the blood and haemorrhage (*On the action of the venom of the viper on the blood of animals*) [24]. Nevertheless, he was not the first who observed a bleeding after a snake envenomation. It should be noted that a practically unknown publication by Geoffrey and Hunauld (1737) recorded that the blood of cats and dogs bitten by vipers did not coagulate [4].

In his further work, Fontana asked himself "what is the quantity of the venom needed to kill an animal of a certain size", and stated that "the bite of the viper is not absolutely mortal to man and, that those have been mistaken who have regarded the disease caused by [European] viper's venom as one of the most dangerous, and from which it is impossible to recover" [24]. He was also interested on the venom's *Action on nerves* and *Examination of the remedies employed against the bite of the viper*, however, he failed to find a specific antidote against experimental envenoming [24].

Fontana's work must, undoubtedly, be considered the foundation of modern toxinology and his Treatise, the first modern text on the subject. But, despite his groundbreaking work, additional 100 years were to elapse before the study of animal venoms and haemostasis was resumed [4].

Only an occasional reference is seen in the intervening period. Dr P. Russell, who apart from working on the system of snakes' classification (*An Account of Indian Serpents collected on the Coast of Coromandel,* 1796) studied the clinical signs and symptoms of their envenomations. He noted, again, a possible recovery of an envenomed animal in some experiments, and on the other hand, blood issuing from the mouth and nose as well as great swelling of the bitten area of a dog which died

soon after being bitten by an Indian viper [25]. His clinical descriptions of envenomations suggest the presence of neurotoxic and hemorrhagic elements that are now known to be present in Indian viper venom. Following the work of Russell the Indian viper Ketuka Rekula Poda was named Russell's viper (*Vipera (Daboia) russelli*) [25].

Toward the end of the XIXth century, a number of researchers recorded that animals envenomed by snakes had incoagulable blood [4]. In 1860, S.W. Mitchell reported in the United States that blood rapidly mixed with rattlesnake venom coagulated first and then completely dissolved when kept for 24 hours at room temperature. With this statement he mentioned for the first time the fibrinolytic activity of snake venoms *in vitro* [4]. Following Mitchell's work, the most significant developments in the field of snake venoms and haemostasis over the next hundred years were made by C.J. Martin (1866-1955), R.G. MacFarlane (1907-1986) and H.A. Reid (1913-1983) [4].

Sir C.J. Martin had a major influence on experimental biology in Australia, after discovering that, contrary to work done in the United States, there had been no investigations of the chemistry of Australian snake venoms. By 1884, he had demonstrated that the venom of the red-bellied black snake (*Pseudechis porphyririacus*) contained heat-coagulable protein that did not survive prolonged boiling [4, 26]. Martin investigated further the "paradox" described by Fontana, finding out that the same venoms, which induced thrombosis, at lower doses, render the blood incoagulable. He also observed that small doses of snake venom injected into animal first led to thrombus formation, while thereafter blood did clot "only after some hours, or not at all". These stages were referred to as the positive and negative phases of coagulability [4, 26]. In 1903, Dr Lamb in Bombay found that the venoms of *Vipera russelli* and *Echis carinatus* were able to clot citrate plasma *in vitro* without the requirement of calcium ions. Martin repeated his experiments using the venoms of *Notechis scutatus* and *Pseudechis porphyririacus* and concluded that Australian elapids contain in their venoms even more powerful coagulants than the Indian vipers. His observations were revised in 1909 by Mellanby demonstrating that *N. scutatus* and *P. porphyririacus* venoms contain prothrombin activators which require cofactors, including calcium ions, for their optimal activity [26]. So the first calcium independent and dependent prothrombin activators were identified. Clinical symptoms of envenomations by the Australian elapids pointed out also a presence of neurotoxic components in their venoms. Thus, after investigating the neurotoxicity

mechanism, Martin created a clever device to separate the large clotting factors from the smaller neurotoxic components contained in the snake venoms. On the basis of molecular size, he was able to explain the marked effects of subcutaneous and intravascular injections of the venom, whereby the large clotting factors and the smaller neurotoxic components diffused from subcutaneous tissues into the bloodstream at very different rates. The neurotoxins were much quicker, thus dominating the clinical picture [4, 26].

Following Martin's pioneering work on Australian snake venom procoagulants, there were a number of isolated references to snake venoms and haemostasis in the early part of the XXth. century. However, it was the work of R.G. MacFarlane that would elevate Russell's viper venom (RVV) to international consideration within the field of haemostasis [4].

R.G. MacFarlane was regarded as the most distinguished and senior haematologist in Britain and to him we owe much in terms of our present thinking on blood coagulation mechanisms [4]. In 1934, encouraged by H. Hartridge, Professor of Physiology at Barts, MacFarlane together with the Curator of Reptiles at the London Zoo milked some 20 assorted species of snakes and tested the crude venoms on haemophilic blood *in vitro*, observing the "great coagulant power" of the RVV. The same year he managed to convince a haemophilic patient undergoing tooth extraction to have his tooth socket plugged with RVV. The venom was thus used clinically for the very first time [27]. Following its use in the tooth extraction, the RVV was commercially marketed as Stypven and MacFarlane continued to work on the coagulant properties of the venom, discovering the importance of lipids (lecithin or cephalin) for its action [4, 27-29]. After discovery of the coagulation factor V [30], and factor X [31, 32] MacFarlane showed that RVV reacted with factor X in the presence of factor V and phospholipids [27, 28, 33].

While MacFarlane's investigations into the properties of RVV were underway, others were busily engaged researching similar snake venom fields resulting in the discovery of several varieties. For example in 1958 Habermann first isolated a thrombin-like enzyme from the venom of *Bothrops jararaca* [34], Denson began to work on snake venoms as diagnostic tools in the early 1960s [35, 36] and Reid carried out his pioneering work on the thrombin-like enzyme in the Malayan pit viper

(*Calloselasma (Agkistrodon) rhodostoma*) [37] which would lead to a new era of therapeutic possibilities with snake venoms acting on haemostasis [4].

H.A. Reid started his work on snake venoms with an extensive study of the toxic effects of sea snakes bites. He demonstrated the presence of myotoxic phospholipases $A_2$ in the venom of the *Enhydrina schistose* snake and succeeded in having the treatment of snake bites in Malayan Government hospitals recorded separately from other bites and stings [4, 38]. This enabled him to undertake an epidemiological study on the victims of Malayan pit viper (*Calloselasma rhodostoma*) bites. Following the bite, the blood remained incoagulable for up to 3 weeks if untreated. This coagulation defect was caused by a severe reduction in plasma fibrinogen resulting from the presence of venom's thrombin-like component, which produced a continuing low level of microthrombi containing non-cross-linked fibrin. Thus, fibrin was removed quickly and effectively by fibrinolysis [4, 38]. The general well being of the envenomed patients with all but very severe *C. rhodostoma* envenomation particularly impressed Reid, who assumed that the venom contained a constituent that could be used for an anticoagulant treatment. The further studies were carried out in collaboration with MacFarlane and his group in Oxford. In the first stage, Dr Esnouf isolated from the venom of *C. rhodostoma* a defibrinating fraction free from lethal and haemorrhagic activities [39]. The stable thrombin-like protease, first known as Arvin was then characterized by Twyford Laboratories in London. Reid and Chan [37] described the protease as an anticoagulant that "removed the fibrinogen powder keg from the blood rather than merely stepping on the fuse" like heparin and oral anticoagulants. Subsequently, the name was changed to Ancrod, as "Arvin" had already been a trademarked for automobile components [4]. The early trials on Ancrod showed much promise, particularly in the treatment of venous thrombosis [40-42], as did trials with a similar enzyme (Defibrase®, Reptilase®) isolated from the venom of *Bothrops* species [43]. However, the results of subsequent studies have not justified this early promise. High costs combined with the problems of protein-derived antigenicity remain unresolved issues. Knoll Pharmaceuticals (Ludwigshafen, Germany) continued to manufacture Ancrod (Viprinex®) from a collection of several hundred vipers. The drug, however, has not gained FDA approval in the United States [4].

In the last fifty years the investigations performed on different animal venoms have increased rapidly. Snake venoms were further studied in regard to their activity on

blood platelets. It was, however, not until 1960s that this began to attract interest, probably as a result of reports of thrombocytopenia in envenomed patients. This resulted in purification of convulxin, thrombocytin, botrocetin, significant number of C-type lectins and numerous disintegrins [4]. Also, many snake venom components affecting haemostasis have been utilized in a range of diagnostic and laboratory settings [44], receiving more and more opportunities for both clinical and laboratory use.

As stated, by Marsh [4] the International Society on Thrombosis and Haemostasis (ISTH) has played a key role in the development of the knowledge regarding animal venoms and the blood coagulation system. Firstly, in July 1985, a Symposium on Animal Venoms and Haemostasis was held in San Diego as part of the Xth. Congress of the ISTH; and secondly, the ISTH has contributed extensively to the information on, and nomenclature of, the venom toxins through the Registry of Exogenous Haemostatic Factors, a section of the Society's Scientific and Standardization Sub-Committee.

## 1.5 Haemostasis in a nutshell

The term haemostasis comprises all the reactions which are activated by a vascular injury. They lead to occlusion of the damage and result in the arrest of bleeding. Coagulation is the transformation of blood from its liquid state into a gel and is only one part of haemostasis. Another part is fibrinolysis, which involves the interactions of tissue, cell and plasma proteins leading to an enzymatic degradation of the blood clot [45]. Thus, the perfect haemostasis means: no bleeding and no thrombosis. The haemostatic events can be divided in three phases: primary haemostasis, secondary haemostasis and fibrinolysis. Primary haemostasis comprises vasoconstriction, platelet adhesion and aggregation; secondary haemostasis – activation of coagulation factors and fibrin formation, while fibrinolysis provides the clot lysis and normally, prevention of pathological thrombus formation [46].

If vascular damage occurs, immediate vasoconstriction reduces the size of the vascular lesion and the affected vessel area. Blood platelets stick to exposed subendothelial cells through collagen and von Willebrand factor, spread along, releasing active substances into blood and forming a surface for coagulation factors, thus promoting thrombin generation. Two alternate proteolytic pathways lead to

thrombin generation and transformation of fibrinogen into fibrin (Fig. 5). The pathways are a cascade of reactions, in which a zymogen of a serine protease is activated to become an active coagulation factor that on its part catalyze the next reaction, ultimately resulting in cross-linked fibrin. The extrinsic pathway of blood coagulation is activated by tissue factor (TF) interacting with FVIIa, forming in the presence of phospholipids (PLs) and calcium ions the, so called, tenase complex. In the intrinsic pathway coagulation is triggered via the contact activation system comprising kallikrein and kininogen, FXII, FXI and FIX. Both coagulation pathways get together in a common part where FXa together with FVa, in the presence of phospholipids (PLs) and calcium ions, forms the prothrombinase complex. This leads to thrombin generation and transformation of fibrinogen into a cross-linked fibrin clot. Once the haemostasis has been achieved and vascular repair is initiated, fibrin polymers are lysed by plasmin generated by the fibrinolytic system.

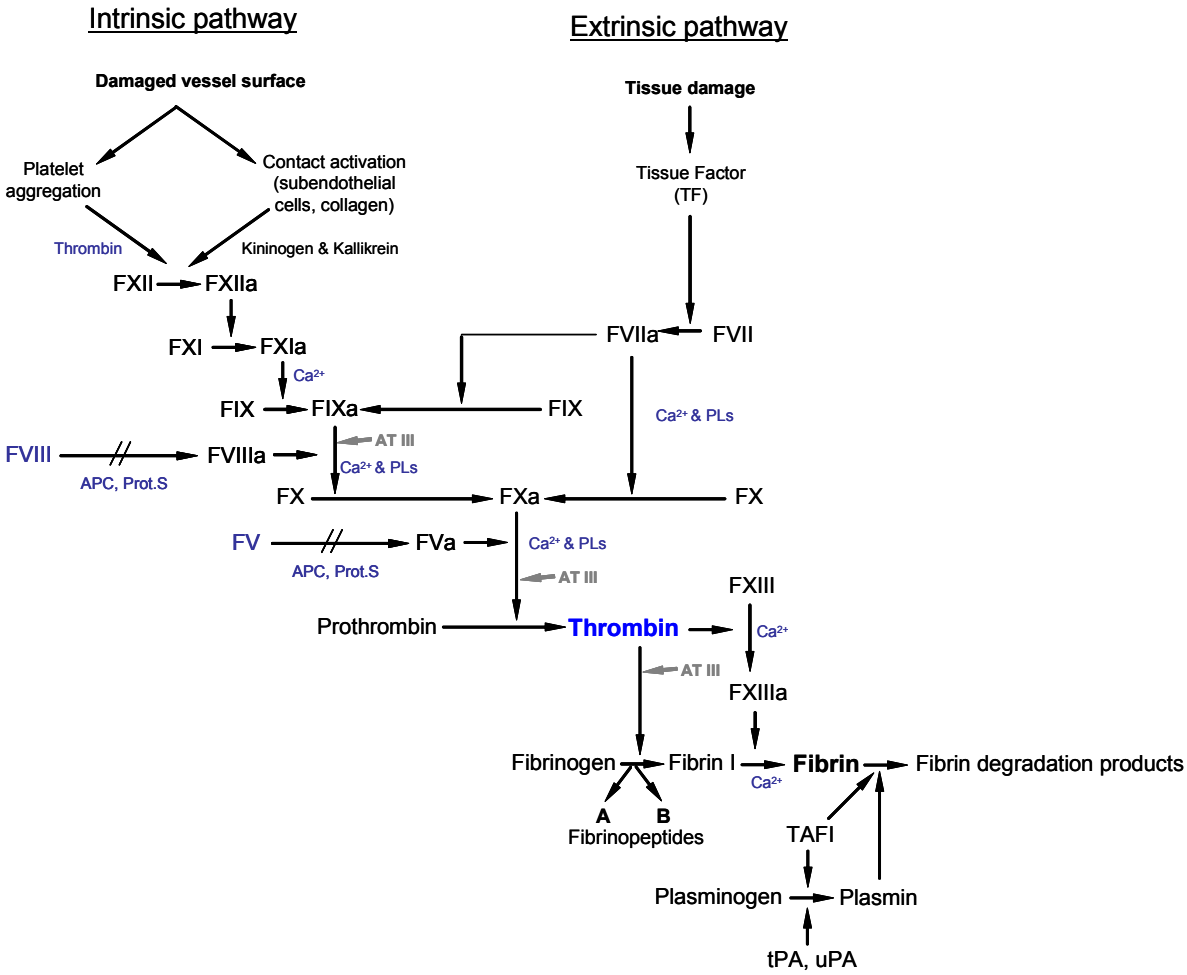


**Figure 5.** Coagulation cascade.

## 1.6 Snake venoms and haemostasis

Interference with the functions of the human haemostatic system is a common mechanism of snake venoms, to a greater or lesser degree depending on the snake family. Snake venom toxins affecting haemostasis have been classified by virtue of their overall effect into the following groups:

- coagulant factors (thrombin-like enzymes, prothrombin activators and FV and FX activators),
- anticoagulant factors (including FIX/FX binding proteins, protein C activators, thrombin inhibitors and phospholipases $A_2$),
- proteins acting on blood platelets (proteases including metalloproteinases, C-type lectins, disintegrins, phospholipases $A_2$ and L-amino acid oxidases),
- factors acting on fibrinolysis (fibrinolytic enzymes and plasminogen activator) and
- haemorrhagins (metalloproteinases degrading the blood vessel extracellular matrix) [44, 47-54].

For almost every factor involved in coagulation or fibrinolysis there is a venom protein that can mimic, activate or deactivate it [48, 49]. Many of these components are insensitive to the commonly used inhibitors of the coagulation system, thus have found uses in diagnoses and treatment of haemostatic disorders.

There are still many research groups all over the world investigating the potential of different snake venoms in order to find novel tools useful in the coagulation field.

### 1.6.1 Coagulant factors

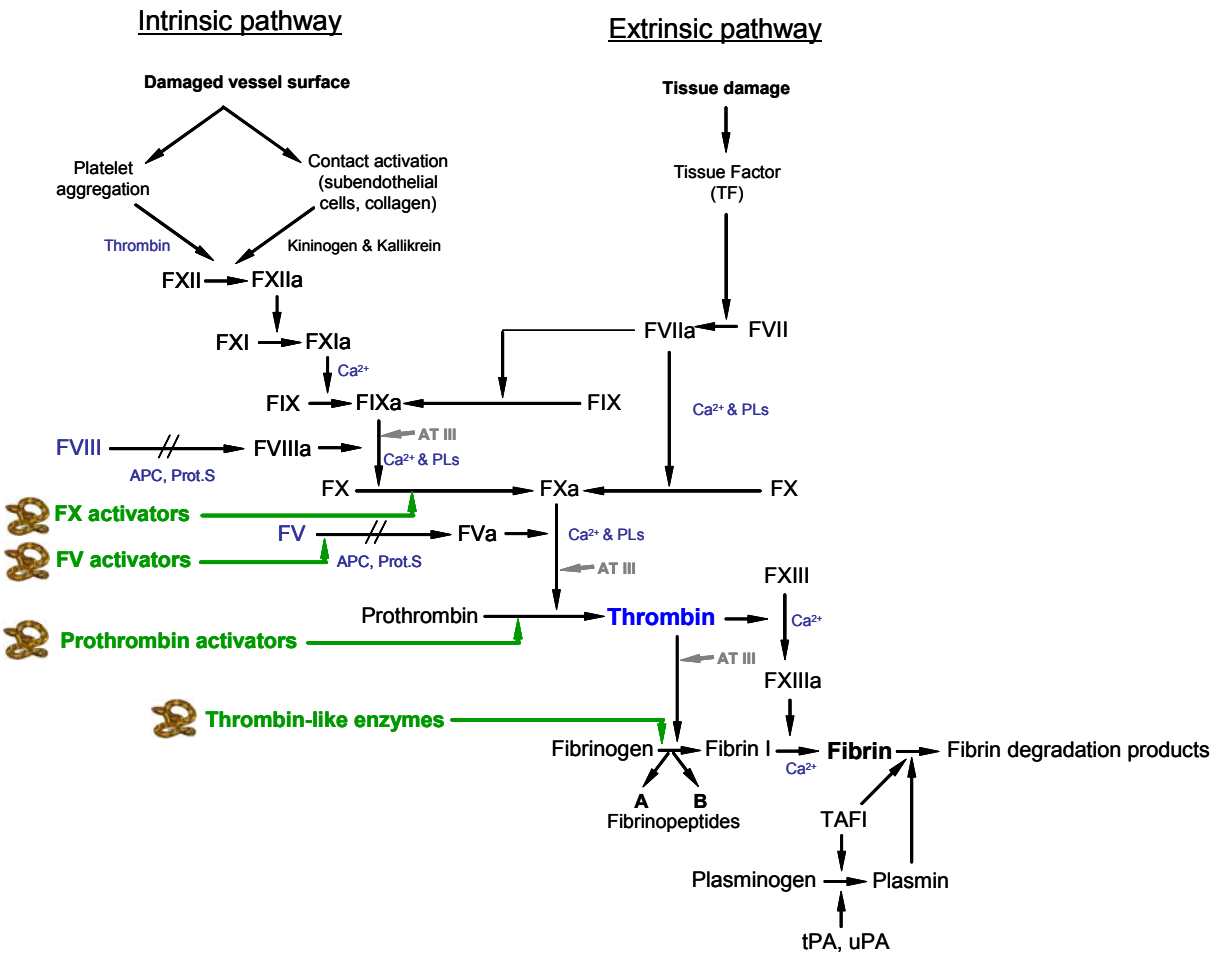


**Figure 6.** Coagulation cascade; coagulant factors derived from snake venoms.

#### 1.6.1.1 Thrombin-like (fibrinogen-clotting) enzymes

Over 90 snake venom thrombin-like enzymes (SVTLEs) have been reported from over 30 snake species [55, 56]. They are widely distributed in several pit viper genera (*Agkistrodon*, *Bothrops*, *Crotalus, Gloydius*, *Lachesis* and *Trimeresurus*), as well as in some true vipers (*Bitis* and *Cerastes*) and the colubrid, *Dispholidus typus* [49, 55, 57]. Sequences of about 30 SVTLEs have been determined and are published in Swiss-Prot and TrEMBL database (http://us.expasy.org/sprot/). Comparison of the primary sequences of SVTLEs with those of classical serine proteases such as chymotrypsin, thrombin, kallikrein and trypsin identifies SVTLEs as single chain serine proteases. The catalytic domain, with His57, Asp102 and Ser195 forming the

catalytic triad, is the most conserved region on these enzymes and, together with susceptibility to classical serine protease inhibitors, is directly responsible for their affiliation to the serine protease group [57]. Although called thrombin-like enzymes, being functionally analogous to thrombin and showing a high level of similarity among each other, SVTLEs share rather a weak sequence identity with thrombin (26-33%) [57]. Most of the SVTLEs are likely to be glycoproteins, each containing a few Asn-N-linked glycosylation sites in nonhomologous positions to one another in the sequence. Their carbohydrate content varies widely from 0 to 30% and is characteristic of N-linked glycans. Fucose, hexose, sialic acid, N-acetyl-D-glucosamine galactose, mannose and N-acetyl-neuraminic acid are also found. Although present in many SVTLEs, the specific importance of the carbohydrate moiety on the structure-function relationship is not yet established [57]. It is supposed that glycans are important structures for protein stabilization rather than for the catalytic function of the venom enzymes [58].

Although being functionally analogous to thrombin and showing *in vitro* blood clotting activity, SVTLEs do not share all properties common to thrombin. Those are more precisely described in chapter 8.

It is interesting to note that SVTLEs do not clot their own plasma or plasma of any other snake in contrast to human or bovine thrombin [59-61]. That confirms that they are not directly equivalent to either human or snake thrombin and describing these venom proteases as ‘thrombin-like’ instead of ‘fibrinogen-clotting’ is rather incorrect [54, 57, 59].

#### 1.6.1.2 Prothrombin activators

Activation of prothrombin to mature thrombin occurs by the proteolytic action of the prothrombinase complex consisting of FXa, FVa, phospholipids (PLs) and calcium ions. A number of exogenous prothrombin activators are found in snake venoms. According to the recent classification (in the previous one, there used to be three groups [52-54, 62]), they are categorized into four classes based on their structures, functional characteristics and cofactor dependency [49, 63-67].

Group A prothrombin activators are metalloproteinases and activate prothrombin efficiently without the requirement of any cofactors. They are widely distributed in many kinds of viper venoms and presumably play the role of toxins in the venom, as

they are resistant to the natural coagulation inhibitors such as antithrombin III [63, 64]. Ecarin, a metalloproteinase from the venom of *Echis carinatus* snake, is the best characterized group A prothrombin inhibitor. It consists of metalloproteinase, disintegrin and Cys-rich domains and cleaves the Arg320-Ile321 in human prothrombin (Arg323-Ile324 in the bovine) producing meizothrombin. Meizothrombin is then finally converted into $\alpha$-thrombin by autolysis [63, 64].

Group B prothrombin activators require calcium ions for their activity. They contain two subunits linked non-covalently: a metalloproteinase and a C-type lectin-like disulfide-linked dimer [63, 64]. The first prothrombin activator of group B, carinactivase-1, was isolated by Yamada *et al.* [65] from *Echis carinatus* venom and was shown to require millimolar concentrations of calcium ions for activity. It has virtually no activity in the absence of calcium.

Group C prothrombin activators are serine proteases found exclusively in Australian elapids, requiring calcium ions and negatively charged PLs, but not FVa for their maximal activity [63, 64, 66]. They are high molecular weight (>250 kDa), multi-subunit protein complexes, containing catalytic and non-enzymatic subunits similar to mammalian coagulation factors Xa and Va, respectively. Pseutarin C isolated from the *Pseudonaja textilis* venom [68] has a powerful procoagulant activity on human plasma. It converts human prothrombin to mature thrombin by cleaving it at peptide bonds Arg271-Thr-272 and Arg320-Ile321 (bovine prothrombin by cleaving: Arg274-Thr-275 and Arg323-Ile324 bonds) corresponding to the same cleavage specificity as mammalian FXa.

Group D prothrombin activators are serine proteases found so far also exclusively in Australian elapid snake venoms. Their activities are strongly dependent on calcium ions, PLs and FVa [63, 64, 66]. As an example of group D prothrombin activator, Trocarin D from the *Tropidechis carinatus* venom can be given [69-71], whose activity is enhanced four orders of magnitude by the addition of cofactors. Similarly to mammalian factor Xa, this serine protease cleaves the bovine prothrombin at two sites, Arg274-Thr275 and Arg323-Ile324, resulting in the mature thrombin. Although there are striking functional similarities between blood coagulation FXa and group D prothrombin activators, they are, unlike mammalian FXa, glycoproteins: the light chains are *O*-glycosylated at Ser52, whereas the heavy chains are *N*-glycosylated at

Asn45 [71]. Nevertheless, the role of the carbohydrate moieties has not been yet determined.

#### 1.6.1.3 Activators of coagulation factors

Several FV activators have been described from *Bothrops atrox*, *Vipera russelli*, *V. labetina*, *V. ursine* and *Naja naja oxiana* venoms [72, 73]. RVV-V from *V. russelli* is a single-chain serine protease, which could be clearly separated from a FX activating protein (RVV-X) also present in this venom [74]. RVV-V is a glycoprotein possessing one glycosylation site near the C-terminus and activates FV to FVa by cleavage of a single peptide bond at Arg1545-Ser1546, which is one of three thrombin cleavage sites in FV [49, 52, 54, 72, 75]. As a result of this cleavage the heavy and light chains of FVa are generated. Unlike thrombin, RVV-V shows no apparent effects on coagulation FVIII, FXIII, fibrinogen or prothrombin [75].

FX activators have been isolated from many Viperide and Crotalide venoms as well as from a few elapid species. They are either metalloproteinases or serine proteases [76, 77]. The best known and most extensively characterized FX activator is RVV-X consisting of a disulfide-linked three-chain glycoprotein with one heavy and two light chains. The heavy chain of RVV-X has metalloproteinase, disintegrin-like and Cys-rich domains [49, 52, 67]. The light chain resemble the C-type lectins [78]. RVV-X contains six *N*-linked oligosaccharides, four in the heavy chain and one in each of the light chains. The carbohydrate structures are different from those known for other snake venom glycoproteins, and they resemble closely those in various mammalian glycoproteins [79]. Gowda *et al.* demonstrated that the complete removal of oligosaccharide chains markedly decreases the functional activity of RVV-X, whereas the removal of peripheral sugars has no effect [80]. His explanation for that phenomenon was that the structural changes are the source of the functional loss, because the deglycosylation caused a decrease in $\alpha$-helix content and an increase in $\beta$-sheet content. The activation of FX to FXa by RVV-X requires a millimolar concentration of calcium ions and involves the specific cleavage of the peptide bond Arg52-Ile53 in the heavy chain of FX. The same bond is cleaved by FIXa and FVIIa during normal blood coagulation [77, 79], however unlike the vitamin K-dependent clotting factors, RVV-X does not require a negatively charged surface such as PLs, but requires the calcium ions for FX activation [78]. Metalloproteinase FX activators from other venoms (*Bothrops atrox* and *Cerastes cerastes*) have similar structure to

RVV-X and probably similar catalytic mechanisms [49, 67]. A few serine proteases that activate FX have also been isolated from the venoms of *Ophiophagus hannah*, *Bungarus fasciatus*, *B. candidus*, *Cerastes cerastes*, and *C. vipera* [67].

RVV-X also activates FIX by cleaving a single peptide bond without affecting molecular mass [49, 52, 81].

So far, snake venom proteins that specifically activate only FVII are not known, although different venoms have been examined by Nakagaki *et al.* for their ability to convert single-chain FVII to two-chain FVIIa [82]. In the same study he showed that oscutarin C activates FVII by cleaving it at a site similar to the natural one. This activation was significantly potentiated by calcium ions and phospholipids and was not dependent upon the presence of the FVa-like subunit of oscutarin [67, 82].

### 1.6.2 Anticoagulant factors

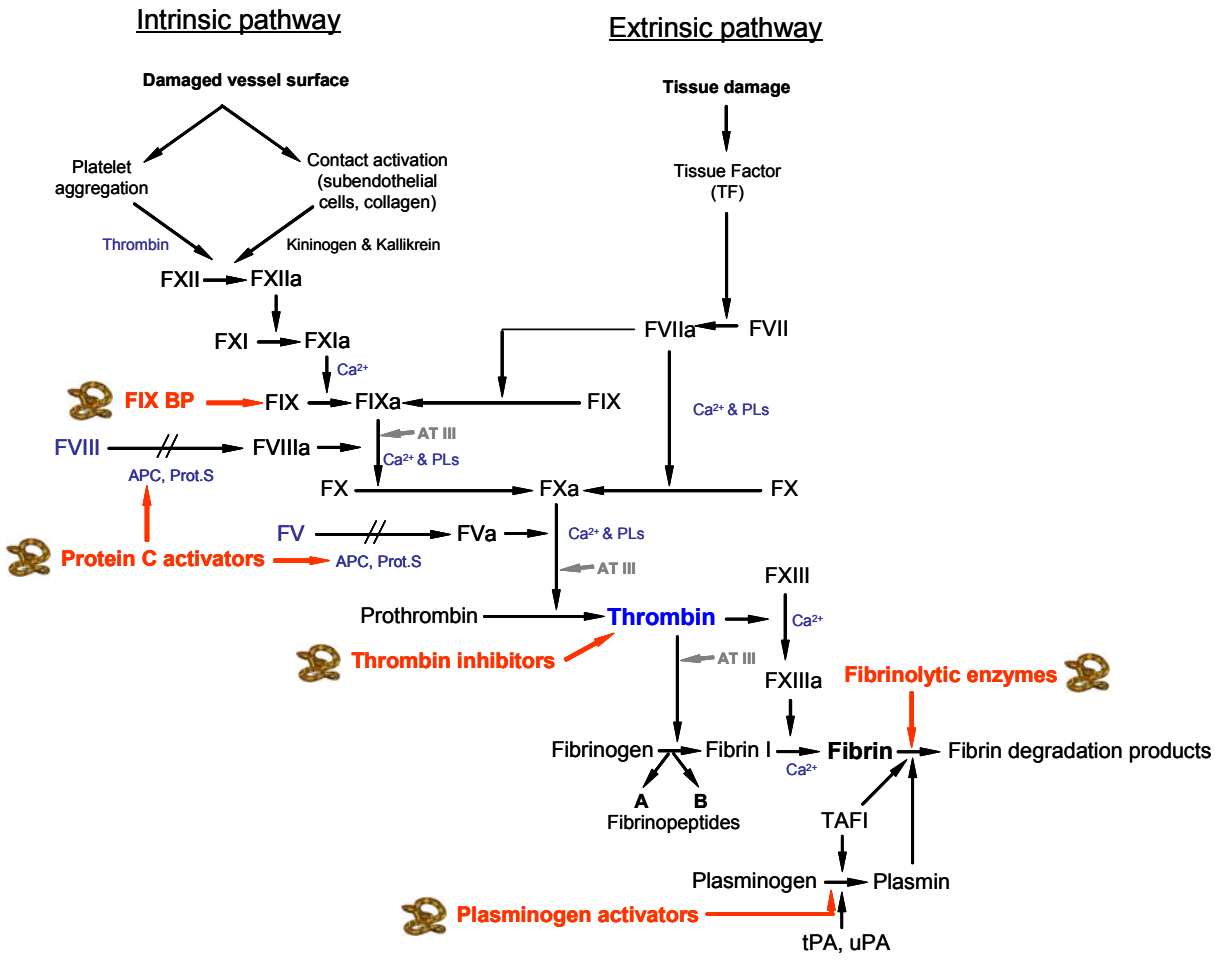


**Figure 7.** Coagulation cascade; anticoagulant factors derived from snake venoms.

### 1.6.2.1 FIX/FX binding proteins

Many anti-coagulant C-type lectin-like proteins, interacting with FIX and/or FX, have been isolated from various snake species [83-85]. Based on their ligand recognition differences, these proteins can be classified as:

- blood coagulation FIX/FX-binding proteins (FIX/X-BPs), interacting with FIX or FX in a 1:1 molar ratio [86],
- FIX-binding proteins (FIX-BPs), which do not interact with FX,
- FX-binding proteins (FX-BPs), binding predominantly to FX [49, 87].

They are heterodimers with highly similar structures, consisting of two homologous C-type lectin-like subunits of 14 and 15 kDa. The anticoagulant activities of these proteins are based on their calcium ions-dependent binding to gamma carboxyglutamic acid (Gla) domains of coagulation factors IX and X [49, 83, 85, 87].

### 1.6.2.2 Protein C activators

Among the serine proteases only the protein C activators exhibit direct anticoagulant effects [83]. Most protein C activators have been purified from *Agkistrodon* venom; others come from *Bothrops*, *Trimeresurus*, or *Cerastes* venoms [49, 83, 88, 89]. They are glycoproteins directly converting protein C (PC) into its active form; they do not require any cofactors, while thrombin needs thrombomodulin for PC activation. High salt concentrations and presence of calcium ions inhibit their ability to activate protein C [49, 83]. Protac® from *A. contortrix contortrix* venom is a fast-acting protein C activator. Unlike the activation product generated by thrombin, Protac®-activated protein C remains unaffected by the plasma protein C inhibitor. In contrast to thrombin, Protac® itself exerts neither a coagulant nor a chromogenic substrate-splitting activity [90-93].

### 1.6.2.3 Thrombin inhibitors

The first specific thrombin inhibitor, bothrojaracin (BJC), was isolated from the venom of *Bothrops jararaca* [94]. This protein belongs to the C-type lectin-like family and interacts with thrombin binding strongly to exosites I and II and forming a stable non-covalent 1:1 complex, influencing but not blocking the proteinase catalytic site. The interaction causes blockage of fibrinogen clotting, platelet aggregation and secretion,

and other effects [85, 95-97]. Competition assays showed that BJC displaces thrombin ligands such as fibrin, hirudin and thrombomodulin. In addition, it inhibits the activation of FV by thrombin, and is therefore a potent inhibitor of the feedback activation of the clotting cascade [96]. Moreover, BJC also displaces the thrombin that is bound to the clot, inhibiting its clotting activity and binds with high specificity to prothrombin, decreasing its proteolytic activation – especially in the presence of FVa and absence of PLs [95-97].

*B. jararaca* as well as other *Bothrops* species produce a large number of BJC isoforms which differ in their primary sequence. That reveals that there is a family of BJC-like molecules present in the venoms of *Bothrops* snakes [96, 98-100].

#### 1.6.2.4 Phospholipases $A_2$

Phospholipases $A_2$ ($PLA_2$s) are esterolytic enzymes which hydrolyse glycerophospholipids at the *sn*-2 position of the glycerol backbone releasing lysophospholipids and fatty acids. Snake venoms are rich sources of $PLA_2$s, often containing a large number of isoenzymes. So far, several hundred of snake venom $PLA_2$s and $PLA_2$-like proteins have been purified and characterized, showing significant similarity in their three dimensional structure [101], however possessing different toxic properties and inducing wide range of pharmacological effects (neurotoxicity, cardiotoxicity, myotoxicity, haemolysis, haemorrhagic, antiplatelet or anticoagulant effects etc.) [102-104]. Among those, diverse influences on the haemostatic system are remarkable.

Snake venom $PLA_2$s can be divided into two groups Asp49 $PLA_2$s with high catalytic activity and Lys49 $PLA_2$s (so called $PLA_2$-like proteins) with very low or no enzymatic activity [105]. Asp49, as a part of a calcium-binding site, together with conserved and conformationally flexible loop of residues Tyr25-Gly-Cys-Tyr/Phe-Cys-Gly-X-Gly-Gly33 [101], is critical for catalytic activity.

Depending on their anticoagulant potency, $PLA_2$s have been classified into:

- strong (e.g. some venom $PLA_2$s from *Naja nigricollis*, *Vipera berus orientale*),
- weak (e.g. some venom $PLA_2$s from *N. mossambica*, *Oxyuranus scutellatus*),
- nonanticoagulant (e.g. some venom $PLA_2$s from *Bitis gabonica*, *Crotalus admanteus*) enzymes [102].

Their catalytic activity, leading to hydrolysis of phospholipid surfaces necessary for coagulation complexes, is essential, however not the only mechanism inducing anticoagulant effects [102, 103, 106]. According to Kini *et al.*, the susceptibility of a tissue to a particular $PLA_2$ depends on the presence of specific 'target sites' on the surface of target cells or tissues [102-104, 107]. These target sites are recognized by specific 'pharmacological sites' on the $PLA_2$ molecule, independent however sometimes overlapping with the active site. The target sites and pharmacological sites are complementary to each other in terms of charges, hydrophobicity and van der Waal's contact surfaces. Further, Kini proposed that the affinity between $PLA_2$ and its target protein is in low nanomolar range, whereas the binding between $PLA_2$ and phospholipids is in the high micromolar range [102, 107]. This explains why the interaction of the $PLA_2$ and its target protein (and not phospholipids) directs the pharmacological specificity and why anticoagulant effects can be triggered even by $PLA_2$-like proteins lacking enzymatic activity.

Blood coagulation FXa is the target protein for strongly anticoagulant $PLA_2$s, acting by both nonenzymatic and enzymatic mechanisms, e.g. CM-IV from the venom of *N. nigricollis* [103, 108, 109]. Apart of hydrolyzing PLs, CM-IV competes with FVa to bind to FXa and thus interferes with the formation of prothrombinase complex. This appears to be the most common mechanism of anticoagulant effects among $PLA_2$s [102]. Comparing the amino acid sequences of strong, weak and non-anticoagulant $PLA_2$s, Kini and Evans identified the anticoagulant region between residues 54 and 77 [110]. This region is positively charged in strong, but negatively charged in weak or non-coagulant $PLA_2$s. The weakly anticoagulant $PLA_2$ enzymes (e.g. CM-I and CM-II from the venom of *N. nigricollis*) inhibit only the extrinsic tenase complex (TF, FVIIa, PLs and calcium ions), simply due to their ability to hydrolyze and destroy or bind the phospholipid surface [102, 106].

## 1.6.3 Fibrinolytic factors

### 1.6.3.1 Fibrinolytic enzymes

In 1998 an inventory of $\alpha$- and $\beta$-fibrinogenases was presented [111], containing 67 direct acting fibrin(ogen)olytic enzymes, which were chracterized from the venoms of Crotalidae, Viperidae, Colubride and Elapidae snakes. The $\alpha$- and $\beta$-chain fibrinogenases can be defined as venom endoproteinases degrading preferentially

either the α- or β-chains of fibrin(ogen), respectively, without requirement of any cofactors. In contrast to SVTLEs, also cleaving fibrinogen chains, fibrinogenases do not release fibrinopeptides A or B and do not induce fibrin clot formation. The majority of venom fibrin(ogen)olytic enzymes belongs to the family of metalloproteinases (zinc metalloproteinases), specifically degrading the Aα-chain, with somewhat lower activity for Bβ-chain of fibrinogen. Most of the metalloproteinases are both fibrinogenolytic and fibrinolytic, some of them possess also a haemorrhagic activity [112]. As an example of a fibrin(ogen)olytic metalloproteinase, alfimeprase, a truncated form of fibrolase, is described in chapter 8.

In contrast to metalloproteinases, fibrino(gen)olytic serine proteases cleave the Bβ-chain of fibrinogen preferentially, with lower activity directed towards its Aα-chain. Like metalloproteinases most of the serine proteases are both fibrinogenolytic and fibrinolytic, however a number of them are not fibrinolytic [112].

#### 1.6.3.2 Plasminogen activators

A direct acting plasminogen activator in snake venom was described from *Trimeresurus stejnegri* venom (TSV-PA) [113]. Plasminogen activators were also reported in venoms of *Lachesis muta muta* (LV-PA) [114] and *Agkistrodon halys* (Haly-PA) [115]. TSV-PA is a single chain glycoprotein, belonging to the family of venom serine proteases, activating plasminogen and generating plasmin by a single cleavage of the Arg561-Val562 peptide bond; acting exactly like the physiological activators u-PA and t-PA. However, unlike t-PA, fibrin fragments do not stimulate action of TSV-PA [52]. TSV-PA does not activate nor degrade FX, protein C or prothrombin and it does not clot nor degrade fibrinogen or fibrin in the absence of plasminogen. As well, it lacks the sequence responsible for the interaction of t-PA (KHRR) and u-PA (RRHR) with plasminogen activator inhibitor 1 (PAI-1) [49, 113, 116].

### 1.6.4 Haemorrhagins (Metalloproteinases)

Haemorrhagic activity is defined as the leakage of blood components through the basement membrane. It is caused by proteolytic degradation of the basement membrane proteins by the venom haemorrhagic proteinases, which affects the interactions between basement membrane components and the endothelial cells [49,

112]. Theses cells then undergo morphological and functional alterations *in vivo*, associated with haemodynamic factors such as shear stress. Eventually, gaps are formed between endothelial cells through which extravasation occurs [117]. With some exceptions, snakes of the family Viperidae induce envenomations characterized by haemorrhage, in addition to other typical symptoms [118].

Snake venom haemorrhagic toxins are zinc metalloproteinases (with the zinc atom bound to three histidine residues). Their haemorrhagic effects depend on proteolytic, zinc-dependent activity and zinc chelation completely abolishes them [118]. Snake venom metalloproteinases (SVMPs) comprise four groups classified according to their domain composition:

- P-I class has a single metalloproteinase domain,
- P-II class has a metalloproteinase followed by a disintegrin domain,
- P-III class has metalloproteinase, disintegrin-like and cystein-rich domains,
- P-IV class comprised by enzymes with two subunits, one constituted by the three domains characteristic of P-III enzymes and another being a C-type lectin protein, linked through disulfide bridges to the first one [49, 118, 119].

In general, P-III SVMPs are more potent haemorrhagic toxins than SVMPs of other groups. The disintegrin-like and cystein-rich domains make them more active by directing the enzyme to critical locations in capillary blood vessels and modulating the catalytic specificity of the enzyme domain on the blood vessel extracellular matrix substrates [49, 118, 119].

### 1.6.5 Proteins acting on blood platelets

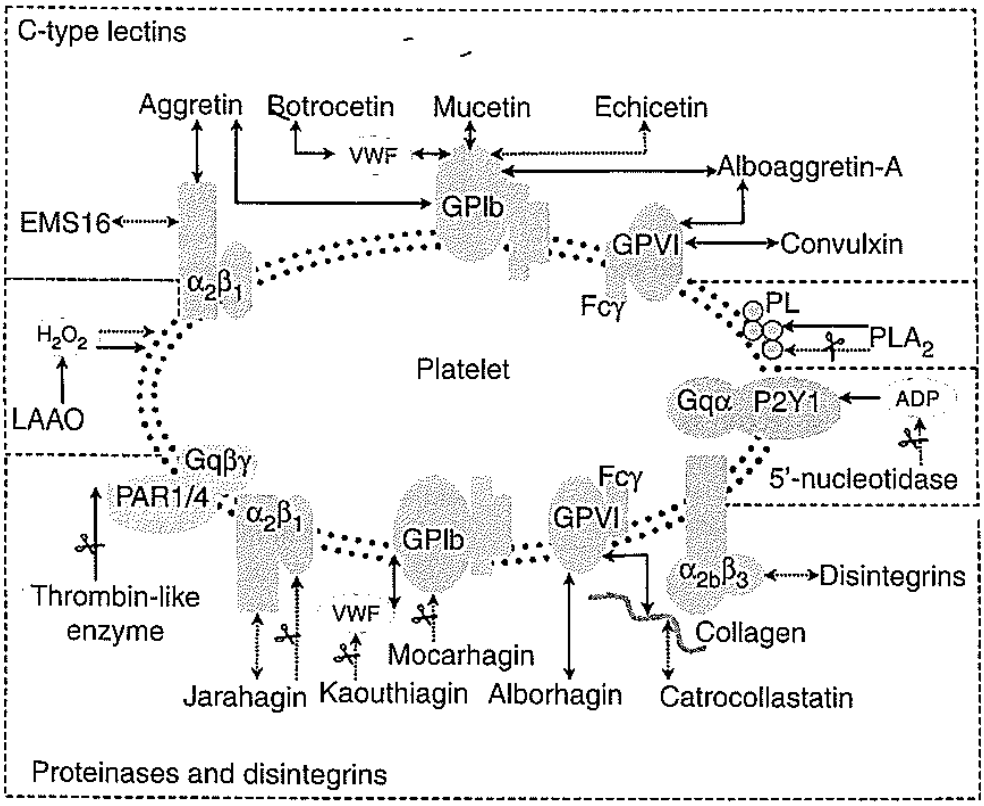


**Figure 8.** Actions of venom proteins on platelets [49].

#### 1.6.5.1 Proteases

One group of proteases acting directly on platelets are the SVTLEs (see 6.1.1.). There is no correlation between the procoagulant activities of these enzymes and their ability to induce platelet aggregation. Some of them mimic thrombin by activating platelets through cleavage of PAR or binding to GPIb, the aggregation is however mainly due to ADP released from platelets. As examples of serine proteases inducing platelet aggregation the following enzymes can be mentioned: bothrombin or PA-BJ from the venom of *Bothrops jararaca*, afaacytin from the venom of *Cerastes cerastes*, or MSP1 and MSP2 from the venom of *B. moojeni* [120].

Other proteases affecting platelet functions are metalloproteinases. Some of them bind to collagen or collagen receptors on platelets by their disintegrin-like or cystein-rich domains to inhibit platelet aggregation. α-Fibrinogenases inhibit platelet aggregation but not β-fibrinogenases.

Some metalloproteinases activate platelets by binding to platelet receptors.

### 1.6.5.2 C-type lectins

A large number of snake venom C-type lectins have been recognized to interact selectively with various platelet receptors, affecting their functions, either promoting or inhibiting platelet aggregation [85]. C-type lectins in snake venoms mostly have a heterodimeric structure with two subunits $\alpha$ and $\beta$ that are often oligomerized to form larger molecules [84]. Simple heterodimers family have been shown to inhibit platelet functions by binding to GPIb blocking von Willebrand factor- (vWF-) and thrombin-induced platelets activation, e.g. echicetin, from *Echis carinatus*, but others activate platelets via the same receptor. Several multimeric (heterodimeric) snake C-type lectin-like molecules have been isolated and characterized with specificity for GPIb that activate platelets with various degrees of efficacy, e.g. flavocetin-A from *Trimeresurus flavoviridis* is a tetrameric heterodimer capable of forming small agglutinates in washed platelets and inducing some low level of activation [85]. Some other snake venom C-type lectins activate platelets by inducing vWF binding to GPIb, e.g. botrocetin from *Bothrops jararaca* and bitiscetin from *Bitis arietans* [84, 85]. Another group of snake C-type lectins activate platelets by binding to GPVI, which is an important collagen receptor. The first such C-type lectin characterized from snake venom was convulxin from *Crotalus durissus terrificus* and it has developed into an important tool for the study of GPVI-specific platelet activation [84, 85]. Other snake C-type lectins acting via GPVI have been described, such as ophioluxin from *Ophiohagus hannah* and stejnulxin from *Trimeresurus stejnegeri* [84]. Several snake C-type lectins interact with platelets also via the integrin $\alpha_2\beta_1$, but none of these are completely specific, involving also other platelet receptors. As examples of snake venom C-type lectins belonging to this group aggretin and rhodocetin from *Calloselasma rhodostoma* can be mentioned. Both of them, apart of $\alpha_2\beta_1$, require also at least GPIb to activate platelets [84].

### 1.6.5.3 Disintegrins

Snake venom disintegrins are cystein-rich low molecular weight polypeptides which contain an RGD, KGD or MGD sequence recognized by integrins. Monomeric disintegrins were first described as unusually potent competitive inhibitors of fibrinogen binding to the GPIIb/IIIa of platelets activated by ADP [121]. As a prototype of this group trigramin, from the venom of *Trimeresurus gramineus*, was

characterized [122, 123]. Monomeric disintegrins contain 48 to 84 amino acids, 8, 12 or 14 highly conserved cysteins and characteristic RGD (KGD or MGD) motif maintained in an appropriate conformation by the disulphide bridge. They are divided into short disintegrins, containing 48-49 amino acids (e.g. echistatin from *Echis carinatus*), medium-size disintegrins containing approximately 70 amino acids (e.g. trigramin) and long disintegrins, containing 84 amino acids, like bitistatin from *Bitis arietans* venom. The RGD (eventually KGD or MGD) motif is crucial for the expression of biological activity of disintegrins, since the substitution of this particular arginine with alanine resulted in almost complete loss of activity by different disintegrins [121]. Also homodimeric (contortrostatin from *Agkistrodon contortrix*) and heterodimeric (EC3 and EC6 from *Echis carinatus*) disintegrin have been isolated and characterized [121].

#### 1.6.5.4 Phospholipases $A_2$

Venom $PLA_2$s can be divided in groups concerning their mechanism affecting platelet functions. At least three mechanisms can be described:

- The first group of $PLA_2$s induce platelet aggregation directly by cleaving platelet membrane PLs resulting in release of arachidonic acid leading to formation of thromboxane $A_2$. The aggregation is not mediated by release of ADP from platelets and can be inhibited by indomethacin and aspirin. As an example a $PLA_2$ from the venom of *Trimeresurus mucrosquamatus* can be given [120, 124].
- The second group of venom $PLA_2$s (e.g. cobra and Russell's viper venoms) induce biphasic effects on platelets aggregation. At lower concentration they initiate a reversible aggregation, whereas at higher concentrations they inhibit platelets aggregation induced by arachidonic acid, ADP or collagen. These biphasic effects are due to the formation of arachidonate and lysophospholipids and can be negated by the addition of serum albumin, which binds and neutralizes the products of enzymatic hydrolysis [120, 124]
- The third group of venom $PLA_2$s inhibits platelets aggregation either by mechanism(s) depending on their enzymatic activity (due to the formation of lysophospholipids, e.g. $PLA_2$ from cobra venom) or by mechanism(s) independent of their enzymatic activity, e.g.$PLA_2$ enzymes from *Pseudechis*

*papuanus* or *Ophiophagus hannah* [49, 125].

#### 1.6.5.5 L-amino acid oxidases

Venom L-amino acid oxidases (LAAOs) are homodimeric flavoenzymes, which catalyse the oxidative deamination of an L-amino acid substrate to a $\alpha$-keto acid along with ammonia and hydrogen peroxide. They are widely distributed in Viperidae, Crotalidae and Elapidae venoms [126], being usually homodimeric glycoproteins, with diverse isoelectric points possibly determining their different pharmacological properties [126]

The reported effects of LAAOs on platelet function are quite controversial. LAAO from *Echis colorata* inhibits ADP-induced platelet aggregation, while LAAOs from *Agkistrodon halys blomhoffii* and *Naja naja kaouthia* venoms inhibit agonist- or shear stress-induced platelet aggregation. It is suggested that the interaction between activated platelet integrin GPIIb/IIIa and fibrinogen was inhibited by the continuous generation of $H_2O_2$ [49, 126]. LAAOs from other snakes have been reported to have totally the opposite effect on platelets. LAAO from *Eristocophis macmahoni* was reported to induce human platelet aggregation, as well as LAAOs from *Ophiophagus hannah, Bothrops alternatus and Trimeresurus jerdonii* triggered platelet aggregation through formation of $H_2O_2$ by a still unclear mechanism [49, 126].

## 1.7 Diagnostic use of snake venoms in the field of thrombosis and haemostasis

Snake venom toxins affecting haemostasis are nowadays widely used in the coagulation field and have facilitated extensively the routine assay of haemostatic parameters, such as [44, 47, 49, 56, 127-131]:

a) fibrinogen and fibrinogen breakdown product assay and the detection of fibrinogen dysfunction, performed by the use of SVTLEs (batroxobin, Reptilase®, Ancrod);

b) antithrombin III, as well as other haemostatic variables in heparin-containing samples can be assayed by means of SVTLEs, which are not inhibited by heparin (Reptilase® reagent);

c) for prothrombin assays, studies of dysprothrombinaemias as well as for preparation of meizothrombin and non-enzymic forms of thrombin different snake venoms prothrombin activators are utilised, depending on their cofactor dependence (Ecarin time, Noscarin, Oscutarin),

d) blood clotting factors V, VII and X and, importantly, lupus anticoagulants (LA) can be assayed by means of toxins contained in Russell's viper venom: RVV-V and RVV-X;

e) for the screening of LA a number of snake venom activators are now regularly employed, including RVV-X and prothrombin activators: Oscutarin, Textarin, and Ecarin;

f) protein C and activated protein C resistance, which is one of the major causes of thrombophilia, can be measured by means of RVV and Protac®;

g) von Willebrand factor (vWF) and related syndromes (Bernard-Soulier disease and Type IIa von Willebrand disease) can be studied with Botrocetin® from *Bothrops jararaca* venom, which is a platelet aggregating protein, requiring vWF for its effect on platelets

h) also platelet glycoprotein receptors, notably, GPIIb/IIIa and Ib can be studied by means of the disintegrins and the C-type lectins.

Snake venom toxins affecting haemostasis are also used in therapeutic settings, which will be discussed more precisely in chapter 8. Other snake venom proteins show future promise in the treatment of a range of haemostatic disorders. Several thrombolytic metalloproteinases that do not cause haemorrhaging have been isolated from snake venoms and are potentially interesting as an alternative for medical use in thrombolysis [17, 53]. Within those agents belong: afaacytin from horned viper (*Cerastes cerastes*) venom, atroxase from western diamondback rattlesnake (*Crotalus atrox*) venom and the fibrinogenase from *Vipera labetina* venom, which dissolve blood clots *in vivo* and *in vitro*. Additionally, substances influencing blood platelets like bitistatin form *Bitis arietans* venom are shown to be most promising in imaging both thrombi and emboli [44, 132, 133].

## 1.8 Medical use of snake venoms in the field of thrombosis and haemostasis

(Part of a book chapter: “Schlangengifte” in Hämostaseologie 2nd Edition; Perchuc A.M. and Wilmer M. (in press))

The development of clinically useful agents from natural products has received increasing attention in recent years. In medicine, the most widely used SVTLEs are those from *Bothrops moojeni* (batroxobin as a defibrinogenating agent under its trade name Defibrase® Pentapharm, Switzerland), *Bothrop atrox* (batroxobin as a hemostatic in a mixture with thromboplastin-like enzyme under its trade name Reptilase®, Pentapharm, Switzerland, in lyophilized form or under its trade name Hemocoagulase, Pentapharm, Switzerland, in form of injectable solution) and *Agkistrodon rhodostoma* venoms (Ancrod under its former trade name Arvin® and recently Viprinex®, Nordmark Pharma and Neurobiological Technologies, Inc.) [48].

In the process called ‘defibrinogenation’, the SVTLEs convert fibrinogen into a non-clottable form of fibrin which is then removed by various enzymatic processes [44, 134]. Although Ancrod and batroxobin are distinct proteins, exerting a species-specific action and showing characteristic immunological properties [131] (If patient treated with Defibrase® develops a resistance to that defibrinogenating agent, if necessary, the therapy can be continued with Ancrod [135]), they show the same mode of action, which could be explain using batroxobin as an example [136]. Unlike thrombin, cleaving both fibrinopeptide A and B (FPA and FPB) from fibrinogen and activating FXIII, the SVTLEs usually cleave FPA alone; only a few release FPB or FPA and FPB together. Based on this feature, SVTLEs have been classified as FP-A, FP-B or FP-AB [137], or venombin A, B or AB [52].

After the s.c. or i.v. administration batroxobin splits the Arg16-Gly17 bond in the A$\alpha$–chain of fibrinogen and thereby causes the release of fibrinopeptide A and the formation of fibrin I monomer, which spontaneously aggregates “head-to-tail” into a clot of fibrin I. Moreover batroxobin induces the release of plasminogen activator (tPA) from endothelium. Fibrin I potentiates plasminogen activation by tPA and is in turn rapidly degraded by fibrinolysis into non-colagulable fragments; the resulting fibrinogen degradation products are excreted with the urine. By this mechanism, batroxobin injection leads to a dose-dependant decrease of plasma fibrinogen

concentration and to a reduction in the viscosity and in the coagulability of blood [45]. Unlike thrombin, most of SVTLEs neither activate other coagulation factors nor influence protease-activated receptors (PAR) on blood platelets inducing platelet aggregation and release reactions. With exception of Ancrod, they are not inhibited by thrombin inhibitors like antithrombin III, heparin and hirudin [45, 57, 138, 139]. Thus, although SVTLEs 'resemble' thrombin to an extent, they are structurally and functionally dissimilar to the coagulation factor [67, 83]

Defibrase® is a defibrinogenating drug which contains highly purified batroxibin originating from *B. moojeni* snake venom as the active component. It prevents thrombus formation and propagation, increases capillary circulations, improves tissue oxygenation and supports physiological and medicamentous fibrinolysis. The platelet count and the concentration of coagulation factors other than fibrinogen are not affected by the treatment [45].

Reptilase® is a hemostatic drug containing hemocoagulase, the hemocoagulant enzyme system including batroxobin and thromboplastin-like component from *B. atrox* venom. As a topical hemostatic agent it exerts a dose-dependent fibrinogen coagulant effect upon local application on the site of bleeding. Upon injection of a small dose batroxobin the formation of fibrin I monomer is induced and sustained over a prolonged time, increasing the coagulation capacity of the blood. Fibrin I, being a preferential substrate for thrombin as well as for plasmin, is rapidly converted into a fibrin clot at the site of a vascular injury, where thrombin is formed via the intrinsic and extrinsic pathways or it is rapidly degraded within the intact vascular system, where plasmin is generated [45]. Reptilase is applied when the loss of blood has to be limited or stopped, e.g. bleeding in surgery, internal medicine, dentistry, etc. It can be also used before surgical interventions to decrease the tendency to bleed, to avoid spilling of blood in the operation field as well as postoperative blood losses.

Ancrod acts at different stages of coagulation and blood circulation by depleting the substrate needed for thrombus formation: the depletion of fibrinogen reduces blood viscosity, which improves blood circulation and enhances thrombolysis by stimulation of endogenous plasminogen activators [140]. Anticoagulation induced by this means is rapid, occurring within hours of it being administered. Ancrod does not activate

plasminogen and does not degrade preformed, fully cross-linked thrombin fibrin and consequently can be used postoperatively.

Alfimeprase (Bayer HealthCare, Germany and Nuvelo Inc., USA) [112, 141-144] is a recombinantly produced, truncated form of fibrolase, a known direct fibrino(geno)lytic zinc metalloproteinase that was first isolated from the venom of the southern copperhead snake (*Agkistrodon contortrix contortrix*) [143]. Fibrolase cleaves the Aα chain of fibrinogen preferentially at position Lys413-Leu414; it also cleaves the Bβ chain at the slower rate. Incubation of fibrolase with plasminogen yields no evidence of plasmin formation [112].

The alfimeprase protein is currently produced by recombinant technology using a synthetically created gene that was incorporated into genomic DNA of *Pichia pastoris* [144]. The truncation of fibrolase to produce alfimeprase has improved pharmaceutical product uniformity [142].

The alfimeprase molecule differs slightly from fibrolase at the N-terminus where alfimeprase contains 201 amino acids with an N-terminal sequence of SFPQR---, in contrast to the N-terminus of fibrolase, which starts with <EQRFPQR---, where <E denotes a cyclized glutamine residue (pyroglutamate), and comprises 203 amino acids. Alfimeprase was shown to cleave the fibrinogen Aα chain and to be bound and neutralized by serum α2-macroglobulin, forming non-dissociable macromolecular complexes [143, 144]. In contrast to the plasminogen class of thrombolytic drugs, alfimeprase and fibrolase do not rely on the endogenous fibrinolytic system (conversion of plasminogen to plasmin). They can be distinguished from the plasminogen activators by their unique mode of action and are defined as directly fibrinolytic agents [143]. When measured, in contrast to the plasminogen activator class of thrombolytic agents, time to clot lysis in the majority of studies with alfimeprase (or fibrolase) has been very rapid in comparison to the agents such as tissue-type plasminogen activator (tPA) or urokinase (uPA), in some cases achieving resolution of thrombus 6-times faster than the reference agent. In 2001, alfimeprase was tested in clinical trials. Phase I and II studies in individuals with arterial or venous thrombotic events, such as peripheral arterial occlusion (PAO) and central venous access device (CVAD) thrombosis, indicate that alfimeprase is active and generally well tolerated [142]. Phase III studies in both PAO and CVAD were undertaken and clinical trials in acute ischaemic stroke and deep venous thrombosis were being

planned [142]. Medical News Today [145] announced that phase III trials of alfimeprase in patients with acute PAO did not meet primary endpoints.

The structure of disintegrins, which are platelet aggregation inhibitors, has been used as a template to design compounds that bind to endogenous fibrinogen with higher affinity. This resulted in the introduction of two drugs eptifibatide under its trade name Integrilin® (Glaxo Group Ltd, Great Britain) and tirofiban under its trade name Aggrastat® (MSD Sharp & Dohme GMBH, Germany). Eptifibatide is a cyclic heptapeptide inhibitor of GP IIb/IIIa, with an active pharmacophore (KGD) that is derived from the structure of barbourin, a GF IIb/IIIa inhibitor from the venom of the southeastern pygmy rattlesnake (*Sisturus miliarus barbouri*) [146-148], while tirofiban is a synthetic compound that mimics RGD and was designed from echistatin from the venom of saw-scaled viper (*Echis carinatus*) [149]. Both drugs have been approved for the therapy of acute coronary ischaemic syndrome and the prevention of thrombotic complications in patients undergoing percutaneous coronary intervention such as balloon angioplasty and stenting [48].

## 1.9 The *Bothrops moojeni* snake and known components of its venom

The South and Central American pit vipers of the genus *Bothrops* belong to the family of Viperidae, subfamily of Crotalinae snakes (pit vipers) [150, 151]. Pit vipers are characterized by possessing of facial pit organs on either side of the head between the eye and the nostril, which are temperature-sensitive receptors facing forward and allowing the snake to localize nearby objects of a higher temperature than the surrounding [6, 151]. They can differentiate between temperature changes of less than 0.003°C [6, 7]

**A.** **B.**

**Figure 9.** A. *Bothrops moojeni*; B. *Bothrops atrox.*

*Bothrops sp*. are responsible for more fatalities in the Southamerican countries than any other group of venomous snakes. Only in Brazil they cause more than 20,000 accidents per year which is 90% of all recorded snake-bites [17, 152]. Their envenomation is characterized by prominent tissue damage, including haemorrhage, necrosis and oedema as well as disturbance in the blood coagulation system. The local effects, haemorrhage and necrosis represent a serious clinical problem since the administration of antivenom may not prevent their occurrence unless given immediately following envenomation. In case of severe poisoning these effects can lead to complete destruction and subsequent loss of a limb [153]. However, death is an unusual outcome of *Bothrops* envenomation (0.3-0.5% of cases) and is caused by acute renal and respiratory failure, haemorrhage, shock and /or sepsis [154, 155].

Bothropic venoms have been analysed since the early part of the XXth century, drawing scientists' attention to their coagulant activities [156]. The thrombin-like (fibrinogen coagulant) activity, resulting from the presence of a serine protease called batroxobin in the crude venoms of *Bothrops* species, was first isolated and semi-purified from *B. jararaca* venom by von Klobusitzky and König [156]. The same properties were further demonstrated by Eagle (1937) in *B. atrox*, *B. jararaca* and *B. nunmifer*, by Janszky (1950) in *B. atrox*, by Stocker and Barlow (1975) in *B. moojeni* and by Nahas (1979) in many species [157]. Later, the thrombin-like enzymes were also described for the venoms of *B. insularis* [158], and Peruvian: *B baretti*, *B. pictus* and *B. hyoprorus* [159, 160] and it was observed that among *Bothrops* species only *B. erythromelas* adults as well as the newborns do not possess thrombin-like activity in their venoms [14]. In 1958 Blomback and Yamashina [161] demonstrated that the thrombin-like enzyme (batroxobin) of *B. atrox* venom

releases only the fibrinopeptide A of fibrinogen molecule. The whole action of batroxobin was already precisely described above, in chapter 8. Further, in 1976 Stocker and Barlow [139] observed variations in molecular weight between batroxobins of *B. atrox*, *B. moojeni* and *B. marajoensis*, which were due to different carbohydrate contents of those thrombin-like enzymes. *B. moojeni* batroxobin was shown to contain only 5.8% of neutral sugar [139, 162] and the precise carbohydrate structure analysis has revealed that this snake venom glycoprotein is characterized by a unique oligosaccharide pattern [162].

Stocker *et al.* [138] described also that the interaction of the thrombin-like venom enzymes, fibrinogen and plasma inhibitors is species dependent, as batroxobin isolated from *B. moojeni* was a better defibrinogenating agent when compared to the one from *B. atrox*. The main explanation for the stronger and more persistent effect of *moojeni* batroxobin was found in the different susceptibility to human plasma inhibitors ($\alpha$2-macroglobulin and possibly others). In the same work [138] he also demonstrated that, contrary to thrombin, batroxobin was inhibited neither by the heparin-antithrombin III complex nor by hirudin. No age-dependent correlation in thrombin-like activity of different *Bothrops* venoms was observed [14]. Up to now, batroxobin is one of the best characterized proteins from snake venoms.

The venom of *B. moojeni*, however, does not belong to the most frequently studied bothropic venoms. Thus, apart of the main thrombin-like activity of batroxobin, not many other components have been isolated and described in this venom untill now.

The main feature of *B. moojeni* venom is its high proteolytic activity, responsible for most of the local and systemic effects during envenomation by this snake. However, the high proteolytic activity is associated with low hemorrhagic effects. Assakura *et al.* has isolated the *moojeni* protease A, which is the major proteolytic enzyme purified to homegenity from the crude *B. moojeni* venom [163]. This protease is a 22.5 kDa metalloproteinase, pI 7.7. It is unstable at acidic pH and very sensitive to heat treatment. Protease A was shown to hydrolyze casein quite strongly with the activity increasing in the presence of calcium ions. The proteolysis of human and rabbit immunoglobulins by *moojeni* protease A was also demonstrated [164], as well as degradation of calf skin type I collagen, guinea-pig skin gelatin, A$\alpha$- and B$\beta$-chains of fibrinogen, $\alpha$-polymer and $\alpha$-chains of fibrin and oxidized B-chain of insulin [165]. Similarly, for MPB, a non-hemorrhagic metalloproteinase isolated also form the

venom of *B. moojeni* and showing in SDS-PAGE two protein bands at 55 kDa and 65 kDa [166]. MPB, showing traces of blood-clotting activity, hydrolyzed casein, type I collagen, oxidized B-chain of insulin, gelatin and fibrin, while in fibrinogen digestion it also degraded the γ-chain. Additionally it showed proteolytic activity on fibronectin [165-167].

Moreover, two basic serine proteases MSP1 and MSP2 have been isolated from the *B. moojeni* venom [168]. Both enzymes are basic glycoproteins possessing esterolytic and amidolytic activities, as well as the ability to hydrolyse gelatines, type I collagen, fibrinogen, fibrin and oxidized insulin B-chain, however at lower rates [166-168]. Isolated MSP1 showed two close protein bands on SDS-PAGE under reducing and non-reducing conditions, corresponding to molecular weights of 34 kDa and 32.5 kDa, while MSP2 consisted of a single chain of 38 kDa. MSP1, but not MSP2, proved also to be a potent platelet aggregating enzyme, also amplifying the aggregation induced by ADP [168].

Another basic non-hemorrhagic serine protease, called MOO3, was purified by Oliviera *et al.* from the crude *B. moojeni* venom [169]. In the SDS-PAGE it appeared as a single-chain protein with an approximate molecular weight of 27 kDa, under both reducing and non-reducing conditions. MOO3 exhibited only traces of coagulant activity; nevertheless it caused quite rapid degradation of Aα- and Bβ-chains of fibrinogen and cleavage of casein, with no amidolytic activity toward synthetic substrates.

The above results show that non-hemorrhagic venom metalloproteinases and serine proteinases may contribute to local tissue damage; as well they might be also involved in the defibrinogenation occurring *in vivo* after envenomation by *Bothrops* species [166].

Further, to the most important components of the *B. moojeni* venom belongs for sure the $PLA_2$ family. It is mostly responsible for different toxic effects, such as myonecrosis, blood anticoagulation, platelet aggregation inhibition and inflammatory symptoms after an envenomation by a *Bothrops* snake. Many $PLA_2$s possessing, as well as missing the enzymatic activity have been described for other *Bothrops* snakes [170-172], e.g. *B. atrox* [173, 174], *B. jararacussu* [175, 176], *B. pirajai* [177-179], *B. neuwiedii* [180-182], etc. For *B. moojeni* just one $PLA_2$ enzyme and two $PLA_2$-like myotoxins have been described so far. MOO-1 myotoxin possesses high

phospholipase activity as reported by Moura-da Silva *et al.* [183, 184]. The two other myotoxins, MjTX-I (Swiss-Prot Accession number AN: P82114) and MjTX-II (Swiss-Prot Accession number AN: Q9I834), are described as catalytically inactive Lys49 variants. Their amino acid sequences [185-188], as well as the native structures and the complex of MjTX-II with stearic acid [186, 187, 189, 190] have been determined, revealing high homology with various Lys 49 $PLA_2$-like proteins from other *Bothrops* venoms.

Generally *Bothrops* myotoxins are classified in group II $PLA_2$s, together with all crotalid/viperid venom lipases and the secreted non-pancreatic mammalian $PLA_2$ [191]. Moreover, they are divided into two major classes according to the residue at position 49 in the sequence, being a key residue for binding of the $Ca^{2+}$-cofactor. The Asp49 enzymatically active and the Lys49 enzymatically non active myotoxins can be distinguished [172, 185]. It has also been reported, that there is a higher homology between Lys49 myotoxins of different genera (*Bothrops*, *Agkistrodon* and *Trimeresurus spp.*) than between Lys49 and Asp49 proteins of the same species [191, 192]. Asp49Lys variant together with additional mutations in the calcium-binding loop precludes an effective coordination of calcium ions and, consequently, is responsible for the lack of enzymatic activity [172, 191, 193]. However, the enzymatic activity is apparently not necessary for their toxic actions. Lys49 myotoxins display also bactericidal properties, being lethal for a broad spectrum of gram-negative and gram-positive bacteria [194, 195]. Very recently, Stabeli and coauthors [196] have shown that *B. moojeni* myotoxin II displays antiparasitic and antitumoral activities, suggesting the presence of multiple bioactive sites in this protein.

Another enzyme isolated from *B. moojeni* venom and contributing to its toxicity is an L-amino acid oxidase, called BmooLAAO-I (Swiss-Prot Accession number AN: Q6TGQ8) [197-199]. BmooLAAO-I is a dimeric glycoprotein with a molecular weight of 130.8 kDa, consisting of two identical subunits of 64.9 kDa. Recently, Stábeli *et al.* reported the isolation and functional/structural characterization of that enzyme with special reference to its antitumor, trypanocidal, bactericidal and platelet-aggregating effects [198]. All of these effects seemed to be mainly dependent on the production of $H_2O_2$ by the enzymatic reaction. Nevertheless the exact molecular mechanisms of these effects are still being investigated. In addition, some preliminary studies conducted by Stábeli and co-workers indicate that BmooLAAO-I presents also anti-HIV properties [198].

Recently, a novel fibrin(ogen)olytic zinc metalloproteinase (class P-I of SVMPs), called BmooMPα-I (Swiss-Prot Accession number AN: P85314) has been described in the venom of *B. moojeni* [200]. The enzyme of molecular weight of about 24.5 kDa cleaves the Aα-chain of fibrinogen first, followed by the Bβ-chainand shows no effects on the γ-chain. The action of BmooMPα-I on fibrin was less effective than on fibrinogen, cleaving the FPB preferentially. BmooMPα-I was found to cause defibrinogenation when administered i.p. on mice. Thus, it is expected to be of medical interest as a therapeutic agent in the treatment and prevention of arterial thrombosis.

Bourguignon *et al.* has isolated a few novel peptides from the venom of *B. moojeni* [201]. Three of them were homologues with the biologically active *B. jararaca* bradykinin potentiating peptides (BPP). And one, with the molecular mass of 1370.6 Da was submitted to the Swiss-Prot Protein database as a non-pGlu peptide associated with a methylated Lys2 isoform at 1384.7 Da (Swiss-Prot Accession number AN: P84747).

To other components, described in *B. moojeni* venom, however never precisely characterized belongs a FX-activating metalloproteinase [14, 76, 202] and a bothrojaracin-like protein [98] already described in chapter 6.2.3.

## 1.10 Aims of the "*Bothrops moojeni* venom proteomics project" and Botmo Thesis

Early studies in the 1930s on the venom of South-American Lancehead snakes of the *Bothrops* genus have led to the discovery of compounds very active in haemostasis like batroxobin. Batroxobin obtained from the venoms of *B. moojeni* and *B. atrox*, produced and sold by Pentapharm Ltd. under its trade names Defibrase® and Reptilase® is still widely used in pharmaceutical and diagnostic applications. For the collection of major 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 a large amount of venom collected from different animals makes this venom an abundant, stable and homogenous source for further investigations. However, most of the *Bothrops moojeni* venom material needed for its production is not further processed. But like all

snake venoms it contains still a large number of biologically active compounds not yet discovered or fully described.

**Figure 10.** Pentapharm do Brasil.

The aim of the "*Bothrops moojeni* venom proteomics" project was to develop and use biochemical and proteomic strategies in order to further investigate crude *Bothrops moojeni* venom. This was expected to allow identification and molecular characterisation of a wide range of bioactive compounds.

The goal of "*Botmo Thesis*" was the purification and precise characterization of novel components in the *Bothrops moojeni* venom with main focus on the substances influencing blood coagulation system. Such original compounds were intended to be used as diagnostic tools or further developed for pharmaceutical applications.

For this reason the "function to structure" approach was mainly performed using a newly developed coagulation high-throughput platform. The interesting activities found by means of that platform were further investigated, leading in some cases to the elucidation of the full protein sequence.

Crude venom was analysed by several transversal techniques in order to have an overview of its major components that can be divided in the following classes:

- Proteins of more than 15 kDa, most likely with enzymatic activities; examples described in the following Botmo publications:

  "Platelet-active substances in the venom of *Bothrops moojeni* snake – a novel evaluation method using whole blood aggregometry."

"A protease possessing blood coagulation factor VIIa-like activity isolated from venom of *Bothrops moojeni* snake."

- Mini-proteins between 1 and 15 kDa with disulfide bonds, and most likely toxins possibly acting as ion channels modulators or specifically binding to receptors or other targets; example described in the following Botmo publication:

  "Isolation and characterization of two new Lys49 $PLA_2$s with heparin neutralizing properties from *Bothrops moojeni* snake venom."

- Linear peptides between 200 and 5'000 Da, with no disulfide bonds, possibly acting as enzyme inhibitors, cell-penetrating peptides, neuropeptides, etc.; examples described in the following Botmo publications:

  "Precursor ion mass spectra for efficient screening of Pyroglutamate and Bradykinin-potentiating peptide (BPP)-type substances in *Bothrops moojeni* snake venom using liquid chromatography/tandem mass spectrometry."

  "Screening of Acetylcholinesterase Inhibitors in Snake Venom by Electrospray Mass Spectrometry."

- Others, mainly small compounds of non peptidic structure such as histamine, polyamines, alkaloids, steroids or other organic structures that have not been studied till now but are all also possibly of high interest.

Apart of the above findings a new heparin neutralising feature derived from two Lys49 myotoxins was found. Its unique inhibitory action towards low molecular weight heparin (LMWH) gained special attention and will be precisely presented in the part called: "Heparin neutralizing properties of synthetic peptides derived from C-terminal end of the novel *Bothrops moojeni* Lys49 phospholipase $A_2$ (Mj-TX-III)."

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# 2 Screening for Proteolytic Activities in Snake Venom by means of a multiplexing electrospray ionization mass spectrometry assay scheme

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## 2.1 Abstract

A multiplexed mass spectrometry based assay scheme for the simultaneous determination of five different substrate/product pairs was developed as a tool for screening of proteolytic activities in snake venom fractions from *Bothrops moojeni*. The assay scheme was employed in the functional characterization of eight model proteases. Time-resolved reaction profiles were generated and the relative reaction progress at each time point was determined. These were used to semi-quantitatively sort the catalytic activities of each enzyme towards the respective substrates into six classes. The resulting activity pattern served as an activity fingerprint for each enzyme.

The multiplex assay scheme was then applied to a screening for proteolytic activities in fractions of the pre-separated venom from *B. moojeni*. Activity patterns of each fraction were generated and used to sort the fractions into three different categories of activity. By comparison of the fingerprint activity patterns of the venom fractions and the model enzymes, a compound with proteolytic properties similar to activated protein C was detected.

## 2.2 Introduction

Enzymatic catalysis plays a key role in the regulation of all kinds of processes in life. Serine proteases like thrombin play a major role in the regulation of the blood coagulation system and are therefore an important target for the development of anti-thrombosis drugs [1]. Besides thrombin, a large number of other proteases and factors are involved in the regulation of the blood coagulation cascade, which can also be a potential target for pharmaceutically active compounds. Snake venom is accounted to be a major natural source for these substances, typically containing at least several hundred of different biologically active components [2-4]. In order to explore the different enzymatic activities found in these highly complex mixtures, a vast number of samples has to be analyzed. Therefore, the demand for a rapid and rugged activity screening method is high.

Typically, the determination of enzymatic activities is performed in single-substrate-single-enzyme assay schemes by either UV / vis-absorbance-, fluorescence- or radioactivity-based detection methods. A substrate, which serves as probe for a certain enzymatic activity, is brought to reaction with the enzyme of interest and the conversion is monitored by detecting either the product formation or the decay in the substrate concentration. To allow the detection by means of optical methods, the spectroscopic properties of the substrate have to be changed significantly during the conversion. Unfortunately, most naturally occurring substrates do not possess any distinct spectroscopic features or these are not significantly altered during the enzyme-catalyzed reaction. Therefore, chromogenic or fluorogenic moieties have to be introduced synthetically into their molecular structure. However, a change of the substrate structure often leads to a considerable change in the enzymatic recognition, thus resulting in different kinetic characteristics of the reaction [5]. An elegant way to circumvent this problem is the use of substrates which contain a radioactive isotope. These compounds are chemically similar to the natural substrates, thus the reaction kinetics are not influenced. However, the necessary radioactive substrates are often not readily available and have to be synthesized laboriously. Additionally, by using radioactively labeled substrates, the problem of handling ‘hot’ material arises. For those reasons, an alternative detection method,

which does not need synthetically prepared substrates or requires the handling of radioactive compounds, is desirable.

Mass spectrometry (MS) can provide these features. Since the detection relies solely on the mass-to-charge (m/z) ratio of a compound, the spectroscopic properties of an analyte are no limiting factor. Therefore, it is in principle possible to perform multi-substrate assay formats, as long as the substrates and respective products are different in their m/z ratios.

First approaches to employ electrospray ionization mass spectrometry (ESI-MS) as means of detection in enzymatic bioassays by Henion and co-workers [6, 7] were initially followed by only a small number of further publications; however, in recent years, interest in this promising field has been growing steadily [8-16]. Up to now, the predominant number of papers deals with single-substrate-single-enzyme assay schemes. A first multiplexing assay system was presented by the group of Gelb and colleagues. A number of different target-specific substrate conjugate compounds were used to probe simultaneously for certain enzymatic activities in cell lysates allowing the diagnosis of enzyme deficiency related diseases. The substrate conjugates were built up from an affinity tag, a linker and the enzyme target-specific substrate moiety. The affinity tag was used for the selective extraction of the substrates and products by means of affinity chromatography. Subsequently, the trapped analytes were eluted to the ESI-MS system [17-23]. Pi and Leary [24] and Zea *et al.* [25] presented multiplexing assay schemes for the competitive determination of enzyme / substrate specificity constants. Basile *et al.* [26] developed an *in vivo* multiplexing assay scheme for the differentiation of bacteria. A mix of four different substrates was incubated with living bacteria. Unique mass tags were released from each substrate during a reaction. The reaction mixture was filtered in order to remove the bacteria and transferred to the mass spectrometer. By determining the amount of each mass tag type released in the mixture, a reactivity pattern for each stem of bacteria could be generated. These activity profiles were used as fingerprints in order to differentiate the different types of bacteria [26]. Yu and co-workers [27] employed a multiplexing assay scheme for the activity screening of different glycosidases. Small libraries containing nine mass-different carbohydrates were incubated with several glycosidases. The reaction mixture was analyzed by means of flow injection analysis (FIA) ESI-MS. A general enzymatic activity towards a substrate was assessed upon decreasing substrate signal intensity and increasing

signal intensity of the respective product [27]. A matrix-assisted laser desorption / ionization (MALDI)-MS-based assay scheme for the activity profiling of kinases was presented by Min and colleagues [28]. A mixture of four different target-specific substrates was immobilized on a MALDI target plate using a self-assembled monolayer. Several kinases were incubated separately on the target plate. After rinsing of the plate in order to remove the enzyme and addition of matrix, the MALDI-MS analysis took place. Enzymatic activity was assessed upon decrease of a substrate signal and build up of the corresponding product signal.

In this paper, an ESI-MS-based multiplexing assay for the profiling of proteolytic activities is presented. The assay development was based on the scheme presented in by Liesener and Karst [29]. Five different substrates were employed as activity probes. The assay scheme was used to generate activity fingerprints for eight different model proteases. Subsequently, it was applied to screen for and characterize the proteolytic activities in fractions of the pre-separated venom from *Bothrops moojeni*. The venom of *B. moojeni* snake although not yet completely characterized, is known to target mainly on the blood coagulation cascade of its prey [2]. Therefore, it is reasonable to assume the presence of a variety of proteases which might be of pharmaceutical interest due to their potent activity in the blood coagulation process.

## 2.3 Experimental

### *Chemicals*

The substrates, enzymes and snake venom fractions used in this study were provided by Pentapharm (Basel, Switzerland). Human factor VIIa, human factor IXa alpha, human factor Xa, bovine factor Xa, human factor XIa, human factor alpha-XIIa and human activated protein C were obtained in the highest purity available from Enzyme Research Laboratories (South Bend, USA) and thrombin from bovine plasma was purchased in the highest purity available from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands). The p.a. grade ammonium acetate was obtained from Merck (Darmstadt, Germany). All solvents were purchased in LC-MS grade quality from Biosolve (Valkenswaard, The Netherlands).

***Sample preparation***

All enzyme and substrate solutions for the ESI-MS assays were prepared and stored at –18 °C until use. The five substrates (see Table 1) were dissolved in buffer (15 mM ammonium acetate / ammonia, pH 7.5) yielding a substrate mix solution with concentrations of 5 x $10^5$ mol/L of each compound. The model enzymes activated human factor VII, activated human factor IX, activated human factor X, activated bovine factor X, activated human factor XI, activated human factor XII, activated human protein C and human thrombin were dissolved in buffer to yield solutions with a content of 100 μg/mL.

The venom of *B. moojeni* was fractionated by preparative gel chromatography yielding fractions with venom components separated by their molecular masses. The gel fractions (F28 – F46) were frozen and kept at –18 °C until use.

***Instrumental set-up of the FIA/ESI-MS system***

For the FIA experiments, a flow injection system comprising a binary gradient HPLC pump HP1100 model GF1312A and an autosampler HP1100 model G1313A (both Agilent, Waldbronn, Germany) was connected to the mass spectrometric detector. The carrier stream was set to 0.3 mL/min of 75:25 (v / v) mixture of methanol and water.

***Mass spectrometric detection***

For detection, an Esquire 3000+ ion trap mass spectrometer (Bruker Daltonik, Bremen, Germany) equipped with a standard ESI source was used. All measurements were performed using the positive ion MS mode. Mass spectra were recorded over the range from m/z 100 – 1000 in full-scan mode. Instrumental settings were as following: nebulizer gas ($N_2$) pressure 40 psi, drying gas ($N_2$) flow 10.0 L/min, drying gas ($N_2$) temperature 365°C, capillary high voltage 5000 V, capillary exit voltage 156.6 V, skimmer voltage 15.0 V, octopole 1 voltage 11.69 V, octopole 2 voltage 3.26 V, octopole amplitude 127.9 Vpp, lens 1 voltage -6.1 V, lens 2 voltage -49.8 V, and trap drive level 57.0. The resulting data were analyzed using DataAnalysis software version 3.1 (Bruker Daltonik).

***ESI-MS assay procedure***

All ESI-MS-based assays were performed according to the following general procedure: 1400 μL of the substrate mix solution was pipetted into a 1.5 mL vial. The

reaction was initiated by addition of 100 μL of the respective enzyme solution or gel fraction. Every 2 min over a period of 40 min, a 50 μL aliquot was sampled from the reaction mixture and quenched in 950 μL methanol, thus stopping the reaction by rapid denaturing of the enzyme. The quenched samples were subsequently injected in triplicate into the FIA / ESI-MS system and analyzed. The injection volume was set to 5 μL.

**Table 1.** List of substrates used in the multiplex activity assays and the respective signal traces used for detection of substrates and corresponding products.

| Substrate | Substrate Structure | Substrate Signal Trace (m/z) | Product Signal Trace (m/z) |
|---|---|---|---|
| XII | H-D-CHT-Gly-Arg-pNA | 505 | 385 |
| IX | H-D-Leu-PHG-Arg-pNA | 541 | 421 |
| X | $CH_3OCO$-D-CHA-Gly-Arg-pNA | 563 | 443 |
| VII | $CH_3SO_2$-D-CHA-Abu-Arg-pNA | 611 | 491 |
| PC | H-D-Lys(Cbo)-Pro-Arg-pNA | 654 | 534 |

## 2.4 Results and Discussion

An ESI-MS-based multiplex assay scheme for the screening and characterization of proteolytic activities in snake venom was developed. In the first step, the eight model proteases were assayed individually with a mixture of five different substrates. In order to monitor the simultaneous conversion of the substrates to the respective products, sample aliquots were taken from the reaction mixture every 2 min over a time span of 40 min. The conversion in the sample aliquots was stopped by quenching with a 19-fold excess of methanol, thus conserving the state of product formation and substrate consumption at each time point. The samples were analyzed by means of direct FIA / ESI-MS.

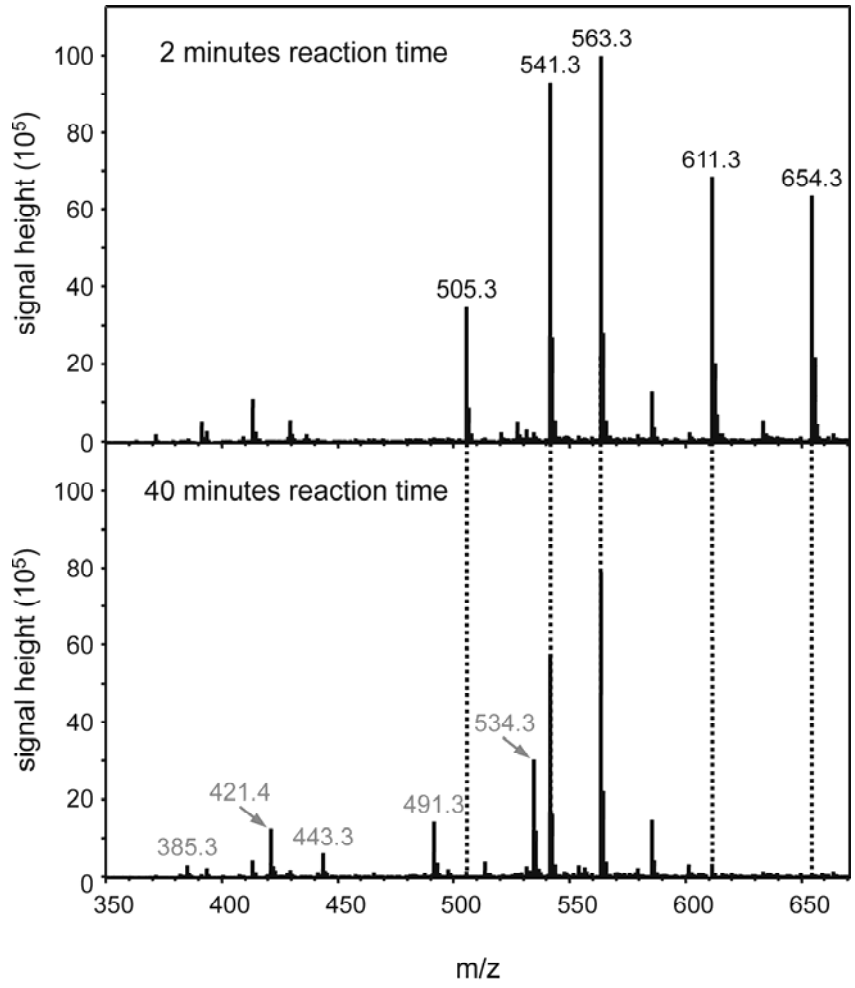


**Figure 1.** Mass spectra obtained from samples of the thrombin reaction mixture after reaction times of 2 and 40 min. The mass spectrum after 2 min reaction time shows only signals for the substrates PC (m/z 654.3), VII (m/z 611.3), X (m/z 563.3), IX (m/z 541.3) and XII (m/z 505.3). After 40 min reaction time, signals for the respective products PCcleaved (m/z 534.3), VIIcleaved (m/z 491.3), Xcleaved (m/z 443.3), IXcleaved (m/z 421.4) and XIIcleaved (m/z 385.3) are observed, while the intensity of the product signals is decreased (position of product signals indicated by the dashed lines).

Figure 1 shows the mass spectra obtained for different reaction times in the assay with the model protease thrombin. At the beginning of the reaction (2 min reaction time), there are only signals found which correspond to the five substrates (see Table 1). At the end of the reaction (40 min reaction time) the signals for the substrates PC and XII are no longer present, while the signals of the respective products PCcleaved and XIIcleaved can be found, indicating the complete conversion of these two substrates. The signal heights for the substrates VII, X and IX are also decreased

over the course of the reaction and the respective product signals can be found, but the conversion of the substrates is not yet completed.

In order to generate time-resolved reaction profiles, the signal traces of the substrates and products are extracted and the area underlying the peaks is integrated resulting in values for the respective signal intensities. Plotting the peak areas for each substrate and the according product versus the reaction time yields the time-resolved reaction profiles of the respective conversions.

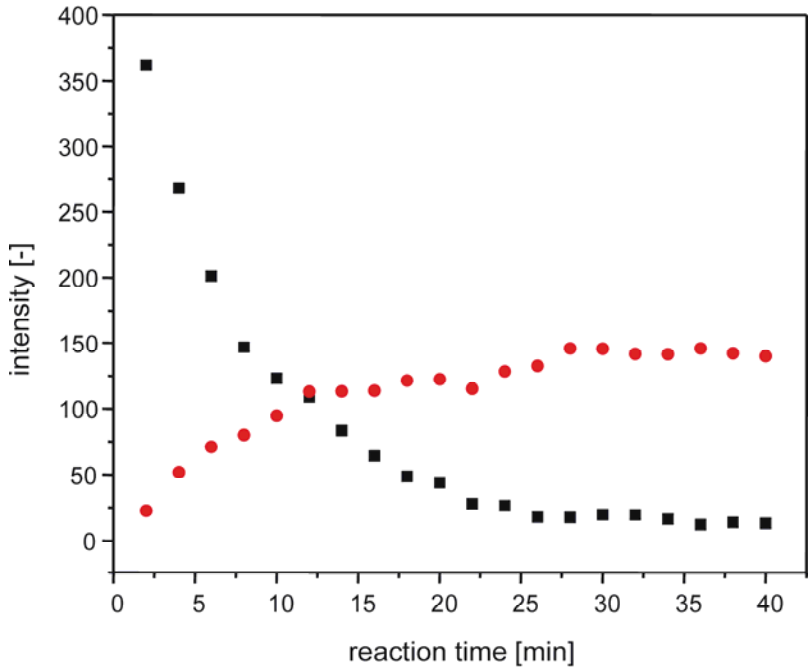


**Figure 2.** Time-resolved reaction profile of the conversion of substrate XII catalyzed by human factor XIIa. Substrate consumption ( ■ ) and product formation ( ● ) curves are shown.

Figure 2 shows the complete reaction profile of the human factor XIIa-catalyzed conversion of substrate XII. The conversion is completed after 30 min reaction time, since the curves for both substrate consumption and product formation are reaching a stable plateau value.

Quantification of the analytes in MS is often achieved by addition of an internal standard. The concept of using the substrate as internal standard for the relative quantification of the product compound was discussed by several authors for the use in MALDI-MS assay schemes [30-32]. Based on their approach, the relative reaction progress (RRP) at a time point was assessed by the ratio product and substrate in

the sample. The RRP in percent is expressed as the product signal intensity ($S_P$) as a fraction of product plus remaining substrate signal intensity ($S_S$).

$$RRP(\%) = \frac{S_P}{S_S + S_P} x100 \quad (1)$$

The values for the RRP range from 0% (no product formed) to 100% (complete conversion of the substrate). Plotting the RRPs for the five parallel substrate conversions catalyzed by one enzyme versus the reaction time results in an 'activity map' for the respective enzyme. It must be noted that the ionization properties of the individual substrates and their respective products are prone to differ. Therefore, no direct proportionality of the RRP value to molar concentration of the analytes can be extracted. However, the RRP values can compensate up to a certain extent for changes in the signal response, thus enhancing the shot-to-shot reproducibility, but a reliable quantification is not possible. Another issue is the simultaneous detection of several analytes in varying concentrations. A mutual influence of these compounds cannot be ruled out, resulting in ion suppression or enhancement of the individual analyte. Therefore, this methodology is not considered a suitable tool for a reliable quantification of the analytes, but remains a semi-quantitative approach, allowing for a sufficiently accurate classification of the reaction progress, which was the main goal of the study.

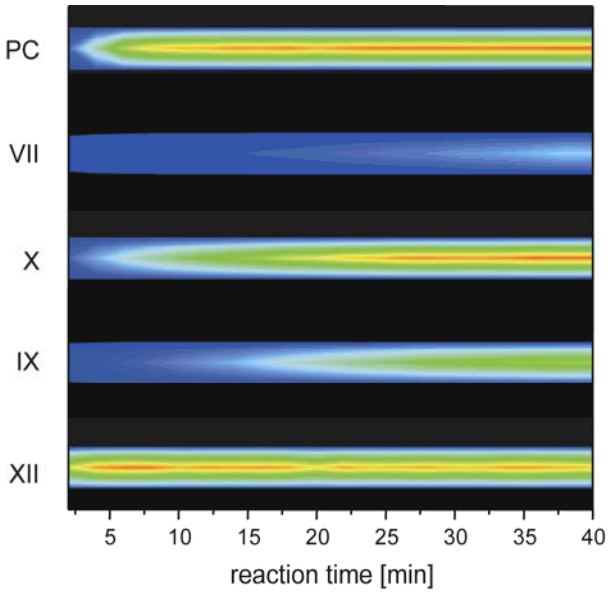


**Figure 3.** Reactivity map of human factor XIIa showing the relative reaction progress (RRP) at each point in time for the conversion of the respective substrates (red → 100% RRP; dark blue → 0.2% RRP).

Figure 3 shows the activity map of human factor XIIa as a heat map, where red color represents high values (complete conversion) and blue color represents low values (no conversion; noise). In the y-axis, the different substrate conversions observed are listed. The plot is extrapolated between the respective measurement points. The width of the signal traces promotes a better perceptibility of the slopes (rate of color change), which indicate the respective reactivity. High enzymatic activity (fast reaction) is characterized by a steep increase in the RRP value. Similar reactivity maps were obtained for the other enzymes as well.

On the basis of these maps, the individual catalytic activity of the enzymes towards the respective substrates could be assessed. Therefore, the RRP values for each conversion at reaction times of 20 and 40 min were taken into account.

**Table 2.** Criteria for classification of enzymatic activities by their time-dependent RPP values.

| **Activity classes** | **RRP value after 20 min** | **RRP value after 40 min** |
|---|---|---|
| Very high activity | ~80% | ~100% |
| High activity | ~40% | ~100% |
| Medium activity | ~10% | ~50% |
| Low activity | ~3% | ~20% |
| Very low activity | ~1% | ~3% |
| No activity | ≤1% | ≤1% |

As summarized in Table 2, the data result in six classes of enzymatic activity. The complete overview of the activities of the eight model proteases is presented in Table 3.

**Table 3.** Summary of catalytic activity classes of each enzyme with respect to the different substrates (++++: very high activity; +++: high activity; ++: medium activity; +: low activity; +/-: very low activity; -: no activity).

| Model Enzyme | PC | VII | X | IX | XII |
|---|---|---|---|---|---|
| Human Protein Ca | **++++** | **++++** | **+** | **++++** | **++++** |
| Human Thrombin | **++++** | **+++** | **+** | **+** | **+++** |
| Human Factor VIIa | **-** | **+/-** | **-** | **-** | **-** |
| Human Factor IXa | **+/-** | **+/-** | **++** | **-** | **+/-** |
| Bovine Factor Xa | **++++** | **++++** | **++++** | **++++** | **-** |
| Human Factor Xa | **++++** | **++++** | **++++** | **+++** | **-** |
| Human Factor XIa | **++** | **++++** | **+++** | **+** | **-** |
| Human Factor XIIa | **++++** | **+** | **+++** | **++** | **++++** |

This classification of the respective activities characterizes the enzymes and can be used as activity fingerprint. It is important to note that the resulting activities of an enzyme towards the respective substrates are the results of a multi-substrate assay. Due to competition effects in the enzymatic reaction caused by the presence of several different substrates, the resulting activity profile might differ from results obtained by single-substrate assay. Therefore, the activity fingerprint can only be used to compare assays which have been performed under the same experimental conditions.

In the second part of this work, fractions of the venom from *B. moojeni* were screened for their proteolytic properties. Each fraction was assayed under the same conditions as the model enzymes, which allows a direct comparison of activities between the individual fractions and the model enzymes. The complete activity maps of all fractions are presented in Fig. 4.

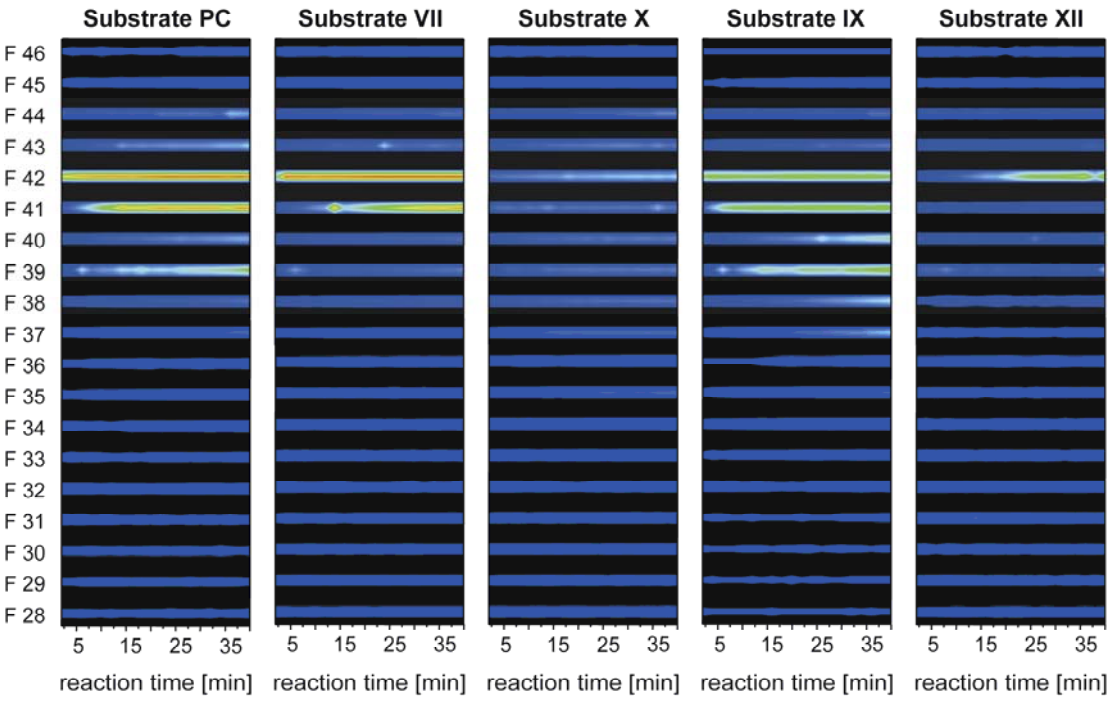


**Figure 4.** Complete activity maps for all venom fractions (F28 – F46) showing the time-resolved relative reaction progress (RRP) of the respective substrate conversions mediated by the individual venom fraction (red → 100% RRP; dark blue → 0.2% RRP).

In the y-dimension, the different gel fractions of the snake venom are listed from F28 to F46. The RRP time profiles for the conversions of each substrate are presented in the corresponding columns. Thus, each line through the five columns represents the activity map for the individual venom fraction. According to Table 2, the catalytic activity of each venom fraction towards the five substrates is assessed and classified. The results of this procedure are given in Table 4.

**Table 4.** Summary of catalytic activity classes of each venom fraction with respect to the different substrates (++++: very high activity; +++: high activity; ++: medium activity; +: low activity; +/-: very low activity; -: no activity).

| Venom Fraction | PC | VII | X | IX | XII |
|---|---|---|---|---|---|
| F 46 | - | - | - | - | - |
| F 45 | - | - | - | - | - |
| F 44 | + | +/- | + | +/- | - |
| F 43 | ++ | + | +/- | + | +/- |
| F 42 | ++++ | ++++ | + | ++++ | ++++ |
| F 41 | ++++ | +++ | +/- | ++++ | +/- |
| F 40 | + | +/- | +/- | ++ | - |
| F 39 | ++ | + | +/- | +++ | - |
| F 38 | +/- | +/- | +/- | ++ | - |
| F 37 | +/- | - | + | + | - |
| F 36 | - | - | - | - | - |
| F 35 | - | - | +/- | +/- | - |
| F 34 | - | - | +/- | - | - |
| F 33 | - | - | - | - | - |
| F 32 | - | - | - | - | - |
| F 31 | - | - | - | - | - |
| F 30 | - | - | - | - | - |
| F 29 | - | - | - | - | - |
| F 28 | - | - | - | - | - |

On the basis of this classification scheme, the venom fractions can be sorted into three different categories. Generally, proteolytic activity seems only to occur in the fractions F37 to F44. Consequently, the first category comprises the fractions with no proteolytic activity (F28 – 36, F45, F46). The second category, fraction F42, is characterized by significant catalytic activity towards the substrates PC, VII, IX and, most importantly, XII, while the activity towards substrate X is only low. In fact, F42 is the only fraction, which efficiently catalyzed the hydrolysis of substrate XII. The fractions F43, F44 and especially F41 show some similarities to the activity profile of F42 and are therefore also contributed to the same category. The third category is characterized by the activity pattern of F39. This fraction exhibits some catalytic

activity towards the substrates PC and IX and low activity towards substrates VII and X, but, most importantly, no activity towards substrate XII. A related pattern can be observed for the fractions F37, F38 and F40, which are therefore also sorted to category three.

The fact that several neighboring fractions seem to have a similar activity pattern with respect to the main fraction of a category can be explained by the separation. Gel permeation chromatography separates compounds by their molecular masses. However, the separation efficiency is not high and, therefore, compounds of similar molecular masses are not separated well. This results in some active compounds being distributed in different amounts over several fractions. This conclusion is supported by the fact that the characteristic enzymatic activities all are decreasing around the dominant fraction.

The characteristic enzymatic activity fingerprints generated in these screening assays for the venom fractions can be compared with the activity profiles of the model proteases. As a result, one perceives a high correlation in the pattern of F42 and activated protein C. Therefore, the conclusion can be drawn that a compound with properties similar to protein C is present in a significant amount in the venom fraction F42.

## 2.5 Conclusions

The opportunity for multiplex analysis of enzymatic activities by means of ESI-MS-based assay schemes offers great possibilities in the functional characterization of enzymes. The successful application of such a multiplex assay was demonstrated for the characterization of eight different proteases. The main advantage of this methodology lies in the application as a screening tool for enzymatic activities in complex biological samples. The assay conditions (pH value, buffer concentration, temperature, etc.) were chosen to allow a broad range of proteases to be active. Thus, the assay conditions were not at the optimum for all enzymes studied. However, since the aim is to generate activity patterns for the model enzymes and venom fractions and perform a comparative analysis of these profiles, it is only important that the assay conditions remain the same for all single experiments. By using this assay scheme, it was possible to rapidly identify and classify proteolytic activities in the venom fractions of *B. moojeni*. The classification was carried out

semi-quantitatively by determination of the relative reaction progress at defined reaction times. The resulting activity patterns could be used as fingerprints to characterize the respective venom fractions. Additionally, it was possible to find model-like activities in the venom fractions by comparison of the fingerprints.

This study clearly indicates the high potential of employing multiplex ESI-MS assays in enzymatic activity screening processes. However, it will be advantageous for the characterization of the enzymatic activities and thus the unique character of the fingerprint activity patterns to widen the scope of substrates used in an assay. Further work will be directed to employ a broader range of different substrates to derive more detailed information from the multiplex screening.

## Acknowledgements

We thank the Fonds der chemischen Industrie (Frankfurt / Main, Germany) and the Nederlandse Organisatie voor Wetenschappelijke Onderzoek NWO (Den Haag, The Netherlands) for the financial support of the work.

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# 3 The potential of *Bothrops moojeni* venom in the field of hemostasis. Established use and new insights

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## 3.1 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 focused on enzymatic and other activities in the field of haemostasis 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.

## 3.2 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 the 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.

## 3.3 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.

## 3.4 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 $CH_3COONH_4$, 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 successfullyperformed.

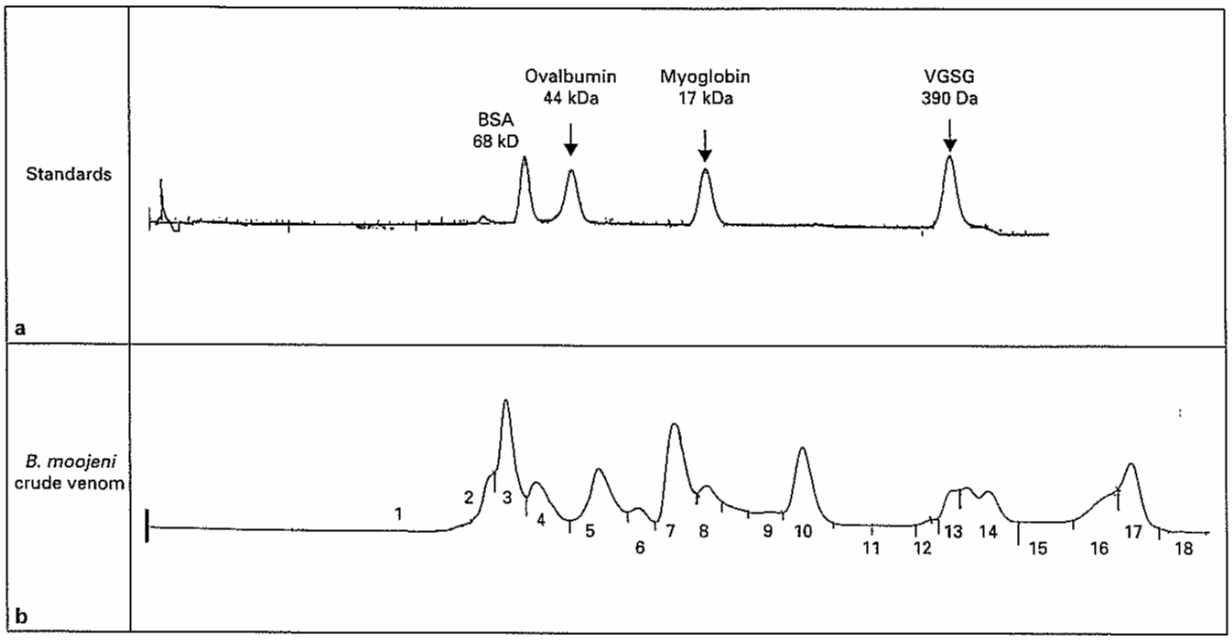


**Figure 1.** Comparison of the GF profiles of standards and of the crude venom of *Bothrops moojeni*. Chromatogram obtained with 300 mg of *B. moojeni* crude venom.

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.

By use of on-line Liquid Chromatography-Electrospray Ionisation-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 $C_{18}$, 10 mm x 250 mm); see figure 2.

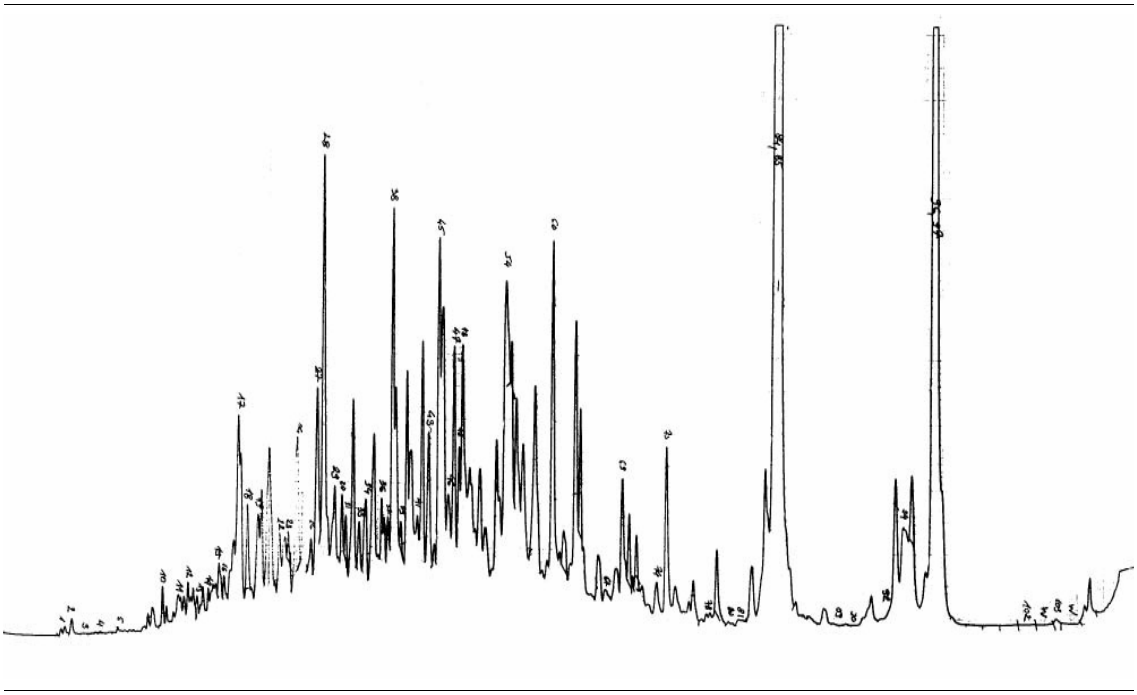

**Figure 2.** Semi-preparative HPLC profile of GF 12 fraction.

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 Ionisation-Time of Flight-Mass Spectrometry (MALDI-TOF-MS) using 4-HCCA ($\alpha$-cyano-4-hydroxycinnamic acid) matrix, resulting in about 1,200 measured molecular masses in the range of 500 to 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 $A_2$ ($PLA_2$) enzymes were found in the low-molecular-weight fractions of *B. moojeni* crude venom. According to the MALDI-TOF-MS and LC-ES-MS about 20 molecular masses corresponding to the $PLA_2$ mass range were identified in the crude venom and the HPLC sub-fractions. Not all of these fractions showed enzymatic activity typical for $PLA_2$, 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 $PLA_2$. 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.

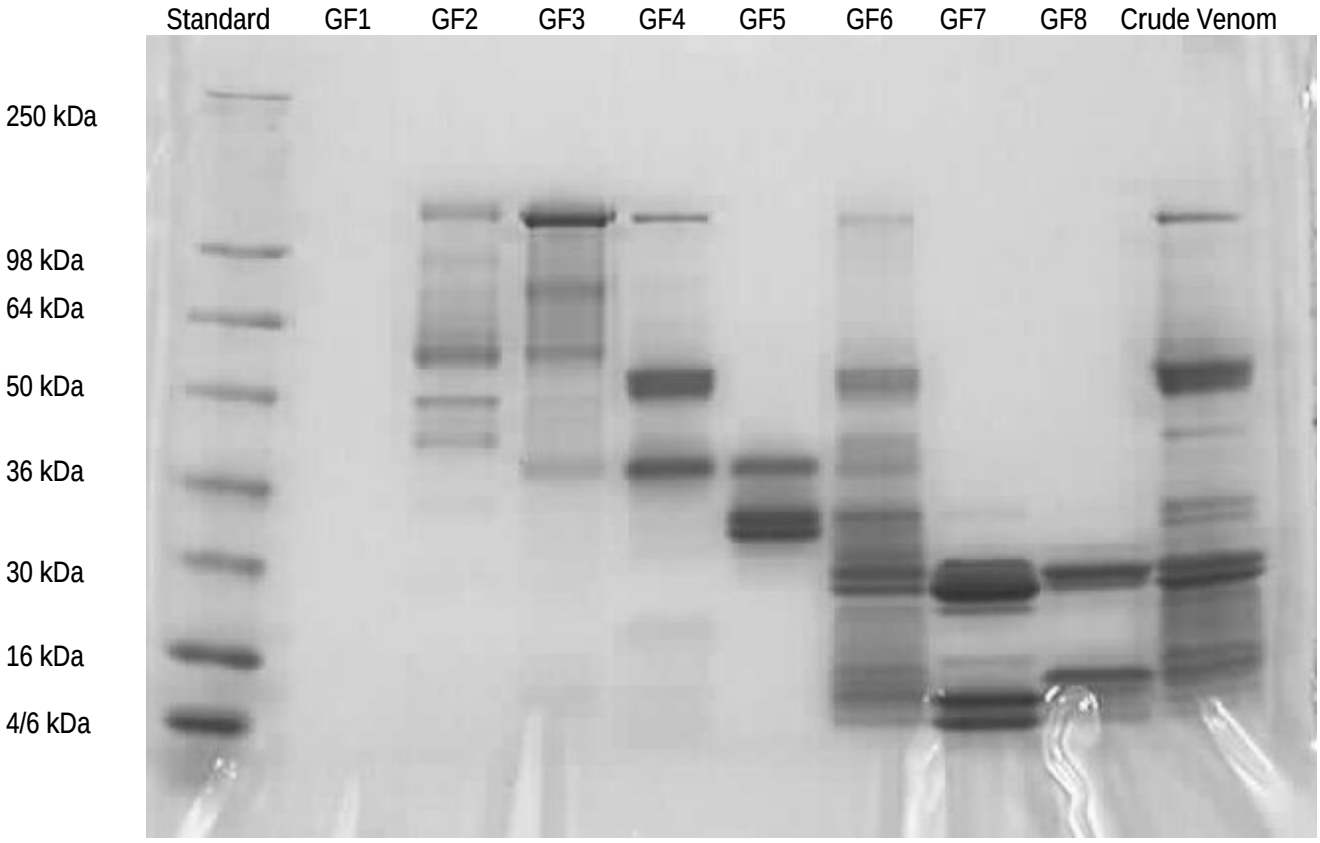


**Figure 3.** *B.moojeni* GF fractions 1-8, SDS-PAGE analytical scale, batroxobin is present in GF 5.

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 clot-inducing properties of the compound contained in this fraction were compared with those of ecarin in plasma containing unfractionated heparin (UFH). The experiments showed that both substances are insensitive to the presence of UFH in plasma and do not need any additional cofactor to be enzymatically active (table1).

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

| **Prothrombin Activator** | **Clotting Time in sec** | | | |
|---|---|---|---|---|
| | **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 |

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-amino acid oxidase fragments, 1 peptide with homology with the N-terminal part of a small myotoxin, different $PLA_2$ fragments and small peptides. Also 6 bradykinin potentiating peptides could be characterized (data not shown).

## 3.5 Summary

All gel filtration 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 and structures of identified compounds 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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# 4 Screening of Acetylcholinesterase Inhibitors in Snake Venom by Electrospray Mass Spectrometry

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## 4.1 Abstract

An electrospray ionization mass spectrometry (ESI-MS)-based assay for the determination of acetylcholinesterase (AChE)-inhibiting activity in snake venom was developed. It allows the direct monitoring of the natural AChE-substrate acetylcholine and the respective product choline. The assay scheme was employed in the screening for neurotoxic activity in fractions of the venom of Bothrops moojeni. AChE inhibition was assessed in two fractions. As a positive control, the established AChE-inhibitor 1,5-Bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide (BW284c51) was used, a dose-response-curve for this compound was generated and the $IC_{50}$-value for the inhibitor was determined to be 1.60 ± 0.09 $*10^{-9}$ mol $L^{-1}$. The dose-response-curve was used as “calibration function" for the venom inhibition activity resulting in equivalent BW284c51-equivalent concentrations of $1.76*10^{-9}$ mol $L^{-1}$ and $1.07*10^{-9}$ mol $L^{-1}$ for the two fractions containing activity. The ESI-MS-based assay scheme was validated using the established Ellman reaction. The data obtained using both methods were found to be in good agreement. The ESI-MS-based assay scheme is therefore an attractive alternative to the standard colorimetric assay.

## 4.2 Introduction

The catalytic activity of cholinesterases in the degradation of acetylcholine (AC, figure 1a) to choline is of central importance in the treatment of Alzheimer's disease (AD).

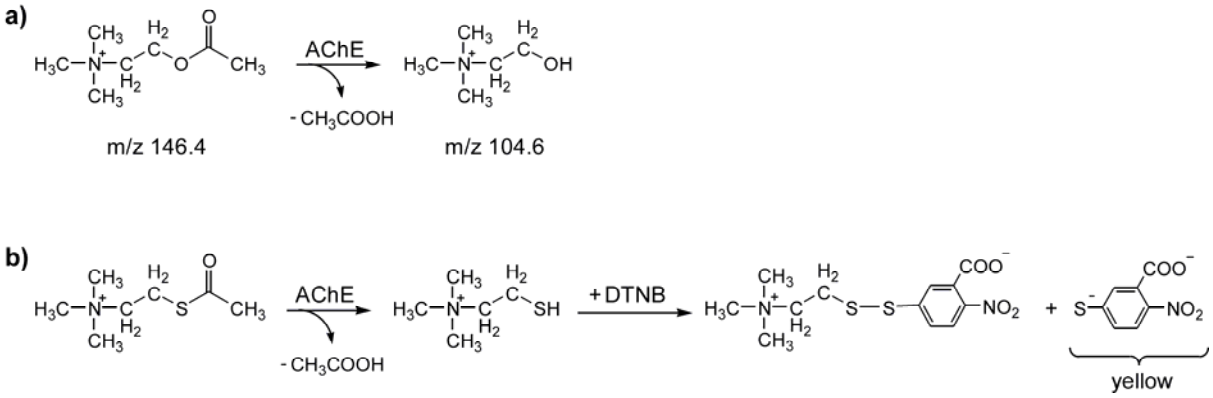


**Figure 1.** Reaction schemes underlying the assays for the determination of AChE activity; a) AChE-catalyzed conversion of acetylcholine to choline; detection by means of ESI-MS on the respective signal traces of m/z 146.4 (AC) and 104.6 (choline); b) AChE-catalyzed conversion of acetylthiocholine to thiocholine; interception of the thiol by DTNB releases the colored anion, which can be colorimetrically detected.

As a general symptom of this disease, reduced cholinergic neurotransmission is observed in the brain of AD patients [1, 2]. This deficiency seems to be caused by either reduced activity of choline transferase or by enhanced activity of acetylcholinesterase (AChE) resulting in lower levels of acetylcholine [3 – 5]. Therefore, the predominantly used strategy in the treatment of AD patients is aimed at the potentiation of AC in the brain by lowering its degradation rate. This is achieved by administering potent inhibitors of AChE, such as tacrine, donezepil, galantamine and rivastigmine. The most significant therapeutic effect is the stabilization or even improvement of the cognitive functions of the patient for at least one year's time [6 – 10]. Since the use of AChE-inhibitors is up to now the most effective therapeutic approach in AD treatment, the demand for alternative inhibitors is high.

Identification of inhibiting activity is performed by assessing the enzymatic activity in presence of the potential inhibitor. The most widely used method for the determination of AChE activity is a colorimetric assay scheme developed by Ellman *et al.* in 1961 [11]. Since AC and its product choline do not possess any characteristic spectroscopic properties, an alternative substrate has to be used. To allow the detection by means of light absorption, a secondary reaction is coupled to the enzyme-catalyzed conversion. Acetylthiocholine (ATC) serves as substrate for AChE and is hydrolyzed yielding the respective thiol compound. This reactive thiol is intercepted by 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) under formation of a disulfide bond with thiocholine and the yellow $TNB^{2-}$ anion is released (figure 1b). The formation of this compound can be monitored by measuring the absorption of the reaction mixture at a wavelength of 412 nm [11].

Mass spectrometry (MS) is a possible alternative to this colorimetric assay scheme. As in MS, analytes are detected depending on their mass-to-charge (m/z) ratio, the spectroscopic properties of the substrate or product compounds are not important. Therefore, MS detection allows the use of natural occurring substrate compounds without any modifications to the molecular structure or the need of coupling a secondary reaction for detection. In case of the determination of AChE activity, the natural substrate acetylcholine and its product choline are detected at their respective m/z ratios.

The concept of employing electrospray ionization MS (ESI-MS) as means of detection in enzymatic bioassays was introduced by the group of Henion [12, 13]. A first approach to identify potential inhibitors and determine their IC50 values by means of ESI-MS was presented by Wu *et al.* in 1997 [14]. Thomas *et al.* and Lewis *et al.* developed a MS assay scheme based on matrix assisted laser desorption/ionization from porous silicon surfaces (DIOS) for the monitoring of the AChE-catalyzed hydrolysis of AC. Using the DIOS-MS assay scheme, they also investigated the inhibition of AChE by several known inhibitors [15, 16]. Wall and co-workers employed both, a DIOS-MS and an ESI-MS assay scheme for the determination of kinetic parameters of the AC hydrolysis mediated by AChE and studied also the inhibition of this reaction by tacrine. The values obtained from both methods were found to be in good agreement [17]. Shen and colleagues presented a high-throughput approach based on the DIOS-MS system for the screening of potential AChE-inhibitors from a compound library reporting a throughput of 4000

samples in a five-hour measurement period [18]. An alternative high-throughput screening approach based on mass spectrometry was presented by Ozbal *et al.* [19]. All of these investigations on potential AChE inhibitors by means of MS were only conducted with pure compounds and not with natural extracts. Recently Irth and coworkers described the HPLC-MS-based online Assay for screening of AChE inhibitors in a crude extract of Narcissus c.v. “Bridal Crown”. After separation of potential inhibitors on a reversed-phase LC-System, first the enzyme AChE and subsequently, after a reaction time of three minutes, the substrate AC was added. After a reaction time of one minute, substrate and product were detected by ESI-MS. Despite the complicated setup with two post-column reaction coils, active inhibitors were successfully detected in the plant extracts [20].

One of the most promising sources for potent AChE inhibitors are snake venoms. Besides the effects on the haemostatical system, blood coagulation and fibrinolysis, also strong neurotoxic effects, resulting in the immobilization and finally death of the prey, have been reported [21 – 23]. One possible mode of venom action is the selective inhibition of mammalian AChE by, for instance, fasciculins [24].

Recently, an ESI-MS based method for proteolytic activity screening was developed in our laboratory [25 – 26]. As this method is robust and easy to use with any common ESI-MS instrumentation, we applied this concept to screening of snake venom fractions for the presence of AChE inhibitors. The assay scheme was employed in the inhibition screening of the venom of Bothrops moojeni. The results from the ESI-MS assay were validated with a colorimetric assay based on the Ellman scheme.

## 4.3 Experimental

### *Chemicals*

The snake venom used in this study was provided by Pentapharm (Basel/CH) and was fractionated by size exclusion chromatography (SEC) as described by Perchuc *et al.* [27]. Acetylcholinesterase (235 U/mg) from electric eel (AchE), acetylcholine bromide (AC), acetylthiocholine iodide (ATC), 1,5-Bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide (BW284c51) and 5,5’-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased in the highest purity available from Sigma-Aldrich Chemie (Zwijndrecht/NL). The p.a. grade ammonium acetate was

obtained from Merck (Darmstadt/D). All solvents were purchased in LC-MS grade quality from Biosolve (Valkenswaard/NL).

***Sample preparation***

All solutions were prepared in buffer (15 mM ammonium acetate/ammonia, pH 7.5 in water) and stored at –18 °C until use. The AC bromide and ATC iodide were dissolved in buffer yielding substrate solutions of $1^{*}10^{-4}$ mol $L^{-1}$ each. The DTNB was dissolved in buffer to give a $1^{*}10^{-3}$ mol $L^{-1}$ solution. The inhibitor BW284c51 was dissolved to give a stock solution with a concentration of $1^{*}10^{-4}$ mol $L^{-1}$, which was diluted to yield solutions of $3^{*}10^{-5}$ mol $L^{-1}$, $1^{*}10^{-5}$ mol $L^{-1}$, $3^{*}10^{-6}$ mol $L^{-1}$, $1^{*}10^{-6}$ mol $L^{-1}$, $3^{*}10^{-7}$ mol $L^{-1}$, $1^{*}10^{-7}$ mol $L^{-1}$, $3^{*}10^{-8}$ mol $L^{-1}$, $1^{*}10^{-8}$ mol $L^{-1}$ and $1^{*}10^{-9}$ mol $L^{-1}$, respectively. AChE was dissolved in buffer to yield a solution with a content of 5 mg $L^{-1}$, unless stated otherwise.

The venom of Bothrops moojeni was fractionated by preparative gel permeation chromatography yielding 19 fractions with venom components separated by their molecular masses in solutions. The gel fractions (F28 – F46) were frozen and kept at –18 °C until use

***Instrumental set-up of the FIA/ESI-MS system***

For the FIA experiments, a flow injection system comprising a binary gradient HPLC pump HP1100 model GF1312A and an autosampler HP1100 model G1313A (both Agilent, Waldbronn/D) was connected to the mass spectrometric detector. The carrier stream was set to 0.3 mL $min^{-1}$ of 75/25 (v/v) mixture of methanol and water.

***Mass spectrometric detection***

For detection, an Esquire $3000^{+}$ ion trap mass spectrometer (Bruker Daltonik, Bremen/D) equipped with an ESI source was used. All measurements were performed using the positive ion MS mode. Mass spectra were recorded over a range from m/z 100 to m/z 1000 in full scan mode. Ionization of the analytes was achieved in the ESI interface with 40 psi nebulizer gas, 10 L dry gas/min of 365 °C and -5000 V on the capillary inlet. The resulting data was analyzed using DataAnalysis software version 3.1 (Bruker Daltonik, Bremen/D).

***ESI-MS assay procedure***

All ESI-MS-based inhibition assays were performed according to the following general procedure. AChE (50 µL) and 50 µL of the respective venom fraction solution

were combined with 300 µL buffer and pre-incubated at RT for 10 minutes. The reaction was started by adding 400 µL of the substrate AC solution to the mixture. Aliquots of 50 µL were sampled from the reaction mixture every 2 minutes for a complete time span of 20 minutes. The aliquots were quenched in 950 µL methanol to stop effectively the conversion by rapid denaturing of the catalytic enzyme. The quenched samples were subsequently injected into the FIA/ESI-MS system and analyzed in triplicate. The injection volume was set to 5 µL.

For the reference assays without inhibition, 50 µL of buffer were added to the enzyme instead of the venom fraction solution. In case of the BW284c51 assays, 50 µL of the inhibitor solution were pre-incubated with the enzyme. Due to the dilution steps in the assay scheme, the effective concentration of BW284c51 ranged from $6.25^{*}10^{-7}$ mol $L^{-1}$ to $6.25^{*}10^{-11}$ mol $L^{-1}$.

***UV/vis assay procedure***

In order to allow the direct comparison between ESI-MS assays and UV/vis assays, the reaction conditions and ratios of reagents had to be similar. Therefore, the colorimetric inhibition assays were carried out according to the following scheme. 300 µL of the AchE, 300 µL of venom fraction solution and 160 µL of the DTNB solution were mixed, diluted with 1640 µL buffer and pre-incubated at RT for 10 minutes in order to allow the DTNB to react with possibly present thiol functions in the protein content of this mixture. 100 µL of this mixture was transferred to the wells of a 96-well microtitation plate. The reaction was started by adding 100 µL of the substrate ATC solution to each well. The reaction progress was monitored by measuring the increase of absorption at 412 nm wavelengths on a SpectraMax 250 plate reader (Molecular Devices, Munich/D).

For the reference assays without inhibition, 50 µL of buffer were added to the enzyme instead of the venom fraction solution. In case of the BW284c51 assays, 50 µL of the inhibitor solution were pre-incubated with the enzyme. Due to the dilution steps in the assay scheme, the effective concentration of BW284c51 ranged from $6.25^{*}10^{-6}$ mol $L^{-1}$ to $6.25^{*}10^{-11}$ mol $L^{-1}$.

## 4.4 Results and Discussion

For the investigation of inhibitor activity towards AChE in the venom of Bothrops moojeni, the venom fractions are assayed individually against AChE and the natural substrate AC. In the course of the reaction, AC is hydrolyzed by AChE to choline and acetic acid (figure 1a). This is monitored by ESI-MS. Typical mass spectra obtained from quenched samples at the beginning of the reaction and at the end are shown in figure 2.

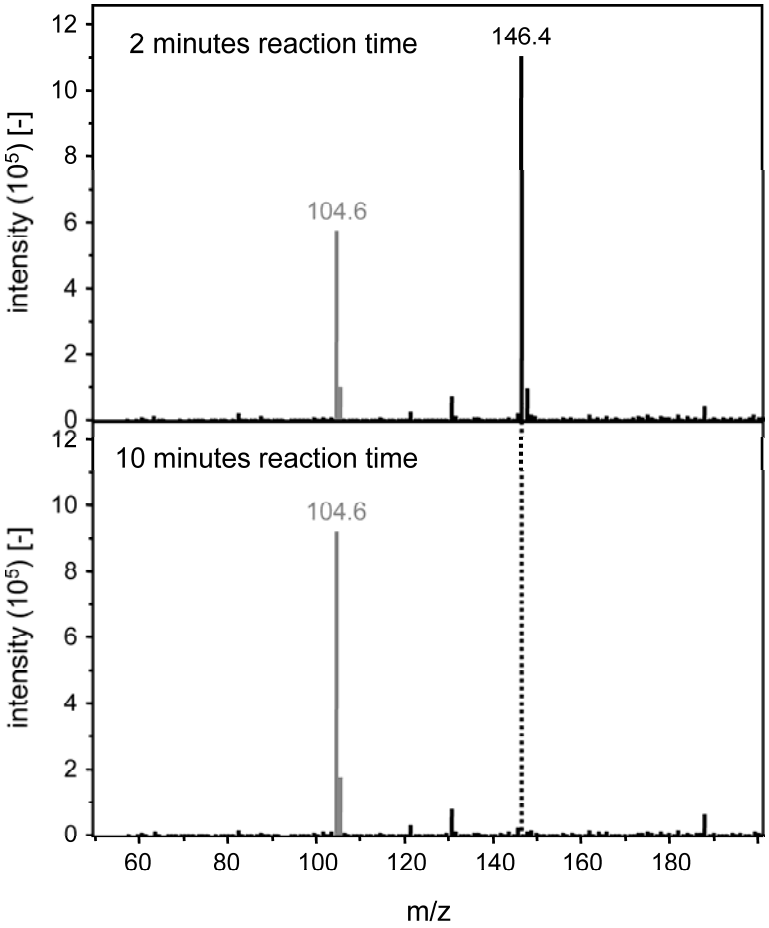


**Figure 2.** Mass spectra obtained from samples of the reaction mixture after 2 minutes and after 10 minutes reaction time. The mass spectrum after 2 minutes reaction time shows signals for both, the substrate compound AC (m/z 146.4) and the product choline (mz 104.6). After 10 minutes reaction time, only the signal for choline is observed, while the substrate signal is completely decreased (position of AC signal indicated by the dashed line).

After 2 minutes reaction time, signals for both substrate and product are observed. After a reaction time of 10 minutes, the signal for AC is not longer perceptible and the

product signal increased significantly. No additional signals appear in the mass spectra, not even at longer reaction times (monitored until 30 min). Therefore, it can be concluded that the conversion of the substrate is completed.

Subsequent injections of the quenched samples allow to monitor the progress of the enzyme-catalyzed hydrolysis (figure 3).

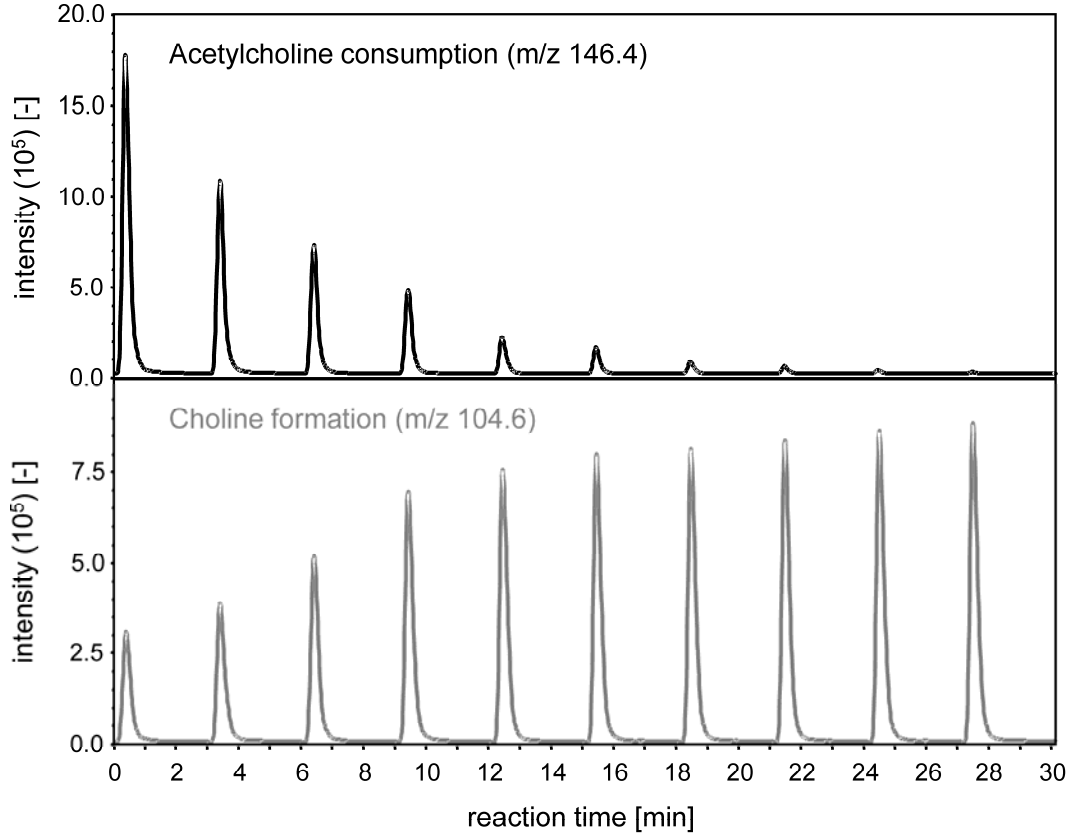


**Figure 3.** Injection profile of enzymatic conversion by AChE (2 mg $L^{-1}$) recorded on the signal traces of substrate and product showing clearly the decrease in AC concentration and the increase of choline concentration in the reaction mixture over the course of time.

Figure 3 shows the signal traces for the substrate and the product. The consumption of the AC and the formation of choline is directly observed by the decrease and increase of the peaks on the respective m/z-traces. Integration of the peak areas gives a measure of the concentration the respective compounds present at a given time. Quantification of the analytes in MS is usually achieved by addition of an internal standard. The concept of using the substrate as internal standard for the relative quantification of the product compound was discussed by several authors for

the use in MALDI-MS assay schemes [28 – 30]. Since absolute quantification of the concentrations of the analytes is not necessary to judge the assay performance, the relative reaction progress (RRP) at any sampling time was determined on the basis of this approach. The RRP is calculated from the ratio of product and substrate in the sample. The RRP in percent is expressed as the product peak area (SP) as a fraction of product plus remaining substrate peak areas (SS).

$$RRP(\%) = \frac{S_P}{S_S + S_P} * 100 \quad (1)$$

The value for the RRP ranges from 0% (no product formed) to 100% (complete conversion of the substrate). Plotting the RRP values versus the reaction time for each assay results in a full time-resolved reaction profile (figure 4).

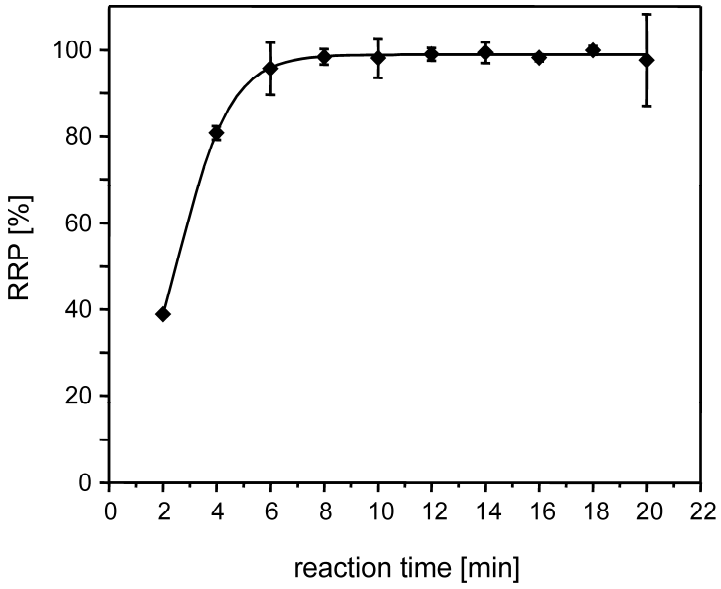


**Figure 4.** Complete reaction profile showing the relative reaction progress at different time points; derived from the ESI-MS assay scheme of the reference assay without any inhibitor present. The relative standard deviation ranges from 1.3% to 10.9% (n = 3).

The profile depicted was derived from the reference assay without any inhibitor present. The profile shows a typical course of an enzyme-catalyzed reaction with a high initial reaction rate. The conversion of the substrate seems to be completed after 8 minutes reaction time.

In order to assess inhibitor activity in the snake venom, the RRP profiles derived from the assays in presence of each venom fraction were compared to the reference profile. This can be investigated closer using a correlation plot (figure 5a).

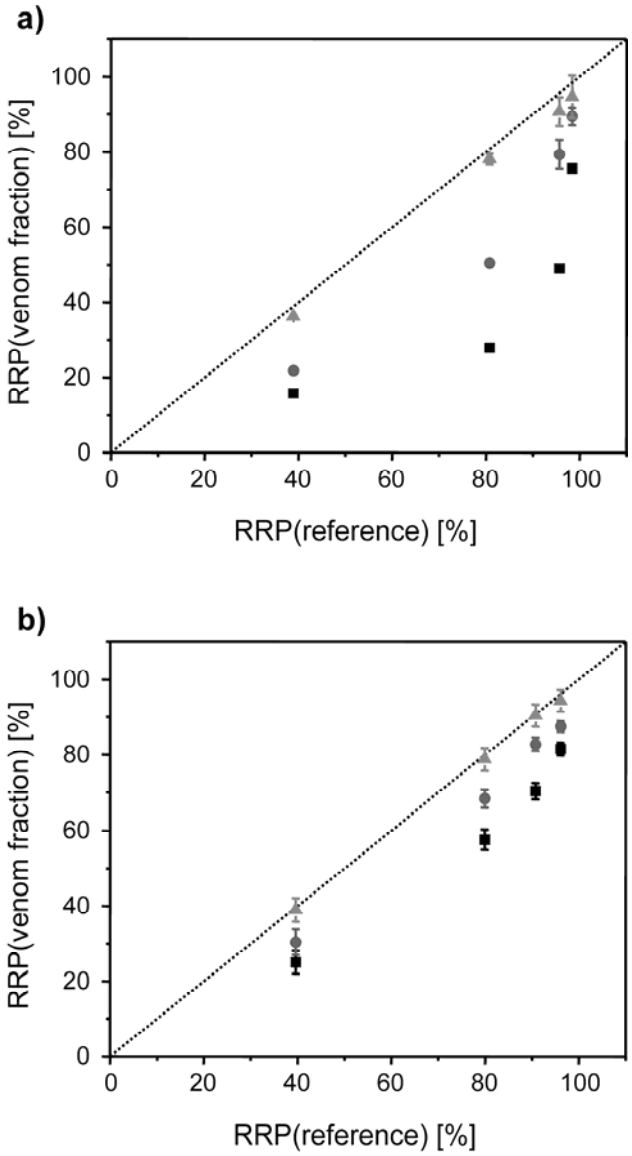


**Figure 5.** Correlation plots derived from a) the ESI-MS assays and b) from the colorimetric assays. The relative reaction progress at the first 8 minutes reaction time in the presence of the venom fractions F 34 ( ■), F 33 (●) and F 29 (▲) is plotted versus the relative reaction progress of the reference assays. The dashed line indicates the position for complete correlation of reference and screening assays.

In this figure, the RRP values of the reactions in presence of the venom fraction F 33 and F 34 are plotted versus the RRP values of the reference assay. Close correlation

(no change in catalytic activity of AChE) is indicated by points close to the dotted line. The correlation plots of the assays in presence of F 33 and F 34 show significant divergences from the line, thus indicating a loss in enzymatic activity in the respective assays. This leads to the conclusion, that in those fractions an inhibitor for AChE must be present. The correlation plots of the assays in presence of the other venom fractions (shown as example for F 29) did not exhibit any significant divergences. Therefore, no inhibitory activity is assigned to be present in those fractions.

To verify this finding, control assays following a modified Ellman procedure were performed. The relative reaction progress in percent at a given time point in the absorption assay was calculated from the ratio of absorbance to maximal absorbance (after completion of the conversion). Plotting the RRP values of the individual assays versus the values obtained from the reference assay results in a correlation plot similar to the one derived from the MS assays (figure 5b).

As assessed from the ESI-MS-based assays, a significant divergence from the reference assay is observed indicating the slower reaction progress in the presence of F 33 and F 34. Also, no significant divergences could be observed in the correlation of the other fractions assays. This validates the conclusion drawn from the ESI-MS assays that only in fractions F 33 and F 34, an AChE-inhibiting compound is present.

In order to further characterize the inhibition potential, the inhibitory activity found in the fractions was related to that of the known inhibitor BW284C51. Therefore, dose-response profiles for the inhibition of AChE by BW284C51 were generated employing both, the ESI-MS- and the UV/vis-absorption-based assay schemes. Assays were performed in the presence of varying concentrations of inhibitor and the respective RRP values were calculated as described above. The RRP values of the inhibition assays after 6 minutes reaction time were related to the RRP value found in the reference assay at the same time point (100% assay performance) and plotted versus the concentration of BW284C51 (figure 6).

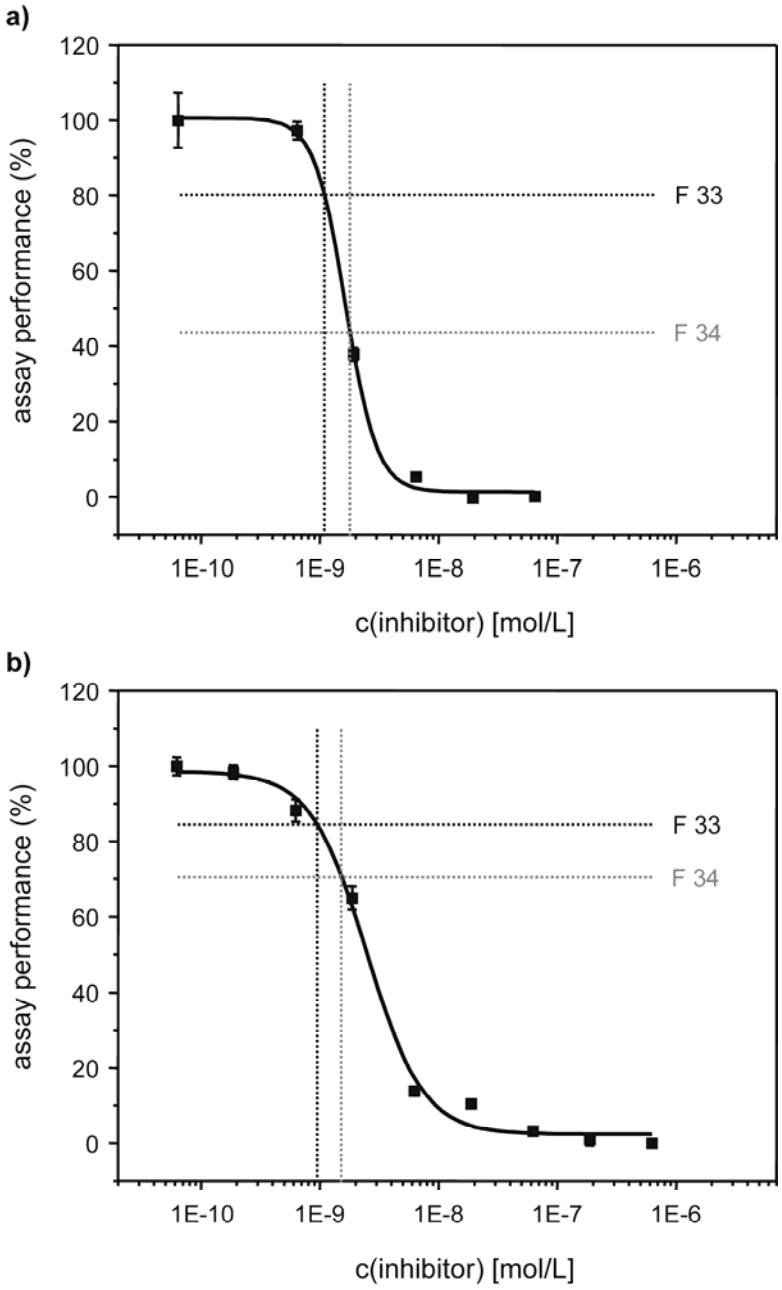


**Figure 6.** Dose-response plots for the AChE inhibitor BW284C51 derived from a) ESI-MS assays and b) colorimetric assays; the respective assay performance in the presence of F 33 and F 34 are indicated by the horizontal dashed lines; the equivalent concentrations of BW284C51 are given by the intersections with the response curve.

From these plots, the $IC_{50}$-values of BW284C51 towards AChE were determined to be 1.60 ± 0.09 $^{*}10^{-9}$ mol $L^{-1}$ (ESI-MS assay) and 2.48 ± 0.24 $^{*}10^{-9}$ mol $L^{-1}$ (UV/vis-absorbance assay), respectively.

The dose-response profiles from both assay schemes were used to relate the inhibitory activity in the venom fractions F 33 and F 34 to the inhibitory activity of BW284C51. Therefore, the assay performance of the respective reactions after 6 minutes reaction time was determined and the corresponding theoretical concentrations of BW284C51 were calculated (table 1).

**Table 1.** Equivalent concentrations of BW284C51 calculated for the inhibition activities found in F 33 and F 34.

| Venom Fraction | **Equivalent Concentration of BW284C51** | |
|---|---|---|
| | ESI-MS | UV/vis-absorption |
| F 34 | $1.76^{*}10^{-9}$ mol $L^{-1}$ | $1.42^{*}10^{-9}$ mol $L^{-1}$ |
| F 33 | $1.07^{*}10^{-9}$ mol $L^{-1}$ | $0.89^{*}10^{-9}$ mol $L^{-1}$ |

Considering the fact that two different assay schemes were employed, one based on the direct detection of product and substrate and the other employing a secondary reaction for the generation of a detectable product, the values calculated for the $IC_{50}$ of BW284C51 and for the equivalent concentrations for the venom fractions correlate well.

## 4.5 Conclusion

A robust ESI-MS-based assay was developed for the rapid screening for AChE-inhibitory activities in complex biological samples. The assay was applied successfully to the inhibition screening of snake venom. The inhibitory activity found in the venom fractions was quantified in relation to the known AChE inhibitor BW284C51 using its dose-response plots as calibration curve, thus giving an independent measure for comparison of inhibition activity in different fractions. All findings resulting from the ESI-MS assay scheme were controlled by colorimetric assays, thus validating the ESI-MS methodology. The results were found to be in good agreement with each other. ESI-MS is therefore an excellent alternative detection method for the monitoring of enzyme-catalyzed assays. The major advantage over the optical method is the possibility to monitor the naturally occurring substrate AC and its product choline, which are not detectable by any optical means.

The ESI-MS assay scheme proved to be generally applicable not only to the investigation of pure substances, but also for highly complex biological samples. Since ESI-MS assays offer the opportunity to monitor several enzymatic conversions in parallel, further work will be directed to the development of a multiplexing inhibition assay scheme

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# 5 High throughput screening of pyroglutamyl and bradykinin-potentiating peptides in *Bothrops moojeni* snake venom using precursor ion mass spectrometry

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## 5.1 Abstract

Snake venoms are known to be an extensive source of bioactive peptides. Bradykinin potentiating peptides (BPPs) are inhibitors of the angiotensin-converting enzyme that have already been identified in the venom of many snake, scorpion, spider, and batrachian species. Their most characteristic structural features are an invariable N-terminal pyroglutamate residue (pGlu or Z) and two consecutive proline residues at the C-terminus. Fragmentation of BPPs by collision-induced dissociation during electrospray tandem mass spectrometry analysis (ESI-MS/MS) generates a predominant signal at *m/z* 213.1 corresponding to the *y*-ion of the terminal Pro-Pro fragment. In addition, signals at *m/z* 226.1 and 240.1 that correspond to the *b* ions of the N-terminus pGlu-Asn and pGlu-Lys, respectively, can often be observed. Based on these structural determinants, the present work describes an original methodology for the discovery of BPPs in natural extracts using liquid chromatography coupled to ESI-MS/MS operated in precursor ion-scan mode. The venom of the *Bothrops moojeni* snake was used as a model and the methodology was applied for subsequent structural analysis of the identified precursors by tandem mass spectrometry on quadrupole-time-of-flight (Q-TOF) and matrix-assisted laser-desorption/ionization time-of-flight tandem mass spectrometry (MALDI-TOF MS/MS) instruments. More than 40 peptides below 2500 Da could be detected, among them 20 were shown to belong to the BPP-like family including the related tripeptides pGlu-Lys-Trp and pGlu-Asn-Trp. A total of 15 new sequences have been identified using this approach.

**Abbreviations:** ACE angiotensin converting enzyme; ACN acetonitrile; BPPs bradykinin potentiating peptides; CID collision-induced dissociation; ESI-MS electrospray ionization mass spectrometry; FA formic acid; HPLC high performance liquid chromatography; MALDI-TOF matrix-assisted laser desorption/Ionization time-of-flight; MS mass spectrometry; pGlu pyroglutamate; Q-TOF quadrupole-time-of-flight; SPE solid phase extraction; SVMPs snake venom metalloproteases; TFA trifluoroacetic acid; TIC total ion chromatogram

## 5.2 Introduction

Snake venoms constitute an incredible source of active peptides among which the small endogenous pyroglutamyl (pGlu) peptides represent an important part (Francis and Kaiser, 1993). The most studied of these peptides are BPPs, proline-rich linear peptides generally composed of less than 14 amino acid residues. They contain an invariable pGlu at their N-terminus, and most of them display a characteristic dipeptidic sequence Pro-Pro at the C-terminus. BPPs first discovered in 1965 (Ferreira and Rocha e Silva, 1965) served as prototypes to design clinically useful ACE inhibitor drugs such as Captopril and analogues thereof that are currently used to treat hypertension in humans (Ondetti *et al.*, 1977; Cushman and Ondetti, 1991) BPPs are known to inhibit the angiotensin-converting enzyme (ACE), which is responsible for the conversion of inactive angiotensin I to the potent vasoconstrictor angiotensin II as well as for bradykinin degradation. Therefore, BPPs prevent the hypertensive effect of the angiotensin II and potentiate the hypotensive effect of the circulating bradykinin. It is interesting to point out that not all BPPs are good inhibitors of ACE (Hayashi and Camargo, 2005). Structure-function relationship studies performed on BPPs from the rattlesnake *Crotalus dursissus terrificus* have shown that the presence of a pyroglutamic acid and a proline residue in each peptide extremity was not sufficient to ensure their characteristic biological activity (Gomes *et al.*, 2007). This further suggests that BPPs could be a broader family of enzyme inhibitors as originally thought.

BPPs have been found in the venom of many snake species from the *Bothrops* (Cintra *et al.*, 1990; Murayama *et al.*, 1997; Ferreira *et al.*, 1998; Hayashi *et al.*, 2003; Ianzer *et al.*, 2004; Hayashi and Camargo, 2005), *Agkistrodon* (Kato and Suzuki, 1970; Tominaga and Stewart, 1975; Chi *et al.*, 1985; Yanoshita *et al.*, 1999; Murayama *et al.*, 2000), *Bitis* (Francischetti *et al.*, 2004; Calvete *et al.*, 2007), *Crotalus* (Graham *et al.*, 2005), *Lachesis* (Soares *et al.*, 2005), *Naja* (El-Saadani and El-Sayed, 2003), *Trimeresurus* (Higuchi *et al.*, 1999; Jia *et al.*, 2003) and *Vipera* genera (Komori and Sugihara, 1990). In addition, BPPs were described from scorpion (Ferreira *et al.*, 1993; Meki *et al.*, 1995; Zeng *et al.*, 2000) and spider venoms (Ferreira *et al.*, 1996) as well as from skin secretions of the tree-frog *Phyllomedusa hypochondrialis* (Conceicao *et al.*, 2006).

The BPP structural family also includes small pyroglutamyl tripeptides which are present at high concentrations (in the mM range) in crotalinae and viperinae venoms and also act as protease inhibitors. pGlu-Asn-Trp and pGlu-Gln-Trp were originally described from *Agkistrodon halys blomhoffi, Crotalus adamanteus, Bothrops jararaca* and *Trimeresurus flavoviridis* venoms (Kato *et al.*, 1966). They were shown to display selective binding properties to snake venom metalloproteases (SVMPs), being competitive but weak inhibitors of their proteolytic activities (Robeva *et al.*, 1991; Francis and Kaiser, 1993). In particular, the pGlu-Lys-Trp and pGlu-Asn-Trp peptides described from many rattlesnakes from the *Crotalus* and *Sistrurus* genera were shown to inhibit SVMPs, thus avoiding autolysis before envenomation and contributing to the natural protein stabilization of the venom (Munekiyo and Mackessy, 2005). Tripeptides have also been described from the venom of the Taiwan habu snake (*Trimeresurus mucrosquamatus*) and the high-resolution crystal structure was obtained of the SVMP (TM-3) cocrystallized with the endogenous inhibitors pGlu-Asn-Trp, pGlu-Gln-Trp and pGlu-Lys-Trp (Huang *et al.*, 1998; Huang *et al.*, 2002).

pGlu peptides have been shown to be most likely released from the large BPPs polycistronic and C-type natriuretic peptide (CNP) cDNA precursor in *Bothrops jararaca* (Murayama *et al.*, 1997). The N-terminal pGlu formation was shown to be catalyzed by the glutaminyl cyclase in several colubrinae snake venoms (Pawlak and Manjunatha, 2006).

Developing a rapid tool for characterization of pGlu peptides in venoms can be of importance for the investigation of new pharmacological lead compounds. Indeed the knowledge of the structural diversity of the BPPs family is essential for understanding the structure-function relationship. Matrix-assisted laser-desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) was shown to be a fast and efficient method for preliminary studies of crude venom mixtures (Wermelinger *et al.*, 2005). Single-step solid-phase extraction (SPE) combined with MS and MS/MS analysis of crude venoms of *Bothrops* and *Crotalus* snakes was developed to compare their BPP content and novel BPP-related peptides were thus found in six out of eight snake species. More recently, individual variability of *B. jararaca* venom peptide content was studied using LC-MS and MALDI-TOF-MS techniques, showing sex-based differences among the BPPs (Pimenta *et al.*, 2007).

The goal of this study was to develop a new and original approach for the screening of pGlu BPP-related compounds in crude snake venoms, and the lance-headed *Bothrops moojeni* venom was chosen for this purpose. *Bothrops* snakes, comprising about 37 species, are responsible for more human deaths in America than any other group of venomous snakes. *B. moojeni* is widely distributed in South and Central America and clinical manifestations caused by such viper bites go from dry-bites to complications such as abscess and necrosis (Jorge and Ribeiro, 1990; Nishioka and Silveira, 1992; Perchuc *et al.*, 2005). Snake venoms contain a wide variety of factors affecting the blood coagulation system through coagulant, anticoagulant and haemorrhagic activities (Marsh, 1994). In particular, the venom of *B. moojeni* has potent proteolytic and phospholipase $A_2$ ($PLA_2$) activities (Assakura *et al.*, 1985). It contains a thrombin-like enzyme termed batroxobin that specifically splits off fibrinopeptide A and is currently used clinically for the treatment of thrombotic diseases (Stocker *et al.*, 1982; Itoh *et al.*, 1987; Lochnit and Geyer, 1995; Gutierrez and Lomonte, 1997; Markland, 1998; Petretski *et al.*, 2000). Two myotoxic Lys 49 $PLA_2$ (MjTX-I and II) were also characterized from the *B. moojeni* venom (Lomonte *et al.*, 1990; Soares *et al.*, 1998; Soares *et al.*, 2000) and crystallized by X-ray diffraction (Nonato *et al.*, 2001; Watanabe *et al.*, 2003; Marchi-Salvador *et al.*, 2005). These myotoxic Lys 49-$PLA_2$s were shown to possess promising antimicrobial, antitumoral and antiparasitic activities (Stabeli *et al.*, 2006).

Our methodology for the screening of BPPs in crude snake venoms is based on the conserved motifs such as Pro-Pro at the C-terminus and the post-translational N-terminus pGlu modification. In particular, developing a precursor ion-scan tandem mass spectrometry method to selectively detect and analyse BPPs looked attractive and promising in order to decrease the apparent sample complexity while increasing the sensitivity and selectivity of the analysis. Bourguignon *et al.* (2001) had characterized a few peptides from *B. moojeni* using MALDI-TOF-MS combined with Edman degradation techniques. Among them, a peptide with a molecular mass of 1370.6 Da was submitted to the SwissProt Protein database as a non-pGlu peptide associated with a methylated Lys2 isoform at 1384.7 Da (Accession Number: P84747). In this study, various MS techniques were applied and compared for the detection, identification and characterization of BPP-like components of the *B. moojeni* venom. The approach described in the current work included (i) direct infusion and liquid chromatography coupled to ESI-MS/MS using a precursor ion-

scan method on a triple quadrupole mass spectrometer (ii) electrospray ionisation MS and MS/MS (ESI-MS/MS) analysis by sample infusion on a quadrupole-time-of-flight (Q-TOF) mass spectrometer and (iii) matrix-assisted laser-desorption/ionisation time-of-flight/ time-of-flight mass spectrometry (MALDI LIFT-TOF/TOF MS).

Developing a powerful methodology to detect and identify BPP-like peptides in complex samples such as venoms and secretions could be useful in particular for the identification of endogenous BPPs in mammals in which such peptides have not been described so far.

## 5.3 Material and methods

### 5.3.1 Venom origin and solid-phase extraction

*B. moojeni* crude venom was provided by the snake farm Pentapharm do Brasil (Uberlandia, Minas Gerais, Brazil). One hundred microliters of crude venom (250 mg/mL) were submitted to solid-phase extraction using a Sep-Pak C18 cartridge (Waters, Milford, MA, USA). Prior to loading, the venom was acidified by a 1:5 dilution in aqueous 0.1 % trifluoroacetic acid (TFA). The cartridge was first conditioned with methanol and then flushed with aqueous 0.1 % TFA. Elution was performed with a mobile phase of 70:30 aqueous TFA (0.1 %): acetonitrile (ACN) and the eluate was freeze-dried in a SpeedVac concentrator (ThermoSavant, Holbrook, NY, USA) and stored at -20 °C. The dried samples were suspended in 500 □L of aqueous 0.1% formic acid (FA) for further analysis.

### 5.3.2 On-line LC-ESI-MS

LC-MS analyses were performed on a Micromass Quattro micro triple quadrupole mass spectrometer (Waters, Milford, USA) interfaced to an Alliance HT 2795 HPLC system (Waters). The sample (15 μL) was diluted (1:5) in aqueous 0.1% FA prior to loading on a 2.1 mm I.D. Atlantis C18 column (Waters). The fractionation was performed using a gradient of aqueous 0.1 % FA (solvent A) and a mobile phase of 10:90 aqueous 0.1 % FA: ACN (solvent B). The slope of the gradient was 0.5 % B per minute and the flow rate 0.2 mL/min. A post-column split allowed the introduction of 15 % of the effluent into the mass spectrometer. The mass spectrometer was operated in positive ionisation mode either in the full-scan or in the precursor ion-

scan mode, as described hereafter. Data were acquired and processed using MassLynx version 4.0 software.

### 5.3.3 Mass spectrometry

#### Detection of BPPs using parent scan mode analysis

ESI-MS analyses were performed on a Micromass Quattro micro triple quadrupole mass spectrometer (Waters). The electrospray ionisation source was operated in the positive ion mode, and experimental parameters were set as follows: capillary voltage 3.0 kV, source temperature 80°C, desolvation temperature 120°C, sample cone voltage 15 V. Data were processed using MassLynx version 4.0 software (Waters). The MS/MS parameters were optimized using a 5 pmoles/μL solution of a synthetic BPP (1347.5 Da) obtained from BIOSYNTAN GmbH (Berlin, Germany). The desalted venom sample was diluted 10 times in the ESI solvent ($H_2O$/ACN/HCOOH, 49.8/50.0/0.2 (v/v/v)) and infused in the mass spectrometer at 10 μL/min flow rate. The product ion mode analysis performed on the main mass-to-charge ratio (*m/z*) signals of the spectrum produced a major *m/z* 213.1 fragment corresponding to the protonated *y*-ion of the C-terminus Pro-Pro moiety. MS/MS parameters were fine-tuned to maximize the intensity of this *m/z* 213.1 fragment, as well as to optimize the resolution and sensitivity of both quadrupole analysers. Precursor ion-scan analysis was performed using a collision energy ramp from 20 to 40 V. In addition to the *m/z* 213.1 fragment, the product ion mode analysis produced minor *m/z* 226.1 and 240.1 ions corresponding to the N-terminal *b* fragments of the dipeptides pGlu-Asp (ZN) and pGlu-Lys (ZK), respectively. LC-MS analysis was performed by scanning from *m/z* 300 to 1500 using a scan time of 4 s applied to the whole chromatographic process. In addition, 3 precursor ion-scan *m/z* 213.1, 226.1 and 240.1 functions with a scanning range of each *m/z* 300-900 in 2 s were programmed in the MS method. For the data analysis, the functions were combined and the new total ion current (TIC) chromatogram obtained was analyzed for its content in precursor ions. External calibration of the mass scale was performed with horse heart myoglobin for MS1 (Sigma-Aldrich, St. Louis, MO, USA) and a sodium iodide (Fluka) and caesium iodide (Merck) mixture at a concentration of respectively 2 and 0.05 μg/μL in 50:50 propan-2-ol: water (Fisher Scientific). The masses above 1000 Da measured on the Quattro micro mass spectrometer are given as average masses.

## De novo MS/MS sequencing

ESI-MS and ESI-MS/MS experiments were conducted on a Q-TOF micro mass spectrometer (Waters, Micromass) operated with the standard ESI source and in the positive ionisation mode. The *B. moojeni* venom prepared by SPE was diluted 1:10 in the ESI solvent and introduced into the mass spectrometer by infusion at a flow rate of 5 μL/min. Single MS analyses were followed by MS/MS experiments on the selected precursor ions. The collision energy was manually adjusted for proper fragmentation. The multiple-charged spectrum was deconvoluted into a single-charged axis using the MaxEnt3 routine from MassLynx 4.0 to allow *de novo* sequence analysis. External calibration was carried out with Glu-fibrinopeptide-b. The masses measured on the Q-TOF micro mass spectrometer and reported here are all monoisotopic masses.

## MALDI-TOF-MS and MS/MS analysis

MALDI-TOF-TOF analysis of the pre-treated venom sample was carried out on an Ultraflex TOF-TOF mass spectrometer operated in positive reflectron mode (Bruker, Bremen, Germany). The sample was deposited on a 384 AnchorChip 600 plate using the affinity preparation method. Briefly, the plate was pre-coated with alpha-cyano-4-hydroxycinnamic acid (CHCA) (0.8 mg/mL in acetone/aqueous 0.1% TFA (97/3)) and 0.5 μL of the venom sample was deposited at 3 different dilutions 1:10, 1:100 and 1:1000. After 3 minutes, a washing step using 2 μL of aqueous 0.1% TFA was performed, followed by re-crystallization by addition of 0.25 μL of CHCA (0.5 mg/mL in ethanol/acetone/aqueous 0.1% TFA (60/30/10)). External calibration was carried out with a mixture of 9 peptides in the 700-3500 Da mass range. The masses reported from MALDI-TOF-MS analysis are monoisotopic masses. MALDI-TOF-TOF experiments were conducted using the LIFT technology and data interpretation performed with the help of BioTools 2.2.

## 5.4 Results

### 5.4.1 ESI-MS analysis of pre-treated *Bothrops moojeni* venom

Infusion-conducted ESI-MS analysis of the pre-treated *B. moojeni* venom revealed the presence of major components at *m/z* 1384.8, 1370.7, 1116.6, 541.3, 443.2 and 429.2 Da (not shown). The SPE preparation of the crude venom allowed a complete removal of proteins such as $PLA_2$s and batroxobin. This sample preparation approach was thus well suited for the study. The product ion analysis performed on the *m/z* 558.9, 686.2 and 693.1 signals corresponding to double-charged species produced a major *m/z* 213.1 fragment corresponding to the protonated *y*-ion of a C-terminus Pro-Pro moiety. Subsequently to the optimization of the precursor ion-scan mode on these major precursors of the *m/z* 213.1 ion present in the venom, the precursor ion *m/z* 213.1 scan mode was applied to the venom sample. A total of 12 components were found to contain the Pro-Pro sequence at the C-terminus, thus expected to belong to the BPPs family (Table 1).

**Table 1.** Molecular masses observed in the *B. moojeni* venom by MALDI-TOF-MS as well as direct infusion ESI-MS and LC-ESI-MS analyses. The ESI-MS data were acquired either in full-mass or precursor ion-scan modes. For precursor ion-scan analysis, the 3 ions *m/z* 213.1, 226.1 and 240.1 were selected and the functions combined using the MassLynx 4.0 tool. The molecular masses in bold have been further characterized by ESI-MS/MS.

| Molecular masses (Da) | Fragment ions | Structural features | | MALDI-TOF MS | ESI-MS (Direct infusion) | | LC-ESI-MS (b) | | |
|---|---|---|---|---|---|---|---|---|---|
| | | N-ter | C-ter | | Full-mass scan | Precursor ion scan *m/z* 213.1 | Full-mass scan | Precursor ion scan *m/z* 213.3 / 226.1 / 240.1 | Rt (min) |
| 304.0 | | | | - | - | - | yes | - | 19.5 |
| 328.1 | | | | - | - | - | yes | - | 8.3 |
| 346.9 | | | | - | - | - | yes | - | 8.1 / 9.3 |
| 403.0 | | | | - | - | - | yes | - | 15.8 |
| 413.3 | | | | - | - | - | yes | yes | 16.5 / 18.4 |
| 414.1 | | | | - | - | - | yes | yes | 8.9 |
| 416.1 | | | | - | - | - | yes | - | 10.0 |
| 420.1 | | | | - | - | - | yes | yes | 13.3 |
| **429.2** | 226.1 | ZN | | - | yes | - | yes | yes | 28.2 |
| 438.1 | | | | - | - | - | yes | - | 11.3 |
| **443.2** | 240.1 | ZK | | - | yes | - | yes | yes | 21.7 / 29.1 |
| 448.2 | | | | - | - | - | - | - | 13.3 |
| 475.2 | | | | - | yes | - | - | - | - |
| 482.1 | | | | - | - | - | yes | - | 15.9 |
| 492.2 | | | | - | yes | - | - | - | - |
| **499.2** | | ZS | | - | yes | - | yes | - | 33.9 |
| 500.2 | | | | - | - | - | yes | - | 14.6 / 16.5 |
| 514.2 | | | | - | yes | - | yes | - | 14.6 / 28.2 |
| 515.1 | | | | - | - | - | yes | - | 9.3 |
| 518.2 | 226.1 | ZN | | - | - | - | yes | yes | 20.7 |
| 526.2 | 226.1 | ZN | | - | yes | - | yes | - | 33.9 |
| 530.2 | | ZT | | - | yes | - | - | - | - |
| **540.3** | 240.1 | ZK | | - | yes | - | yes | yes | 27.5 |
| 559.3 | | | | - | - | - | yes | yes | 20.0 |
| 565.3 | | | | - | - | - | yes | yes | 20.7 |
| 567.2 | | | | - | - | - | yes | - | 14.6 |
| 574.2 | | | | - | - | - | yes | - | 8.9 |
| 581.3 | | | | - | - | - | yes | - | 21.7 |
| 585.2 | | | | - | - | - | yes | - | 20.0 |
| 591.2 | | | | - | - | - | yes | - | 8.9 |
| 600.2 | | | | - | - | - | yes | - | 11.3 |
| 613.4 | | | | - | - | - | yes | - | 20.4 |
| 626.3 | | | | - | - | - | yes | - | 10.0 |
| 642.3 | | | | - | - | - | yes | - | 10.0 |
| 654.3 | | | | - | - | - | yes | - | 17.1 |
| 664.3 | | | | - | - | - | yes | - | 16.5 |
| 672.3 | | | | - | - | - | yes | - | 10.0 |
| 680.4 | | | | - | - | - | yes | - | 10.4 |
| 685.3 | 240.1 | ZK | | - | - | - | yes | yes | 11.3 |
| 717.4 | | | | yes | - | - | yes | - | 11.3 / 20.7 |
| 733.3 | | | | - | - | - | yes | - | 8.9 |
| 733.4 | | | | - | - | - | yes | - | 63.7 |
| 740.3 | | | | - | - | - | yes | - | 13.3 |
| 743.4 | | | | - | - | - | yes | - | 13.3 |
| 760.6 | | | | - | - | - | yes | - | 14.1 |
| 789.4 | | | | - | - | - | yes | - | 13.3 / 21.7 |
| 793.4 | 240.1 | ZK | | - | yes | - | - | - | |
| 805.5 | | | | - | - | - | yes | - | 14.6 |
| 808.4 | 213.1 | | PP | - | - | - | yes | yes | 10.4 |
| 814.4 | | | | - | - | - | yes | - | 21.7 |
| 815.4 | 213.1 | | PP | - | - | - | yes | yes | 11.0 |
| 818.2 | | | | - | | | yes | - | 8.9 |
| 822.5 | 240.1 | ZK | | - | yes | - | - | - | |
| 858.3 | | | | - | - | - | yes | - | 28.2 |
| 872.4 | 226.1 / 240.1 | ZK/ZN | | - | yes | - | - | - | - |
| 882.4 | | | | - | - | - | yes | - | 28.2 |
| **886.4** | 240.1 | ZK | | - | yes | - | yes | yes | 29.1 |
| 888.6 | | | | - | - | - | - | - | 27.5 |
| 889.5 | | | | - | - | - | yes | - | 9.3 |
| 904.6 | | | | - | - | - | yes | yes | 20.0 |
| 932.5 | | | | - | - | - | yes | - | 33.9 |
| 933.8 | | | | - | - | - | yes | - | 21.7 |
| 969.5 | 226.1 / 240.1 | ZK/ZN | | - | yes | - | - | - | - |
| 983.5 | 240.1 | ZK | | - | yes | - | - | - | - |
| 998.5 | | ZS | | - | yes | - | | - | |
| 1008.7 | | | | - | - | - | yes | - | 13.3 |
| 1018.6 | 240.1 | ZK | | - | yes | - | | - | |
| **1074.6** | 213.1 | ZS | PP | - | yes | yes | yes | yes | 40.6 |
| 1098.8 | 213.1 | | PP | yes | - | - | yes | yes | 40.6 |
| 1099.8 | | | | - | - | - | yes | - | 14.1 |
| **1115.6** | 240.1 / 213.1 | ZK | PP | yes | yes | yes | yes | yes | 27.5 |
| 1129.7 | 240.1 / 213.1 | ZK | PP | - | yes | yes | - | yes | 31.0 |
| 1132.6 | 240.1 / 213.1 | ZK | PP | yes | - | - | - | - | - |
| 1137.6 | 240.1 / 213.1 | ZK | PP | yes | yes | - | - | - | - |
| 1145.7 | 240.1 / 213.1 | ZK | PP | - | yes | - | - | - | - |
| 1153.6 | | | | yes | - | - | - | - | - |
| 1158.8 | | | | - | - | - | yes | - | 10.4 |
| 1159.6 | | | | yes | - | - | - | - | - |
| 1175.6 | | | | yes | - | - | - | - | - |
| 1201.7 | 213.1 | | PP | - | yes | yes | - | yes | 31.0 |
| 1220.6 | | | | - | - | - | yes | - | 44.4 |
| 1235.1 | | | | - | - | - | yes | - | 31.2 |
| 1239.7 | | | | yes | - | - | - | - | - |
| 1244.9 | | | | | - | - | yes | - | 44.4 |
| 1272.7 | | | | yes | yes | - | - | - | - |
| 1286.7 | 240.1 | ZK | | yes | yes | - | yes | - | 33.9 |

**Table 1.** Continued

| Molecular masses (Da) | Fragment ions | Structural features | | MALDI-TOF MS | ESI-MS (Direct infusion) | | LC-ESI-MS (b) | | |
|---|---|---|---|---|---|---|---|---|---|
| | | N-ter | C-ter | | Full-mass scan | Precursor ion scan *m/z* 213.1 | Full-mass scan | Precursor ion scan *m/z* 213.3 / 226.1 / 240.1 | Rt (min) |
| 1354.1 | | | | - | - | - | yes | - | 29.1 |
| 1369.7 | 226.1 / 213.1 | ZN | PP | yes | yes | yes | yes | yes | 37.2 |
| 1383.8 | 240.1 / 213.1 | ZK | PP | yes | yes | yes | yes | yes | 31.2 |
| 1391.7 | | | | yes | - | - | - | - | - |
| 1394.1 | 213.1 | | PP | - | - | - | yes | yes | 37.2 |
| 1399.8 | 213.1 | ZH | PP | yes | yes | - | - | yes | 27.5 |
| 1405.8 | | | | yes | - | - | - | - | - |
| 1407.7 | 240.1 / 213.1 | ZK | PP | yes | yes | - | yes | yes | 31.2 |
| 1413.7 | | | | yes | - | - | - | - | - |
| 1421.7 | 240.1 / 213.1 | ZK | PP | yes | yes | - | - | - | - |
| 1424.0 | 213.1 | | PP | - | - | - | yes | yes | 37.2 |
| 1427.8 | 240.1 / 213.1 | ZK | PP | yes | yes | - | yes | yes | 27.6 |
| 1429.7 | | | | yes | - | - | - | - | - |
| 1435.7 | | | | yes | - | - | - | - | - |
| 1437.7 | | | | - | - | - | yes | - | 31.2 |
| 1444.7 | | | | yes | - | - | - | - | - |
| 1449.7 | | | | yes | - | - | yes | - | 33.9 |
| 1450.3 | | | | - | - | - | yes | - | 31.2 |
| 1451.7 | | | | yes | - | - | - | - | - |
| 1459.7 | | | | yes | - | - | - | - | - |
| 1465.7 | | | | yes | - | - | - | - | - |
| 1481.7 | | | | yes | - | - | - | - | - |
| 1504.4 | 213.1 | | PP | - | - | - | yes | yes | 18.4 |
| 1511.0 | | | | - | - | - | yes | - | 10.4 |
| 1515.3 | | | | - | - | - | yes | - | 18.9 |
| 1522.2 | | | | - | - | - | yes | - | 31.2 |
| 1544.8 | 240.1 / 226.1 / 213.1 | ZK/ZN | PP | - | yes | yes | yes | yes | 27.8 |
| 1556.4 | | | | - | - | - | yes | - | 18.9 |
| 1558.9 | 240.1 / 213.1 | ZK | PP | - | yes | yes | yes | yes | 29.1 |
| 1572.6 | | | | - | - | - | yes | - | 18.4 |
| 1655.9 | 240.1 / 213.1 | ZK | PP | - | yes | - | - | - | - |
| 1728.2 | 240.1 / 226.1 | ZK/ZN | | - | - | - | yes | yes | 15.9 |
| 1729.2 | | | | - | - | - | yes | - | 48.6 |
| 1752.3 | | | | - | - | - | | - | 15.9 |
| 1797.6 | | | | - | - | - | yes | - | 29.1 |
| 1798.9 | 240.1 / 226.1 / 213.1 | ZK/ZN | PP | - | yes | yes | - | - | - |
| 1812.9 | 240.1 / 226.1 / 213.1 | ZK/ZN | PP | - | yes | yes | - | - | - |
| 1827.0 | 240.1 / 213.1 | ZK | PP | - | yes | yes | yes | yes | 18.4 |
| 1841.9 | | | | - | - | - | yes | - | 19.5 |
| 1850.9 | 240.1 | ZK | | - | yes | - | - | - | - |
| 1882.1 | 240.1 / 213.1 | ZK/ZS | PP | yes | yes | yes | - | yes | 44.4 |
| 1910.1 | 240.1 / 226.1 / 213.1 | ZK/ZN | PP | - | yes | - | - | - | - |
| 1988.1 | 240.1 / 226.1 / 213.1 | ZK/ZN | PP | - | yes | - | - | - | - |
| 2124.2 | 226.1 | ZN | | - | - | - | yes | yes | 22.4 |
| 2148.2 | | | | - | - | - | yes | - | 22.4 |
| 2165.2 | | | | - | - | - | yes | - | 22.4 |
| 2211.8 | 226.1 | ZN | | - | - | - | yes | yes | 15.9 |
| 2261.0 | | | | yes | yes | - | yes | - | 8.3 |
| 2270.3 | | | | - | yes | - | - | - | - |

The main compounds observed were found at 1074.6, 1115.6, 1369.7, 1383.7, 1544.8, 1558.9, 1827.0 and 1882.1 Da. The presence of these compounds was confirmed by ESI-MS analysis on the Q-TOF micro instrument. About 43 components with molecular masses below 2500 Da were detected in the *B. moojeni* venom by direct infusion into the Q-TOF instrument (figure 1).

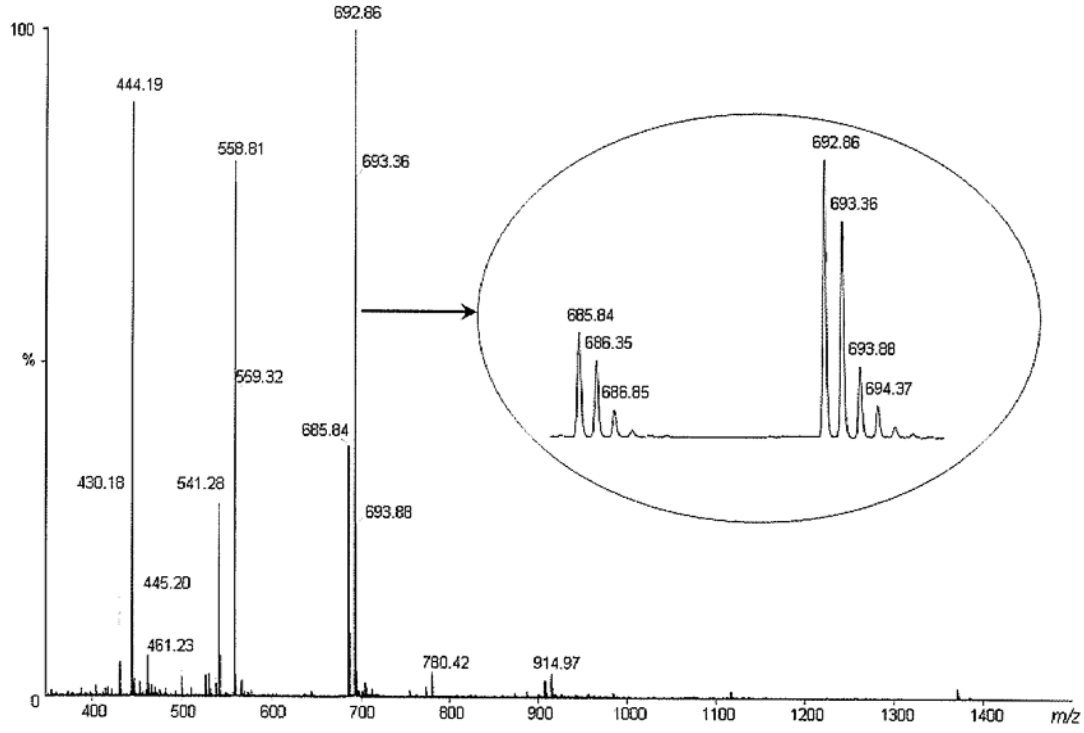


**Figure 1.** ESI mass spectrum of an extract from the crude venom of *B. moojeni*. The sample was desalted on a 2g (12 $cm^3$) Sep-Pak C18 cartridge (Waters, Milford, MA, USA) following the manufacturer's protocol and introduced by infusion at 5 μL/min into a Q-TOF micro mass spectrometer (Waters). About 43 different compounds were detected in the venom by this shotgun analysis. The insert shows the high mass resolution obtained for the $[M+2H]^{2+}$ ion at *m/z* 685.84 and 692.86.

Based on peak intensity, the most abundant peptides were found at 429.19, 443.19, 540.29, 1115.65, 1369.68 and 1383.74 Da (Table 1).

## 5.4.2 LC-ESI-MS analysis

LC-ESI-MS analysis of the *B. moojeni* venom was performed on the triple quadrupole mass spectrometer using both full-scan and precursor ion-scan modes to search for the presence of precursors of ions at *m/z* 213.1, 226.1 and 240.1. The *m/z* 226.1 and 240.1 ions correspond to *b* fragments of respectively ZN and ZK peptides, Z being the pGlu moiety. It should be mentioned that the *m/z* 226.1 signal can also

correspond to the isobaric ZGG dipeptide. The chromatographic profile obtained with our approach was compared to the full-mass scan chromatogram (figure 2).

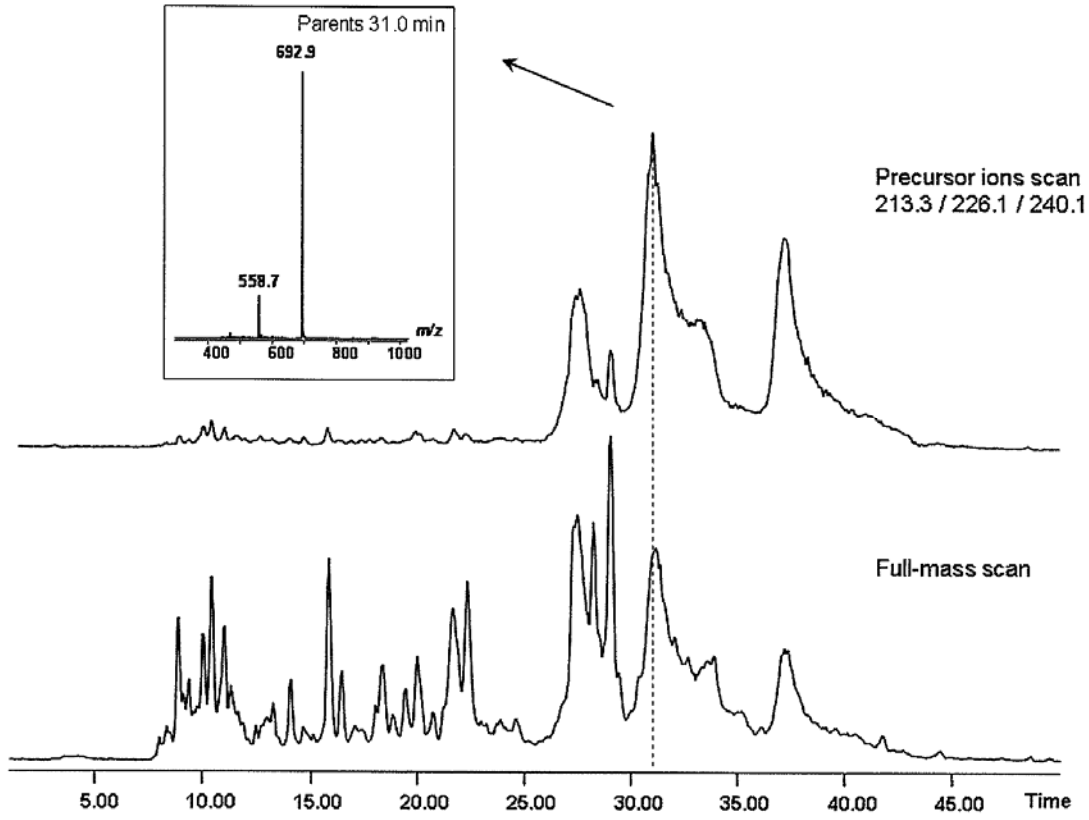


**Figure 2.** LC-ESI/MS chromatograms of the crude *B. moojeni* venom. Lower chromatogram: Total ion current (TIC) in full-mass scan mode *m/z* 300 – 1500. Upper chromatogram: Precursor ions *m/z* 213.1, 226.1 and 240.1 scan chromatograms. Frame box: Parent scan at 31 min. Chromatographic conditions: column Atlantis C18 (Waters); solvent A: aqueous 0.1 % formic acid (FA); solvent B: 10:90 aqueous 0.1 % FA: ACN; gradient slope: 0.5 % B per min; flow rate 0.2 mL/min.

LC-ESI-MS analysis in the full-mass scan mode allowed the detection of 97 compounds with molecular masses in the 300-2300 Da range (Table 1). The profile of the TIC obtained under precursor ion-scan mode comprised much less peaks than in the full-mass scan mode especially in the first part of the elution. As clearly shown, the complexity of the venom has been significantly reduced using the precursor ion-scan mode (figure 2). A total of 34 precursors of the *m/z* 213.1, 226.1 and 240.1 ions

were detected in the *B. moojeni* venom using our approach (Table 1). These results confirmed the high content of BPP-related components in the venom of *B. moojeni*. In addition, the chromatographic profiles obtained show that most of the N-terminal Z / C-terminal PP peptides eluted after 25 min of run. The relatively weak apparent chromatographic resolution observed for the precursor ion chromatogram has been attributed to the high number of isoforms as well as to homo- and hetero-dimer/trimer species observed between BPP-related components.

Based on our data, it appears that mass precursors to fragments at *m/z* 213.1, 226.1 and 240.1 represent about 37 % of the total number of components detected by LC-ESI-MS in the full-mass scan mode. More important, the precursor ion-scan mode allowed the detection of 4 additional masses at 1129.7, 1201.7, 1399.8 and 1882.1 Da compared to the full-scan mode. Additional MS/MS experiments confirmed that these peptides are BPPs (see below).

### 5.4.3 De novo Q-TOF based MS/MS sequencing

Further ESI-MS/MS fragmentations were conducted by direct infusion of the sample into the Q-TOF mass spectrometer and selection of the precursor signals of *m/z* 213.1, 226.1 and 240.1 previously found, in order to confirm their structural features and their belonging to pGlu peptides of the BPPs family. Among the 34 precursors detected in the *B. moojeni* venom by the LC-ESI-MS approach, 19 could be detected by direct ESI-MS infusion and selected for fragmentation by collision-induced dissociation (CID). Most of the additional masses detected in the venom were also submitted to fragmentation.

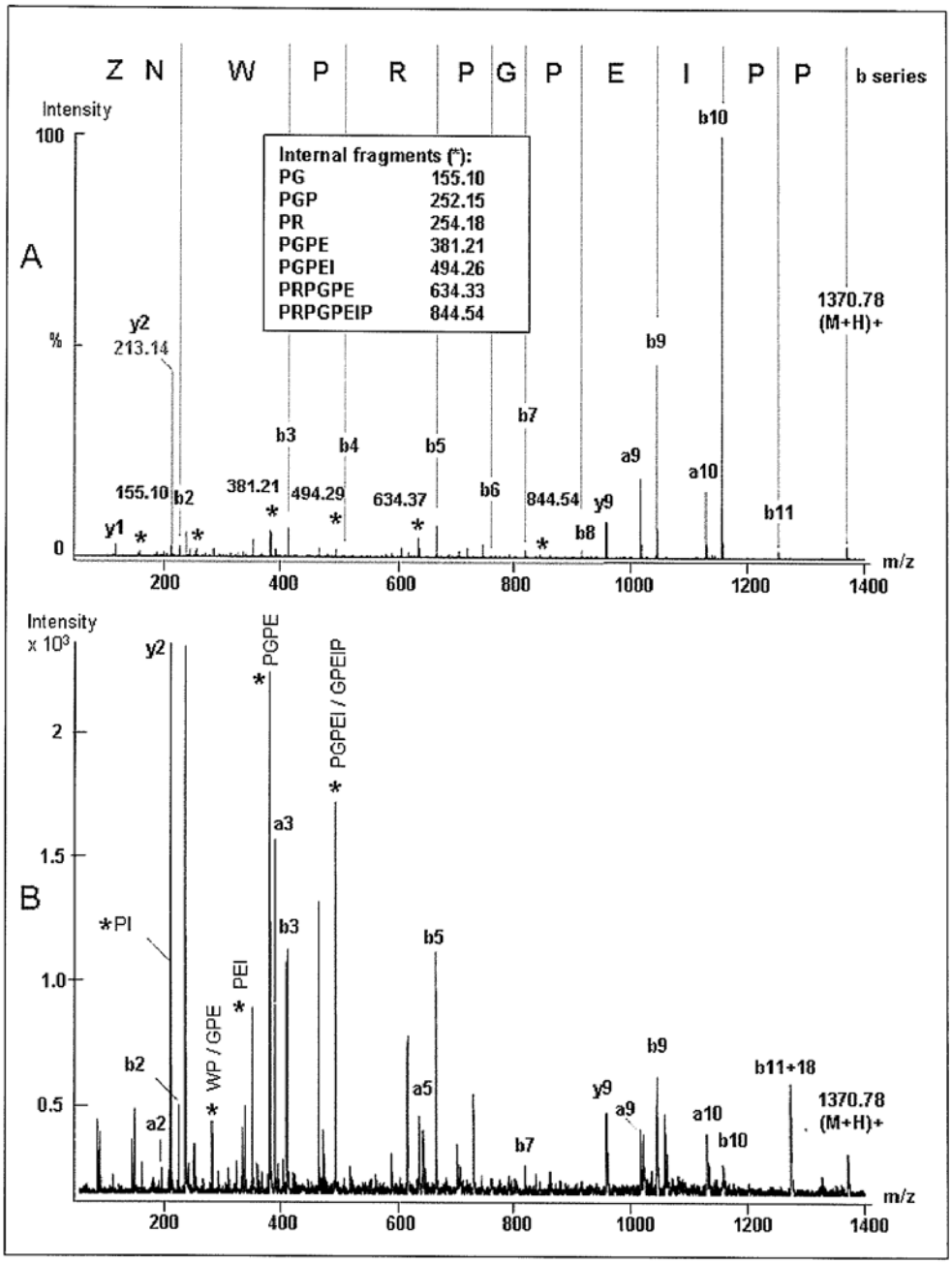


**Figure 3.** *De novo* sequencing of the peptide at 1369.68 Da. Panel A: ESI-MS/MS analysis and product ions resulting from the collision-induced dissociation of the 685.84 $[M+2]^{2+}$ precursor ion. The deduced amino acid sequence is indicated on the top of the spectrum together with *a, b* and *y* ions attribution. Internal fragments are labelled by a star and their corresponding sequence described in the frame: *m/z* 155.10, 252.15, 254.18, 381.21, 494.29, 634.33 and 844.54 ions correspond to, respectively PG, PGP, PR, PGPE, PGPEI, PRPGPE and PPGPEIP fragments. Panel B: MALDI-LIFT-TOF/TOF-MS analysis of the venom carried out on an Ultraflex TOF-TOF mass spectrometer (Bruker, Bremen). The $[M+H]^{1+}$ parent ion at *m/z* 1370.78 was selected for fragmentation. The deduced sequence (*y* series) is shown on the top of the MS/MS profiles.

As an example, figure 3 shows the product ions resulting from the fragmentation of the 685.84 $[M+2]^{2+}$ precursor ion corresponding to the signal of the 1369.68 Da peptide. The deduced amino acid sequence ZNWPRPGPEIPP indicated on the top of the spectrum displays typical features of BPPs. It should be pointed out in this case that the C-terminal PP ion at *m/z* 213.1 is much more intense than the N-terminal ZN fragment at *m/z* 226.1. This is observed in general for most of the peptides having these both N-terminal and C-terminal features. The major *a, b* and *y* ions assignment as well as internal fragments have been labelled by a star on the spectrum: *m/z* 155.10, 252.15, 254.18, 381.21, 494.29, 634.33 and 844.54 ions correspond to PG, PGP, PR, PGPE, PGPEI/GPEIP, PRPGPE and PPGPEIP fragments, respectively. Most of the signals detected by ESI-MS were analyzed by MS/MS in a similar manner and postulated sequences are presented in Table 2.

**Table 2.** Pyroglutamate peptides (pGlu) / Bradykinin-potentiating peptides (BPPs) sequences obtained by *de novo* ESI-MS/MS sequencing of components detected in the *Bothrops moojeni* crude venom. The sequences given are the most probable ones, taking into account some uncertainties due to isobaric residues such as I/L and N=GG.

| | Sequences | Experimental mass* (Da) | Theoretical mass* (Da) | Rt (min) | Comments |
|---|---|---|---|---|---|
| 1 | **ZNW** | 429.19 | 429.16 | 28.2 | N = GG uncertainty |
| 2 | **ZKW** | 443.19 | 443.21 | 22.3 / 29.1 | |
| 3 | **ZSWP** | 499.25 | 499.21 | 33.9 | |
| 4 | **ZNWP** | 526.22 | 526.22 | 33.9 | N = GG uncertainty |
| 5 | **ZTNW** | 530.22 | 530.21 | ND | |
| 6 | **ZKWP** | 540.29 | 540.27 | 27.5 | |
| 7 | **ZKWPRP** | 793.44 | 793.42 | ND | |
| 8 | **ZKWPGPK** | 822.46 | 822.44 | ND | |
| 9 | **DLRPDNAKA** | 998.51 | 998.51 | ND | BNP2_BOTJA precursor (AN: BAA12879) |
| 10 | **ZKWPGPKVP** | 1018.64 | 1018.56 | ND | |
| 11 | **ZSWPGPNIPP** | 1074.58 | 1074.51 | 40.6 | |
| 12 | **ZKWPPGKVPP** | 1115.65 | 1115.61 | 27.5 | |
| 13 | **ZKWPKGPLPP** | 1129.72 | 1129.63 | ND | |
| 14 | **ZKWPHEHPP** | 1137.63 | 1137.53 | ND | |
| 15 | **ZKWALPKVPP** | 1145.68 | 1145.66 | ND | |
| 16 | **ZNWPRPGPEIP** | 1272.74 | 1272.62 | ND | N = GG uncertainty |
| 17 | **ZKWHRNPEIP** | 1286.70 | 1286.65 | 33.9 | |
| 18 | **ZNWPRPGPEIPP** | 1369.68 | 1369.68 | 37.2 | N = GG uncertainty |
| 19 | **ZKWPRPGPEIPP** | 1383.74 | 1383.73 | 31.2 | |
| 20 | **ZHFSE(...)IPP** | 1399.82 | | 27.5 | Partial sequence |
| 21 | **ZKWD(...)GPEIPP** | 1407.68 | | 31.2 | Partial sequence |
| 22 | **ZKWD(...)IPP** | 1427.83 | | ND | Partial sequence |
| 23 | **LEIAKNGLSTTSNPKR** | 1728.12 | 1727.95 | ND | L-amino acid oxidase (fragment, Q6TGQ8) |

Z, pyroglutamic acid; * Monoisotopic masses; (...): missing sequence information; ND: not detected.

The characteristic features of pGlu and BPP-related compounds could be confirmed for 38 detected masses. The others could not be characterized, either because they

could not be detected by direct ESI-MS analysis (518.2, 559.3, 565.3, 808.4, 815.4, 904.6, 1424.0, 1504.4, 2124.2 and 2211.8 Da) or due to a poor quality of their fragmentation spectrum (1098.8 Da). Among the peptides characterized, a total of 13 were shown to be non-covalent homo-/hetero- dimers and trimers of already sequenced pGlu tripeptides and BPPs. Such peptides, among them the masses at 1544.9, 1558.9, 1812.9, 1827.0, 1882.1 and 1988.1 Da were not reported in the Table 2 due to remaining ambiguous sequence assignment.

### 5.4.4 MALDI-TOF-MS analysis and LIFT-TOF/TOF-MS experiments

The MALDI-TOF-MS spectrum of *B. moojeni* pre-treated venom revealed a total of 30 components including the two main BPPs at 1370.71 and 1384.78 $[M+H]^+$ and their $Na^+$ and $K^+$ adducts at 1392.71 $[M+Na]^+$ and 1408.71 $[M+K]^+$, 1406.77 $[M+Na]^+$ and 1422.77 $[M+K]^+$, respectively. The total number of components detected by MALDI-TOF-MS is lower than by direct infusion ESI-MS analysis. In particular, the peptides at 886.41, 1074.58, 1544.84, 1558.85, 1812.93 and 1826.97 Da detected by ESI-MS could not be found by MALDI-TOF-MS, probably due to ion suppression effects. Nevertheless, MALDI-TOF-MS analysis revealed 15 new additional masses, confirming the complementarity of three techniques. The signal at 1370.70 $[M+H]^+$ was analyzed by MALDI-TOF/TOF-MS analysis using the LIFT technology. The fragmentation spectrum is displayed in Fig. 3B, showing some differences in the profile compared to the ESI-MS/MS spectrum (Fig. 3A) with more intense ions visible in the range *m/z* 100 – 800. Some major ions at *m/z* 237.06, 466.05, 617.11, 730.24, 1058.58, 1130.20 and 1273.43 $[M+H]^+$ could not be interpreted as trivial fragment and therefore were not assigned.

## 5.5 Discussion

Three approaches, namely (1) ESI-MS in full-scan mode on a Q-TOF instrument (2) LC-ESI-MS and MS/MS in full-scan and precursor ion-scan mode on a triple quadrupole mass spectrometer and (3) MALDI-TOF-MS and MS/MS analysis, were used to investigate the *B. moojeni* venom content in BPP-related compounds. A total of 134 compounds were detected, among them 33% were detected by ESI-MS, 72% by LC-ESI-MS and 23% by MALDI-TOF-MS. These approaches were complementary since only 9 peptides could be detected by all of them and among

them, 7 were found to be BPP-related compounds. The LC-ESI-MS and MS/MS approach was the most powerful, detecting the highest number of compounds in the full-scan mode, while revealing about 34 precursors of ions at *m/z* 213.1, 226.1 and 240.1. According to the results, it sounds that the best strategy for the venom screening would be (1) to perform ESI-MS/MS and LC-ESI-MS/MS analyses on a triple quadrupole instrument operated in the precursor ion-scan mode to identify the peptides of interest; (2) to go straight to their structural characterization by ESI-MS/MS using more dedicated instruments such as a Q-TOF or by MALDI-TOF/TOF. The MALDI-TOF/TOF instrument enables data to be acquired with high throughput and sensitivity. Our global approach represents a significant gain of time since it allows the pre-selection of the precursors of interest instead of fragmenting all the signals present in a single spectrum.

Furthermore, a total of 36 components could be successfully identified and characterized by *de novo* MS/MS sequencing. The Table 2 reports 21 sequences of pGlu tripeptides and BPP-related components among them 15 are novel. In addition to BPP-related peptides, 2 other components could be characterized: a compound with a molecular mass of 998.51 Da displayed homologies with a fragment of the C-type natriuretic peptide venom gland precursor of *B. jararaca* (Swiss-Prot Accession number AN: BAA12879). The peptide at 1728.12 Da was shown to be a fragment of the *B. moojeni* L-amino acid oxidase (AN: Q6TGQ8). It is interesting to point out that this peptide was found by precursor ion-scan LC-ESI-MS/MS to contain a ZK/ZN feature (Table 1) but its belonging to BPPs was not confirmed by further sequencing. This false positive case may be explained by the presence of two different peptides with the same mass in the sample.

Despite a good confidence in the sequence attribution, some uncertainties remain. For example, the *b*2 ion at *m/z* 226.1 corresponding to the ZN fragment could also be the *b*3 ion of ZGG that should break well under CID conditions. In most of the product ion spectra of this study, the *b*2 ion of ZG could not be observed or its intensity was too low to be able to discriminate between ZGG and ZN. BPPs with the N-terminus ZN sequence have been already described for *B. jararaca* (Hayashi and Camargo, 2005; Wermelinger *et al.*, 2005) but the N-terminus ZGG sequence has also been described for a large number of *Bothrops* species (Cintra *et al.* 1990; Murayama *et al.* 1997; Hayashi and Camargo 2005; Hayashi *et al.* 2003; Wermelinger *et al.* 2005) as well as in *Crotalus* snakes (Wermelinger *et al.* 2005). In addition, the I residue

preceding the PP motif was assigned based on homologies with existing BPPs and could not be experimentally discriminated from the L residue. Finally, the peptide at 1407.71 Da that had been initially attributed to a $K^+$ adduct of the BPP at 1370.71 Da by MALDI-TOF-MS seems rather to be a novel sequence: indeed, the major fragments observed from *de novo* sequencing data correspond to the ZKWD(…)GPEIPP sequence and this peptide co-eluted in LC-ESI-MS experiments with the peptide at 1384.4 Da and not with the 1370.1 Da component (data not shown).

In our study, the major components at 1369.68 and 1383.74 Da were attributed to the sequence ZXWPRPGPEIPP, where X is respectively N and K. The delta masses obtained between measured and theoretical masses were found to be below 0.01 Da. This is in conflict with the sequence PKVSPRWPPIPP submitted by Guedes *et al.* (2005) to Swiss-Prot (AN: P84747), the K residue in position 2 being modified or not in a N6-methylated K. Indeed, the calculated monoisotopic masses for the PKVSPRWPPIPP published sequences are respectively 1369.79 and 1383.80 Da for K and N6-methylated K residues, corresponding to masse differences with the measured values of 0.11 and 0.06 Da, respectively. This mass difference is higher than the 20 ppm mass accuracy generally obtained on the Q-TOF instrument. In addition, the measured mass gap (14.06 Da) between the 2 peptides is more in favour of a K/N isoform (14.052 Da) than an additional methyl group on the K residue (14.016 Da). In addition, the overall fragmentation pattern obtained for the peptide at 1369.68 Da does not support the PKVSPRWPPIPP proposed by Guedes *et al.* (AN: P84747). Finally, the presence of a N6-methylated K in venom peptides has never been described elsewhere.

In this study, 13 *m/z* species gave fragmentation patterns corresponding to multimeric forms of the tripeptides ZNW and ZKW in association with other BPPs present in the venom (data not shown). The dimer of ZKW at 886.43 Da eluted at the same retention time (29.1 min) than the monomer at 443.19 Da, which is in favour of a non-covalent dimerization process of pyroglutamyl peptides. These polymers may be formed in the source during ESI-MS analysis, but we should mention that the compound at 1883.05 Da, most probably corresponding to the dimer of ZSWP (499.25 Da) and ZKWPRPGPEIPP (1383.74 Da) could also be detected by MALDI-TOF-MS. The presence of such level of multimeric forms of tripeptides in association

with BPPs may explain the low chromatographic resolution obtained in their corresponding LC elution zone.

Table 3 presents the sequences of most of the pGlu tripeptides and BPPs characterized so far from different organisms such as snakes, scorpions, spider and batrachian.

It shows the high sequence diversity of BPP-related peptides and the amazing number of different isoforms encountered in the venoms. The reasons of such diversity still remain to be fully understood, but a possible explanation could be linked to intraspecies variability of the snake venom. Our study was conducted on a venom pool from many *B. moojeni* specimens and it might be of interest to investigate individual specimens in the future. Recently, the peptidome of 18 *B. jararaca* venoms was indeed analyzed by LC/MS, MALDI-TOF-MS and *de novo* peptide sequencing (Pimenta *et al.*, 2007). Sex-based peptide variation among sibling and non-sibling snakes could be observed. The peptidome variability observed among eighteen sibling venoms of a single snake species could not be attributed to the environmental, age or diet parameters. Instead, the diversity observed was explained by genetic factors and sex-linked variations in precursor processing pathways. Therefore, our new approach based on rapid detection of BPP-related peptides by precursor ion-scan tandem mass spectrometry techniques will certainly reveal useful for such differential studies.

**Table 3.** Sequences of pyroglutamyl tripeptides and BPPs described from different organisms (snakes, spiders and batrachians), together with the novel sequences obtained from *B. moojeni* in this work. The peptides are classified by increasing masses.

| Sequences | Species | MW (Da)* | References |
|---|---|---|---|
| **ZNW** | *B. moojeni, C. o. oreganus* | 429.16 | this work, o |
| **ZKW** | *B. moojeni, C. o. oreganus* | 443.21 | this work, o |
| **ZSWP** | *B. moojeni* | 499.21 | this work |
| ZKWA | *B. jararaca* | 514.25 | p |
| **ZNWP** | *B. moojeni* | 526.22 | this work |
| **ZTNW** | *B. moojeni* | 530.21 | this work |
| **ZKWP** | *B. moojeni* | 540.27 | this work |
| ZKWAP | *B. insularis; B. jararaca, B. jararacussu* | 611.31 | b, h, p |
| ZSWPGP | *B. jararaca* | 653.70 | i |
| ZKWDP | *Gloydius* (former *A. halys*) *blomhoffi* | 655.29 | w |
| ZWPRP | *B. jararaca* | 665.80 | i |
| **ZARESP** | *C. durissus terrificus* | 669.31 | w |
| ZPHESP | *Gloydius* (former *A. halys*) *blomhoffi* | 676.28 | w |
| ZDGPIPP | *B. jararaca* | 705.80 | i |
| ZNWKSP | *C. durissus terrificus* | 741.34 | w |
| ZNWPHP | *B. jararaca* | 760.33 | r |
| ZNWPRP | *B. jararaca* | 779.37 | r |
| **ZKWPRP** | *B. moojeni* | 793.42 | this work |
| **ZKWPGPK** | *B. moojeni* | 822.44 | this work |
| **ZWPRPTP** | *B. jararaca* | 863.43 | r |
| ZGRPPGPPIP | *Gloydius* (former *A. halys*) *blomhoffi* | 997.53 | l |
| **ZKWPGPKVP** | *B. moojeni* | 1016.56 | this work |
| ZGWPGPKVPP | *V. aspis* | 1044.54 | m |
| ZGLPPGPPIPP | *Gloydius* (former *A. halys*) *blomhoffi* | 1051.57 | j, q |
| ZGGAGWPPIPP | *B. jararaca* | 1058.53 | u |
| ZSAPGNEAIPP | *B. jararaca* | 1062.51 | u |
| **ZGRAPGPPIPP** | *B. jararaca* | 1068.57 | i, p |
| **ZSWPGPNIPP** | *B. jararaca, B. moojeni* | 1074.51 | this work, i, p |
| WPPRPQIPP | *L. m. muta* | 1086.59 | s |
| ZGRPPGPPIPP | *A. halys pallas, B. jararaca, B. jararacussu* | 1094.58 | a, i, u |
| ZWPRPQIPP | *B. jararaca, B. neuwiedu* | 1100.30 | c, u |
| ZGRPRSEVPP | *Protobothrops* (former *T. ) flavoviridis* | 1104.56 | j |
| **ZGLPPGPPIPR** | *Gloydius* (former *A. halys*) *blomhoffi* | 1110.61 | j, q |
| ZGRPLGPPIPP | *T. mucrosquamatus* | 1110.62 | k |
| **ZKWPPGKVPP** | *B. moojeni* | 1115.61 | this work |
| ZGGAPWNPIPP | *C. viridis* | 1116.55 | u |
| ZGGPPRPQIPP | *B. insularis* | 1125.59 | b |
| **ZKWPKGPIPP** | *B. moojeni* | 1129.72 | this work |
| **ZKWPHEHPP** | *B. moojeni* | 1137.53 | this work |
| **ZKWALPKVPP** | *B. moojeni* | 1145.66 | this work |
| ZGRAPHPPIPP | *B. jararaca* | 1148.60 | i, p |
| ZGRPPGPPIPR | *Gloydius* (former *A. halys*) *blomhoffi* | 1153.63 | j, v |
| ZWGQHPNIPP | *B. insularis* | 1155.54 | b |
| ZGPSPRHPIPP | *C. viridis* | 1164.61 | u |
| ZGLPPRPKIPP | *A. blomhoffi* | 1181.69 | j, t |
| ZARPPHPPIPP | *B. jararaca; B. jararacussu* | 1188.65 | h, i |
| ZAPWPDTISPP | *Scaptocosa raptoria* | 1190.56 | f |
| ZNWPHPQIPP | *B. insularis, B. jararaca, B. jararacussu* | 1195.59 | b, h, i, p |
| ZGGWPRNPIPP | *C. adamanteus* | 1200.61 | u |
| ZNWPRPQIPP | *B. jararaca* | 1214.40 | i, u |
| ZFRPSYQIPP | *Phyllomedusa hypochondrialis* | 1214.60 | d |
| ZWPDPSSDIPP | *B. alternatus* | 1220.54 | u |
| **ZKWDPPPVSPP** | *Gloydius* (former *A. halys*) *blomhoffi* | 1229.60 | j, q |
| ZSKPGRSPPISP | *Protobothrops* (former *T. ) flavoviridis* | 1232.65 | j |
| ZKPWPPGHHIPP | *L. m. muta* | 1244.63 | s |
| ZRWPHLEIPP | *C. durissus terrificus* | 1254.68 | w |
| **ZNWPRPGPEIP** | *B. moojeni* | 1272.62 | this work |
| ZEKPGRSPPISP | *T. gramineus* | 1274.65 | j |
| ZQWPPGHHIPP | *C. adamanteus* | 1275.62 | u |
| ZKKWPPGH HIPP | *L. m. muta* | 1277.74 | s |
| ZLGPPRPQIPP | *B. insularis* | 1278.72 | b, p |
| ZWGRPPGPPIPP | *B. jararaca* | 1280.66 | h, i |
| **ZKWHRNPEIP** | *B. moojeni* | 1286.65 | this work |
| ZGGLPRPGPEIPP | *B. jararaca* | 1297.50 | i |
| ZWPRPTPQIPP | *B. jararaca* | 1298.50 | l, u |
| ZWPRPTPQIPP | *B. neuwiedi* | 1298.85 | u |
| ZGGWPRPGPEIPP | *B. insularis, B. jararaca, B. jararacussu, B. neuwiedi* | 1369.68 | b, h, i, p, u |
| **ZNWPRPGPEIPP** | *B. moojeni* | 1369.68 | this work |
| ZKWDPPPISPP | *L. m. muta* | 1374.16 | s |
| **ZKWPRPGPEIPP** | *B. moojeni* | 1383.73 | this work |
| ZEWPPGHHIPP | *L. m. muta* | 1404.60 | s |
| ZGWAWPRPQIPP | *B. jararaca* | 1415.60 | i |
| ZWAQWPRPQIPP | *B. jararaca* | 1485.80 | i |
| KKDGYPVEYDRAY | *Tityus serrulatus* | 1602.76 | e |
| ZWMPEGRPPHPIPP | *Protobothrops* (former *T. ) flavoviridis* | 1620.79 | w |
| ZWSQRWPHLEIPP | *C. durissus terrificus* | 1655.82 | w |
| ZWAQWPRPTPQIPP | *B. jararaca* | 1683.85 | i |
| ZQWSHRWPHPEIPP | *C. durissus terrificus* | 1776.85 | g |
| LRDYANRVINGGPVEAAGPPA | *Buthus occitanus* | 2136.10 | n |

*: monoisotopic masses; *A: Agkistrodon; B: bothrops; C: crotalus; L: lachesis; T. trimeresurus, V: vipera.*

Ref: a: Chi et al. 1985; b: Cintra et al. 1990; c: Coelho et al. 1999; d: Conceicao et al. 2007; e: Ferreira et al. 1993; f: Ferreira et al. 1996; g: Graham et al. 2005; h: Hayashi et al. 2003; i: Hayashi and Camargo 2005; j: Higuchi et al. 1999; k: Jia et al. 2003; l: Kato and Suzuki 1971; m: Komori and Sugihara 1990; n: Meki et al. 1995; o: Munekiyo and Mackessy 2005; p: Murayama et al. 1997; q: Murayama et al. 2000; r: Pimenta et al. 2007; s: Soares et al. 2005; t: Tominaga et al. 1975; u: Wermelinger et al. 2005; v: Yanoshita et al. 1999; w: Gomes et al. 2007.

## 5.6 Conclusion

This paper describes a rapid and original methodology to screen new BPP-related components in snake venom. This is the first time that a tandem mass spectrometry approach using the precursor ion-scan mode is described for the screening of BPPs in snake venom. This method has proved its efficiency since it allowed the identification of more than 20 BPP-related peptides, among them 15 are new sequences. This is an attractive concept, which allows an efficient screening of BPPs in a large number of venoms and thus their more judicious selection for further purification of individual BPP isoforms. In combination with purification/synthesis steps followed by bioassays on purified peptides, this approach could be useful for structure-function relationship studies and for the design of new pharmacological lead compounds. Indeed the knowledge of the structural diversity of the BPPs family is essential for understanding the structure-function relationship. This sensitive methodology could be adapted to the study of BPP-like peptides in tissues, fluids or secretions of mammals for which nothing similar have been described yet. It could also be developed for the discovery of other venom components.

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# 6 Isolation and characterization of two new Lys49 $PLA_2$s with heparin neutralizing properties from *Bothrops moojeni* snake venom.

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## 6.1 Abstract

Among the new proteins and peptides already characterized in *Bothrops moojeni* venom, two novel phospholipases $A_2$ ($PLA_2$) have been purified and fully sequenced by ESI-MS/MS techniques. Both of them belong to the enzymatically non-active Lys49 variants of $PLA_2$. They consist of 122 amino acids and share a characteristic sequence in their C-terminal region composed of clusters of basic amino acids known to interact with heparin. Thus, as already established, heparin can be used as an antidote to antagonize some myotoxic $PLA_2$s from venoms of *Bothrops* genus. The two $PLA_2$ variants were shown to interact *in vitro* with unfractionated heparin (UFH) and low molecular weight heparin (LMWH), neutralizing their anticoagulant properties. Although the influences of $PLA_2$s from snake venoms on the blood coagulation system are known, their use to antagonize the anticoagulant effect of heparin *in vitro* or *in vivo* has never been proposed. These finding recommend diagnostic and therapeutic applications, which are currently investigated.

**Abbreviations:** ACN acetonitrile; aPTT activated partial thromboplastin time; ATIII antithrombin III; CT clotting time; ESI-MS ElectroSpray Ionization-MS; ex- TEM® solution for activation extrinsic pathway of blood coagulation; FXa factor Xa (activated factor X); FVa factor Va (activated factor V); GF gel filtration; 4-HCCA $\alpha$-cyano-4-hydroxycinnamic acid; in- TEM® solution for activation intrinsic pathway of blood coagulation; ISTH/SSC International Society on Thrombosis and Haemostasis/ Scienticic and Standardization Comitee; LMWH low molecular weight heparin; MALDI-TOF Matrix Assisted Laser Desorption Ionisation – Time Of Flight; MS Mass Spectrometry; NHF normal human fibroblasts; rPF4 recombinant platelet factor 4; PiCT prothrombinase induced clotting time; $PLA_2$ phospholipase $A_2$; SA sinapinic acid; star-TEM® buffered $CaCl_2$ solution; UFH unfractionated heparin; TFA trifluoroacetic acid

## 6.2 Introduction

Phospholipases $A_2$ ($PLA_2$) are widely distributed in Nature. They occur in venoms of snakes, scorpions, bees and in mammalian tissues, both as intracellular and extracellular forms (Soares *et al.*, 1998; Kini, 2003). Snake venom $PLA_2$ enzymes share similarity in structure and catalytic function with their mammalian counterparts. However, mammalian phospholipases are generally non-toxic and do not induce potent pharmacological effects. In contrast, snake venom $PLA_2$s, in addition to their role in digestion of the prey, belong to the most toxic and pharmacologically most potent components of venoms (Kini, 2003). Thus, they are able to induce several biological effects such as pre- or postsynaptic neurotoxicity, cardiotoxicity, myotoxicity, inhibition of platelet aggregation, edema, hemolysis, anticoagulation, hemorrhage, convulsion and hypotension, as well as bactericidal activities (Kini and Iwanaga, 1986b; Kini and Iwanaga, 1986a; Paramo *et al.*, 1998; Soares *et al.*, 1998; Kini, 2003; Stábeli *et al.*, 2006). Despite their diverse pharmacological properties, they share 40-99% identity in their amino acid sequences and a significant similarity in the three dimensional structures. Therefore, the functional differences among $PLA_2$ enzymes cannot be easily correlated with their structural differences (Kini, 2003).

*Bothrops* myotoxins are classified in group II $PLA_2$s, together with all crotalid/viperid venom lipases and the secreted non-pancreatic mammalian $PLA_2$ (Gutierrez and Lomonte, 1995). Moreover, they are divided into two major classes according to the residue at position 49 in the sequence, being a key residue for binding of the $Ca^{2+}$-cofactor. The enzymatically active Asp49 and the enzymatically non active Lys49 myotoxins can be distinguished (Soares *et al.*, 1998; Lomonte *et al.*, 2003a). It has also been reported, that there is a higher homology between Lys49 myotoxins of different genera (*Bothrops*, *Agkistrodon* and *Trimeresurus spp.*) than between the enzymatically active Asp49 and inactive Lys49 proteins of the same species (Gutierrez and Lomonte, 1995; Selistre de Araujo *et al.*, 1996). Asp49Lys mutation, together with additional mutations in the calcium-binding loop, precludes an effective coordination of $Ca^{2+}$ ions and, consequently, is responsible for the lack of enzymatic activity (Gutierrez and Lomonte, 1995; Ownby *et al.*, 1999; Lomonte *et al.*, 2003a). However, the enzymatic activity is apparently not necessary for the toxic actions. Lys49 myotoxins display also bactericidal properties, being lethal for a broad

spectrum of gram-negative and gram-positive bacteria (Paramo *et al.*, 1998; Santamaria *et al.*, 2005). Very recently, it has been shown (Stábeli *et al.*, 2006) that *B. moojeni* myotoxin II displays antiparasitic and antitumoral activities, supporting the idea of multiple bioactive sites in the protein.

Three *B. moojeni* myotoxins have been isolated and described so far. MOO-1 myotoxin possesses high phospholipase activity as reported by Moura-da Silva *et al.* (Moura-da-Silva *et al.*, 1991a; Moura-da-Silva *et al.*, 1991b).

The two other myotoxins, MjTX-I and MjTX-II, are described as catalytically inactive Lys49 variants. Their amino acid sequences (Lomonte, Gutierrez *et al.* 1990; de Azevedo W.F. Jr., R.J. *et al.* 1997; Soares, Rodrigues *et al.* 1998; Soares, Andriao-Escarso *et al.* 2000), as well as the native structures and the complex of MjTX-II with stearic acid (de Azevedo W.F. Jr. *et al.*, 1997; Soares *et al.*, 2000; Marchi-Salvador *et al.*, 2005; Watanabe *et al.*, 2005) have been determined, revealing high homology with various Lys 49 $PLA_2$-like proteins from other *Bothrops* venoms.

Lomonte *et al.* (Lomonte *et al.*, 1994a; Lomonte *et al.*, 1994b) first described the binding of heparin to a lysine-rich site comprising residues 115-129 [numbering of (Renetseder *et al.*, 1985)] of the myotoxin Lys49 MT-II from *B. asper,* resulting in the neutralization of its *in vitro* cytolytic and *in vivo* myotoxic activities. The polyanionic heparin can interact in a non-covalent, charge-dependent way with basic myotoxin, forming an inactive acid-base complex, leading to inhibition of its myotoxic activity.

So far, it has been reported that heparin was used *in vivo* and *in vitro* to neutralize different toxic effects caused by some snake venoms and their $PLA_2$s (Melo and Suarez-Kurtz 1988; Melo, Homsi-Brandeburgo *et al.* 1993; Lomonte, Moreno *et al.* 1994; Lomonte, Tarkowski *et al.* 1994; Lin, Lee *et al.* 1999; Melo and Ownby 1999; Ownby, Selistre de Araujo *et al.* 1999; Beghini, Toyama *et al.* 2000; Soares, Guerra-Sa *et al.* 2000; Oshima-Franco, Leite *et al.* 2001; Calil-Elias, Martinez *et al.* 2002; Calil-Elias, Thattassery *et al.* 2002; Ketelhut, de Mello *et al.* 2003; Stábeli, Amui *et al.* 2006). Heparin derivatives with little or no effect on the coagulation system, *i.e.* heparin with low affinity for antithrombin (Lomonte *et al.*, 1994a; Lomonte *et al.*, 1994b) were considered as a complementary treatment of hemorrhages induced by snakes envenomations. Nevertheless, the therapeutic use of the heparin binding domain of a $PLA_2$ to prevent excessive bleeding mediated by heparin treatment had never been described. Indeed, In the present work, two novel Lys49 $PLA_2$ variants

from the venom of *B. moojeni* were isolated and fully sequenced by tandem mass spectrometry. They were found to interact with both unfractionated and low molecular weight heparins and to display promising properties for neutralization of anticoagulant effects.

## 6.3 Materials and methods

### 6.3.1 Crude venom

*Bothrops moojeni* crude venom was collected, pooled and dried at Pentapharm do Brasil. It was transported and stored in desiccated form and reconstituted in deionized water at Pentapharm Aesch. A 30% solution (250 mg/ml) in water was obtained and stored at -80°C.

### 6.3.2 Protein separation

***Size exclusion chromatography***

Crude, reconstituted venom was separated into 18 gel filtration fractions (fractions Botmo GF 1 to 18) on two in-line Superdex-75 (Pharmacia) XK 26 columns. Up to 250 mg of venom was loaded per run and the elution was performed using a buffer of 50 mM ammonium acetate, 150 mM sodium chloride, pH 7.5 at a flow rate of 2.2 ml/min (25 cm/h). The absorbance was monitored at 280 nm. To ensure the good reproducibility of the process, the columns were regenerated every 5 runs using 0.5 M NaOH. Collected fractions were stored at -80°C until further separation steps and various measurements were performed.

***RP-HPLC***

Fractions Botmo GF 10 and Botmo GF 12 (aliquot of 10 mg of protein) were submitted to solid phase extraction after acidification with aqueous 0.1 % trifluoroacetic acid (TFA), pH 2.2 and loading on Sep-Pak $C_{18}$ classic cartridge (Waters™). Solvatation, equilibration, sample application and elution were performed according to the manufacturer instructions. Elution was performed with a mobile phase of 40:60 aq. TFA (0.1 %): acetonitrile (ACN), the collected fraction were immediately dried in a SC210A SpeedVac™ Plus concentrator (ThermoSavant, Holbrook, NY, USA) and stored at -20 °C. The desalted fractions were processed by RP-HPLC on a Waters Alliance 2690 System using a semi-preparative HPLC column

(Vydac #218TP510 protein & peptide $C_{18}$, 10 mm x 250 mm). Freeze-dried fractions were reconstituted in aq. 0.1% TFA and centrifuged at 11,000 rpm before injection. Proteins and peptides were eluted using a 2-50% gradient of 90% ACN in aq. TFA (0.1 %) in 75 min at a flow rate of 3 ml/min. The absorbance was monitored at 225 nm and the fractions were collected manually, following peak absorbance. Collected sub-fractions of interest were called Botmo GF10/71 (MjTX-III) and Botmo GF12/84 (MjTX-IV), stored at -20°C and used for further measurements.

### 6.3.3 Bioassays

***Phospholipase $A_2$ activity detection***

For the determination of $PLA_2$ activity a secretory phospholipase $A_2$ kit (Assay Designs, Inc.; Ann Arbor, Michigan, USA; Catalog No. 907-002) was used following the manufacturer instructions.

***Immunometric assay for detection of type IIA $sPLA_2$***

For the determination of enzymatically non-active $PLA_2$-like proteins an immunoassay kit (Cayman Chemical; Ann Arbor, Michigan 48108, USA; Catalog No. 585000) was used following the manufacturer instructions.

***Influences on blood coagulation system***

The influences of the fractions on different levels of the blood coagulation cascade was studied using the automated Coagulation Test System BCS (Behring Coagulation System, Dade Behring; Marburg, Germany) and ball coagulometer KC4 A micro (Heinrich Amelung GmbH, Lemgo, Germany). Measurements of the effects on the clotting time in heparinized plasma were performed using aPTT (activated partial thromboplastin time) and Pefakit® PiCT® (prothrombinase induced clotting time) assays.

***aPTT*** assay was performed with normal and heparinized plasma. The measurements were conducted as follows: in 50 µl of plasma (ISTH/SSC plasma) normal or spiked with 0.5 U/ml of unfractionated heparin (UFH; WHO Standard, NIBSC; Potters Bar, England) or 0.5 U/ml of low molecular weight heparin (LMWH; WHO Standard, NIBSC; Potters Bar, England), coagulation was activated with 25 µl of aPTT reagent (HemosIL; Milano, Italy). A 50 mM Hepes pH 7.5 buffer was used as control. The reference heparin inhibitor protamine hydrochloride (ICN Pharmaceuticals Germany

GmbH; Frankfurt/Main, Germany) was applied at a concentration of 0.5 U/ml. The sub-fractions Botmo GF 10/71 (MjTX-III) and Botmo GF 12/84 (MjTX-IV) were used at the concentration of 130 μg/ml.

***Pefakit® PiCT®*** (Pentapharm Ltd., Basel, Switzerland) measurements were performed in normal and heparinized plasma. The plasma (50 μl) coagulation was activated by 25 μl of a reagent containing defined amount of FXa, phospholipids and RVV-V, a specific factor V (FV) activator from the venom of the *Daboia russelli* snake. MjTX-III or MjTX-IV (25 μl) was added, allowing their inhibitors to react with heparin during an incubation time of 3 min. Coagulation was triggered by the addition of $Ca^{2+}$ ions (50 μl of 25 mM $CaCl_2$ solution). Protamine hydrochloride 0.5 U/ml (ICN Pharmaceuticals Germany GmbH; Frankfurt/Main, Germany) and 50 mM Hepes pH 7.5 buffer were used as a positive (reference heparin antidote) and a negative control, respectively.

***Thromboelastometry analysis*** (ROTEM® Pentapharm GmbH Diagnostic Division; Munich, Germany) was applied to quantify the clot formation using ROTEM® standard reagents for all experiments: star-TEM® is a buffered $CaCl_2$ solution; in-TEM® and ex-TEM® (all by Pentapharm GmbH Diagnostic Division; Munich, Germany) are solutions for the activation of the intrinsic and the extrinsic pathways of blood coagulation, respectively. Briefly, 20 μl of star-TEM® and 20 μl of in-TEM® or ex-TEM®, as well as 20 μl of purified MjTX-III were added to 200 μl of normal or heparinized ROTROL N (Pentapharm Ltd.; Basel, Switzerland) reconstructed in water. ROTROL N is lyophilized standardized normal plasma pool. ROTROL N was spiked with 5 and 10 U/ml of UFH (WHO Standard, NIBSC; Potters Bar, England). The activity of the MjTX-III was evaluated at a concentration of 10 mg/ml.

***Chromogenic measurements*** were performed using Pefachrome®FXa (Pentapharm Ltd.; Basel, Switzerland), a chromogenic peptide substrate for factor Xa. The inhibition of heparin anticoagulant properties was measured in a 96-well plate after 3 min pre-incubation of 10 μl of a 2 U/ml UFH solution (WHO Standard, NIBSC; Potters Bar, England) or a 2 U/ml LMWH solution (WHO Standard, NIBSC; Potters Bar, England) with 10 μl of protein sample. MjTX-III and MjTX-IV were used at a concentration of 130 μg/ml in 150 μl 50 mM Hepes buffer, pH 7.5. Then, 10 μl of FXa (Hyphen BioMed; France), 10 μl of antithrombin III (ATIII, Grifols Germany; Langen/Hessen, Germany) and 20 μl of Pefachrome®FXa were added and the

absorbance monitored at 405 nm in 20 cycles over a period of 10 min, using the Multiwell fluorescence plate reader Tecan (Tecan Austria GmbH; Grödig/Salzburg, Austria).

***Cytotoxicity assays***

A cytotoxicity Detection Kit (LDH) (Roche Applied Science; Mannheim, Germany; Cat. No. 11 644 793 001) was used for the determination of cytotoxic action of MjTX-III. Normal human fibroblasts (NHF03 cells) were seeded at the density of $5x10^3$ cells/well in 96-well plates (Lindl, 2002) and then cultivated until confluence in a Minimal Essential Medium (MEM, PAN Biotech GmbH; Aidenbach, Germany) supplemented with 10% (v/v) foetal calf serum (FCS), 1% (v/v) gentamycin, 1% (v/v) glutamax, 1% (v/v) MEM non-essential amino acids, 1% (v/v) sodium pyruvate and 2% (v/v) Hepes. All the medium components were purchased from Gibco (Invitrogen; Basel, Switzerland). A positive control (0% toxicity) and a negative control (100% toxicity) consisted of assay medium and 0.1% Triton X-100 (Sigma-Aldrich Chemie GmbH; Steinheim, Germany), respectively. MjTX-III was tested in the following concentrations: 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml, 0.0625 mg/ml, 0.0312 mg/ml, and 0.015 mg/ml, without and with 1 U/ml UFH. After 3 h incubation at 37°C, a supernatant aliquot was collected for determination of lactate dehydrogenase (LDH) activity released from damaged cells.

### 6.3.4 Protein characterization

***MALDI-TOF-MS*** (Matrix Assisted Laser Desorption Ionisation – Time Of Flight Mass Spectrometry) analysis was carried out on a Voyager DE-STR instrument (Applied Biosystems, Foster City, CA, USA) operated in the linear positive ionisation mode, using sinapinic acid (SA) and alpha-cyano-4-hydroxycinnamic acid (4-HCCA) matrices. Fresh 10 mg/ml solutions of SA and 4-HCCA were prepared in 30% ACN containing 0.1% TFA. Target deposition was carried out using the dried droplet sample preparation method (Cohen and Chait, 1996). Briefly, 1 μl of the MjTX-III and MjTX-IV solution was diluted with 9 μl of the matrix and 1 μl was spotted onto the gold 100 well plate and allowed to dry. Five successive dilutions of the samples in the matrix solution were tested for both matrices. Bovine insulin and horse heart myoglobin (Sigma) were used as external calibration standards for the 4-HCCA and SA matrices, respectively. Typical instrument operating settings were: 25 kV

accelerating voltage, 92% grid voltage, 0.1% guide wire voltage, 450 nanosec extraction delay time, 500 Da low mass gate and 256 scans averaged. The acquired mass spectra were treated and analysed using the Data Explorer version 3.5 software (Applied Biosystems).

***ESI-MS*** (Electrospray Ionization Mass Spectrometry) analysis was performed on a Platform LCZ Micromass (Waters™) instrument equipped with a standard Z-spray ion source and operated in positive ionisation mode under control of the MassLynx 3.5 software (Waters) with the following conditions: mass range: *m/z* 500-1700 in 6 sec; capillary: 3.5 kV; cone voltage: 40 V, multiplier: 650 V. External calibration of the mass scale was performed with horse heart myoglobine (Sigma). MjTX-III and MjTX-IV were diluted in the solvent consisting of $H_2O$/ACN/formic acid (49.9/49.9/0.2 v/v/v) and infused at a flow rate of 10 μl/min into the mass spectrometer.

### *Reduction and Alkylation*

For reduction and alkylation, 45 μg of the stock solution (1 mg/ml in water) of MjTX-III or MjTX-IV was mixed with 15 μl of ammonium bicarbonate 2% (Fluka) and 5 μl of 45 mM DL-dithiothreitol (Fluka). Incubation was performed for 2 h at 37°C. 5 μl of 100 mM iodoacetamid (Sigma) was added to the reaction mixture and incubated for 15 min in the dark. The reaction was quentched with 5 μl of 200 mM cysteine (Fluka) and the mixture was acidified with aq. TFA 0.1% prior to HPLC separation. Alkylated proteins were separated by RP-HPLC on a Waters Alliance 2690 System equipped with a Vydac $C_{18}$ (218TP54), I.D. 4.6 x 250 mm column. Proteins were eluted using a biphasic gradient of ACN in 0.1% TFA at a flow rate of 0.8 ml/min. The absorbance was monitored at 214 nm and the fractions were manually collected following peak absorbance. The complete alkylation of proteins was checked by ESI-MS prior to enzymatic digestions.

### *Enzymatic digestion*

Enzymatic digestions of MjTX-III and MjTX-IV were performed using three different enzymes to ensure a good overlap in the sequence information. About 20 μg of alkylated protein was submitted to tryptic digestion by incubation with trypsin (Trypsin modified TRSEQZ) at a 1:50 enzyme/protein ratio for 3 h at 37°C. Another 20 μg of alkylated protein was submitted to digestion by *S. aureus* protease V8 (Staph protease STAP), performed at a 1:100 enzyme/protein ratio for 2 h at 37°C. Finally,

20 μg of alkylated protein was submitted to chymotrypsin (chymotrypsin-TLCK treated) digestion using a 1:50 enzyme/protein ratio during 1 h at 37°C. All the enzymes were purchased from Worthington Biochemical Corporation. The protein digest was fractionated by RP-HPLC on a Vydac $C_{18}$ , 4.6 mm x 250 mm HPLC column (218TP54) using a monophasic gradient of ACN in aq. 0.1% TFA. Peptide fractions were collected manually and freeze-dried before tandem mass spectrometry sequencing.

***ESI-MS/MS sequencing***

Tandem mass spectrometric analysis was carried out on a Q-TOF I (Micromass, Waters™) instrument operated in positive ionization mode under the control of the MassLynx 3.5 software. MjTX-III and MjTX-IV were dissolved in $H_2O$/ACN/formic acid (49.9/49.9/0.2 v/v/v) and analyzed by off-line nano-electrospray after loading of a 5 μl sample into a NanoES spray capillary (Proxeon). The parent ions were selected and the collision energy manually adjusted for proper fragmentation. Fragment spectra were acquired from *m*/*z* 50 to 2000 with a scan cycle of 2 s. External calibration was performed using human [Glu1]-fibrinopeptide B (1570.57 Da) in MS/MS mode. The multiply-charged MS/MS spectra were deconvoluted onto a singly-charged axis using the MaxEnt3 tool from MassLynx3.5 to allow complete *de novo* sequence analysis in both manual and semi-automated modes.

***Edman degradation***

Automated Edman degradation and detection of the phenylthiohydantoin derivatives was carried out with a pulse liquid automatic sequenator (model 473A; Applied Biosystems, Inc.).

# 6.4 Results

## 6.4.1 Fractionation

Size exclusion chromatography of *B. moojeni* crude venom performed on Superdex 75 columns resulted in 18 fractions (Figure 1) named Botmo GF 1 to 18.

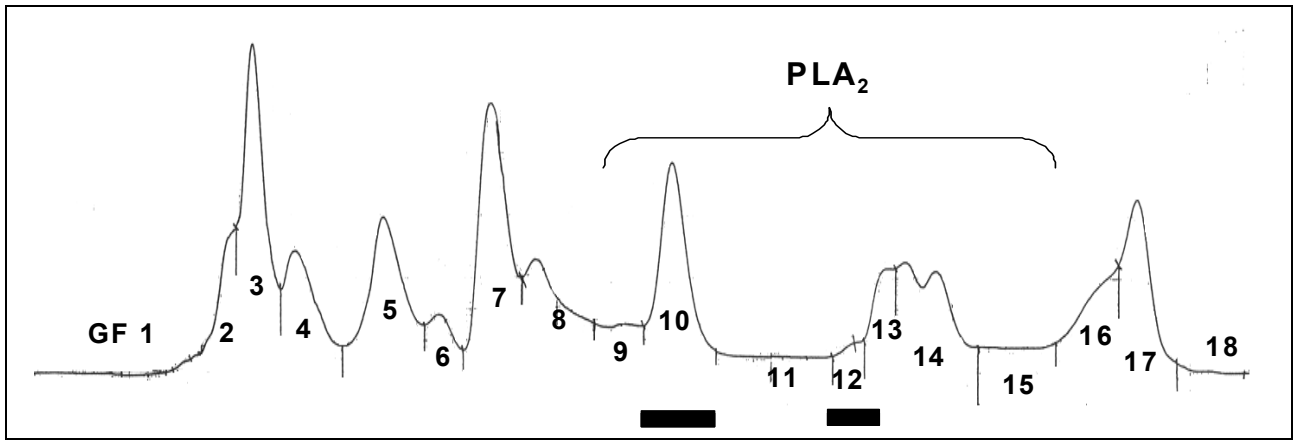



**Figure 1.** Gel filtration profile (GF 1 to GF 18) obtained with 250 mg of crude *B. moojeni* venom. Dark boxes: unfractionated heparin neutralization properties detected by BCS measurements.

Five grams of crude venom were separated by this method in 20 individual runs. Obtained purification profiles were highly comparable, allowing the pooling into appropriate Botmo GF fractions. According to MALDI-TOF-MS measurements performed on the GF fractions (data not shown) Botmo GF 1 to 18 fractions could be divided into high (Botmo GF 1 to 8) and low (Botmo GF 9 to 18) molecular weight fractions. Batroxobin was found in the Botmo GF 5 and neighborly fractions. In the low molecular weight Botmo GF 9 to 15 fractions, compounds in the molecular mass range of $PLA_2$s (13-15 kDa) were identified. Botmo GF 9 to 18 fractions were further processed by RP-HPLC leading to approximately 100 HPLC sub-fractions per GF fraction (e.g. Botmo GF 10/1 to 10/95 and Botmo GF12/1 to 12/104). The RP-HPLC separation profile of Botmo GF 10 is displayed in Figure 2, showing the presence of a major component in the fraction Botmo GF10/71 eluted after 37 min.

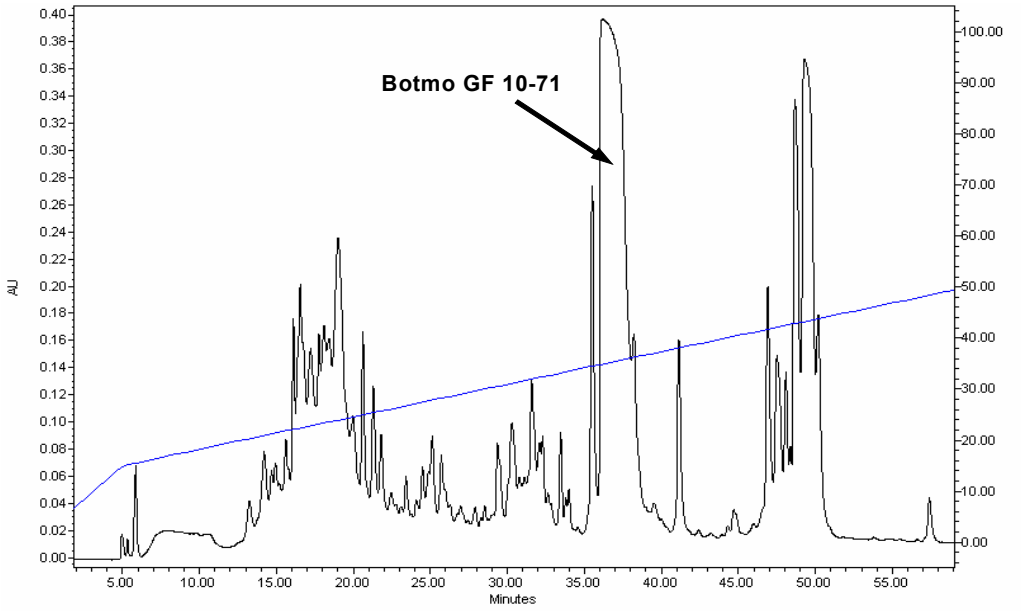



**Figure 2.** Semi-preparative RP-HPLC profile of the Botmo GF 10 fraction. Botmo GF10/71 (MjTX-III) showed the ability to neutralize the anticoagulant action of UFH.

### 6.4.2 Bioassays

The influences of all HPLC sub-fractions on the blood coagulation system were screened using the automated Coagulation Test System BCS (data not shown). The screening method consisted of a set of 14 tests detecting and evaluating different effects on distinct levels and mechanisms of the blood coagulation system and was previously described by Perchuc *et al.* (Perchuc *et al.*, 2005). By means of this screening method, the ability to neutralize anticoagulant properties of unfractionated heparin (UFH) could be identified in the Botmo sub-fractions GF 10/71, GF 12/84 and GF 14/76.

The molecular masses of the purified sub-fractions Botmo GF 10/71, GF 12/84 and GF 14/76 determined by ESI-MS were 13834.8 Da, 13812.9 Da, and 13873.3 Da, respectively. The molecular mass of Botmo GF 14/76 corresponds to the mass of the known Myotoxin II from *B. moojeni* (MjTX-II, Q91834, (Soares *et al.*, 1998). The MjTX-I previously described from the venom of *B. moojeni* (P82114, (Soares *et al.*, 2000) was not found in any of the investigated sub-fractions.

In addition, the phospholipase $A_2$ activity assay and the immunometric assay for the detection of type IIA secreted $PLA_2$ performed on the three sub-fractions, revealed their affiliation to the enzymatically non-active $PLA_2$ family (data not shown).

The blood coagulation bioassays were performed on Botmo GF 10/71 and Botmo GF 12/84 called MjTX-III and MjTX-IV, respectively. The results are only presented for MjTX-III. Similar results were obtained for MjTX-IV.

### *BCS-Test system and aPTT assay*

The ability of MjTX-III to inhibit the anticoagulant activity of UFH was further studied by means of the aPTT assay performed in heparinized plasma. When MjTX-III at a concentration of 0.2 mg/ml was added into heparinized plasma, the clotting time (CT) prolongation caused by UFH was shortened by approx. 50%: CT reduction from 64 s to 48 s. The CT obtained for the control sample performed in the plasma without heparin was 34 s (Figure 3).

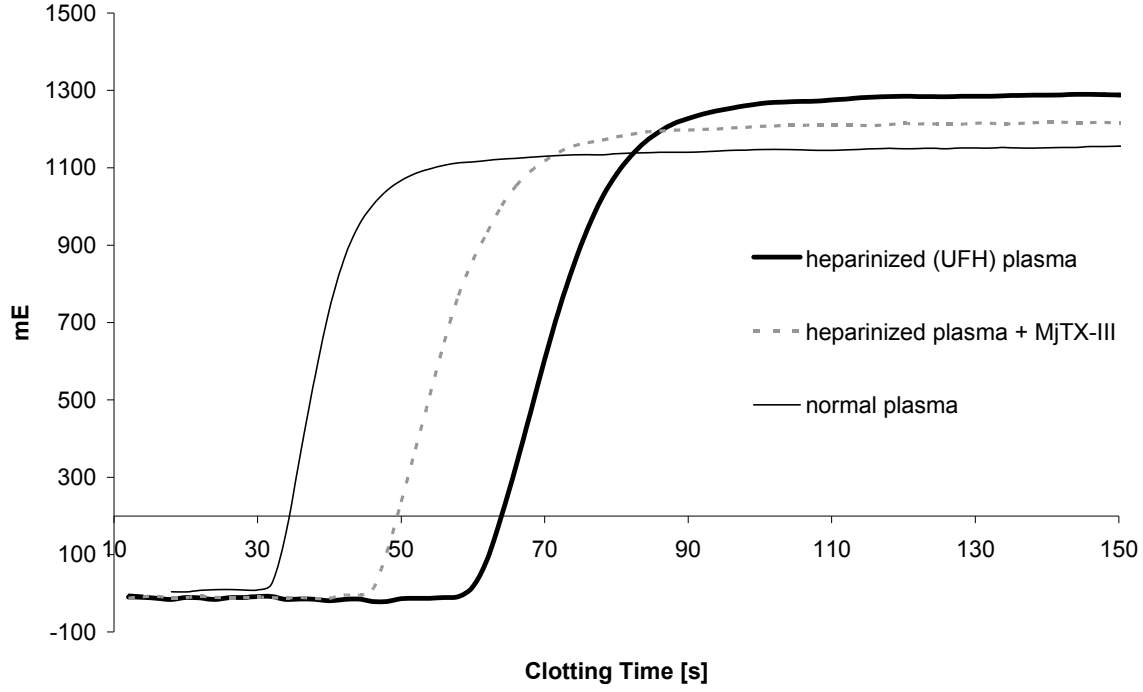


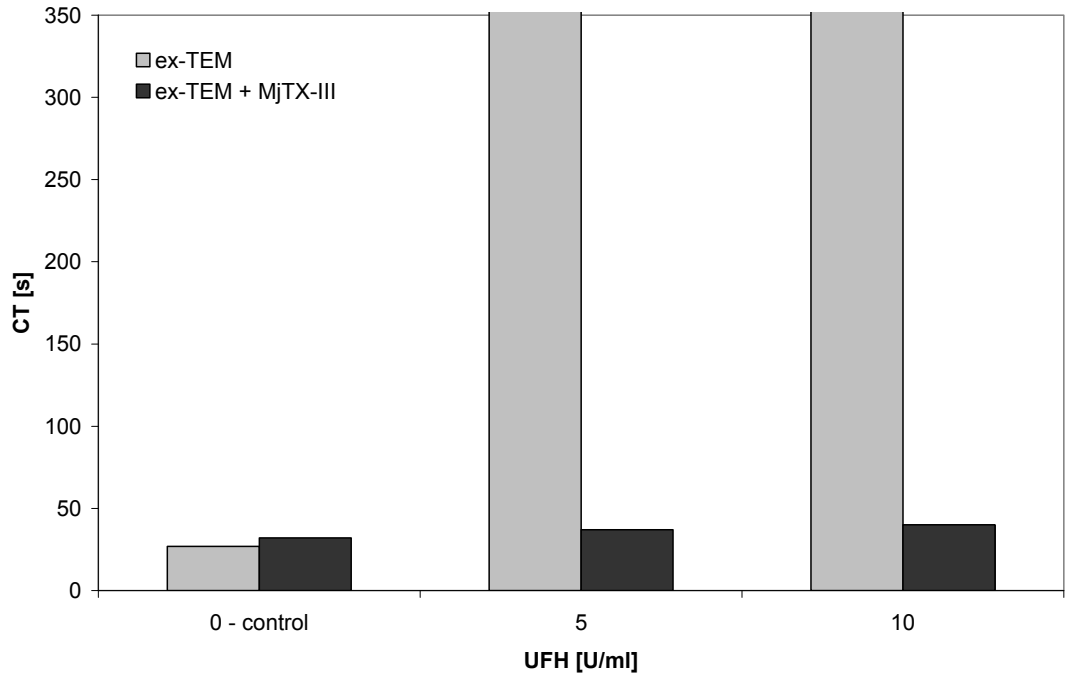

**Figure 3.** BCS-screening: aPTT assay performed in heparanized (UFH 0.5 U/ml) and normal plasma. MjTX-III was used at a concentration of 0.2 mg/ml.

### *Thromboelastometry*

Additional thromboelastometry experiments were performed using the ROTEM® system in order to verify and analyze in more detail the results obtained in plasma using the BCS tool. The heparin neutralizing activity of purified MjTX-III was

evaluated for both extrinsic and intrinsic pathways of blood coagulation, using ex-TEM and in-TEM reagents, respectively.

**A.**




**B.**

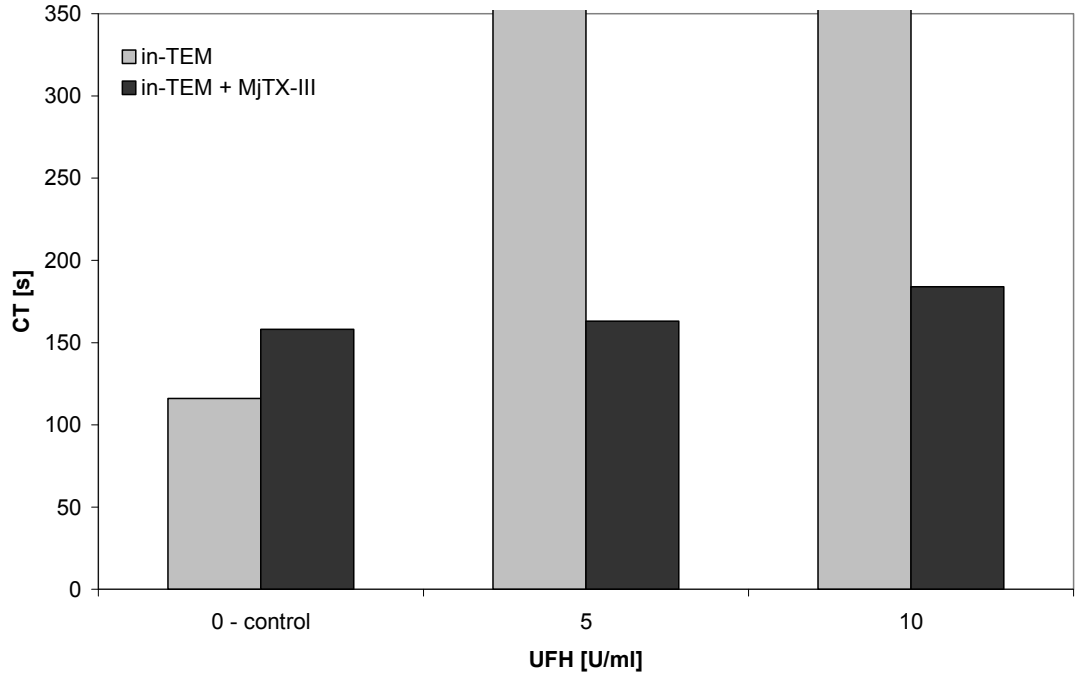



**Figure 4.** Extrinsic activation: Thromboelastometry measurement using the ROTEM® system and the ex-TEM test (diagram A) and in-TEM test (diagram B). Inhibition of UFH by MjTX-III used in the final concentration of 0.77 mg/ml. The inhibition of anticoagulant properties of UFH by MjTX-III was clearly observed in both measurements.

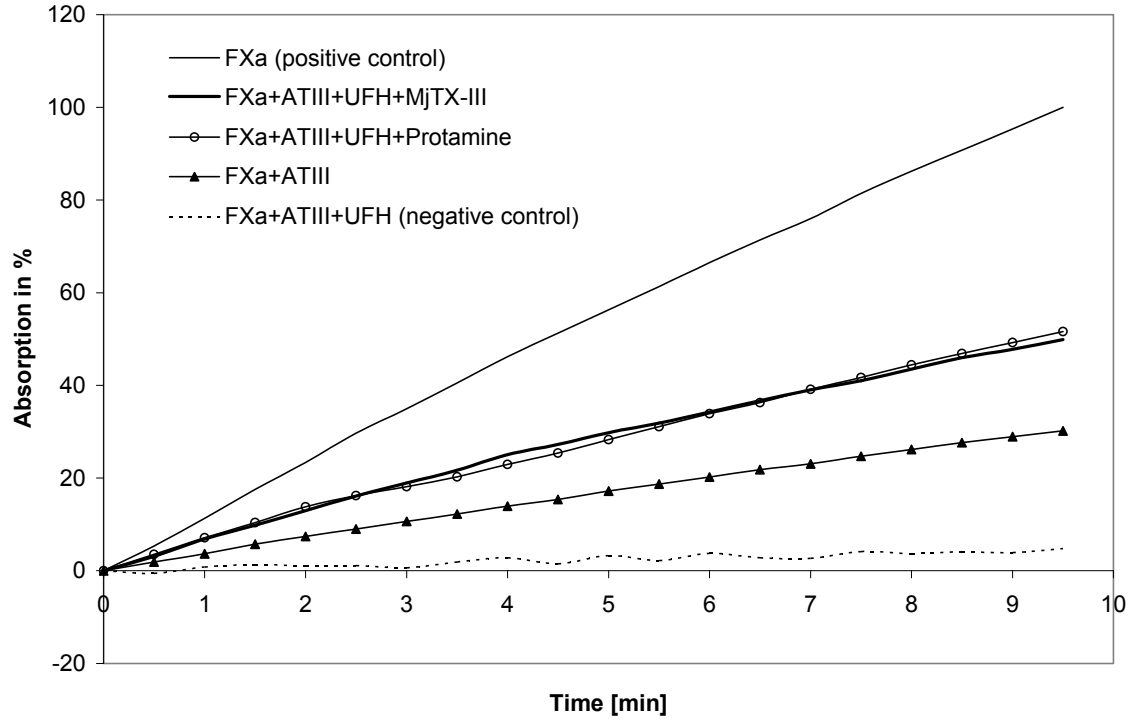
The activity of MjTX-III against UFH was detected by thromboelastometry measurements, however differences between both pathways could be observed. In the extrinsic pathway (Figure 4A), strong neutralization of UFH was detected. While plasma spiked with 5 and 10 U/ml of UFH showed very strong CT prolongations (over the measurement time of 350 s), the addition of MjTX-III at a final concentration of 0.77 mg/ml reduced the CTs significantly, almost to the control CT of 30 s. Interestingly, the addition of MjTX-III shortened the CT for both concentrations of UFH (5 and 10 U/ml) to nearly the same extent. A slight CT prolongation could, however, be observed in the experiments performed in normal plasma without heparin addition.

The intrinsic activation of blood coagulation in the ROTEM® system is weaker than the extrinsic one. The control CTs obtained for normal plasma were 116 and 27 s, respectively (for comparison see Figure 4A and B). In the intrinsic pathway, extreme CT prolongations (over the measurement time of 350 s) could be observed for plasma spiked with 5 and 10 U/ml of UFH. It could, however, be significantly lowered after the addition of MjTX-III (to 163 s and 184 s for 5 and 10 U/ml of UFH, respectively). The CT obtained for plasma spiked with 5 U/ml of UFH could be brought nearly down to the value obtained for MjTX-III in normal plasma (158 s). Interestingly, quite a strong CT prolongation was detected for MjTX-III in the experiments performed in normal plasma (158 s in comparison to the control of 116 s). This was an indication that MjTX-III, although deprived of $PLA_2$ enzymatic activity, prolongs the CT in normal plasma by interacting not just with heparin, but also with other blood coagulation factors. To prove the anticoagulant properties of MjTX-III, the aPTT assay was performed in normal plasma, confirming the previous observations (CT of 34.4 s in normal and 46.6 s in plasma with addition of MjTX-III).

***Chromogenic measurements***

The goal of these experiments was to confirm the ability of MjTX-III to bind and neutralize UFH (Figure 5A), to check its potential to inhibit LMWH (Figure 5B) and to compare those properties to the ones of protamine hydrochloride, the known and commonly used heparin antidote.

**A.**




**B.**

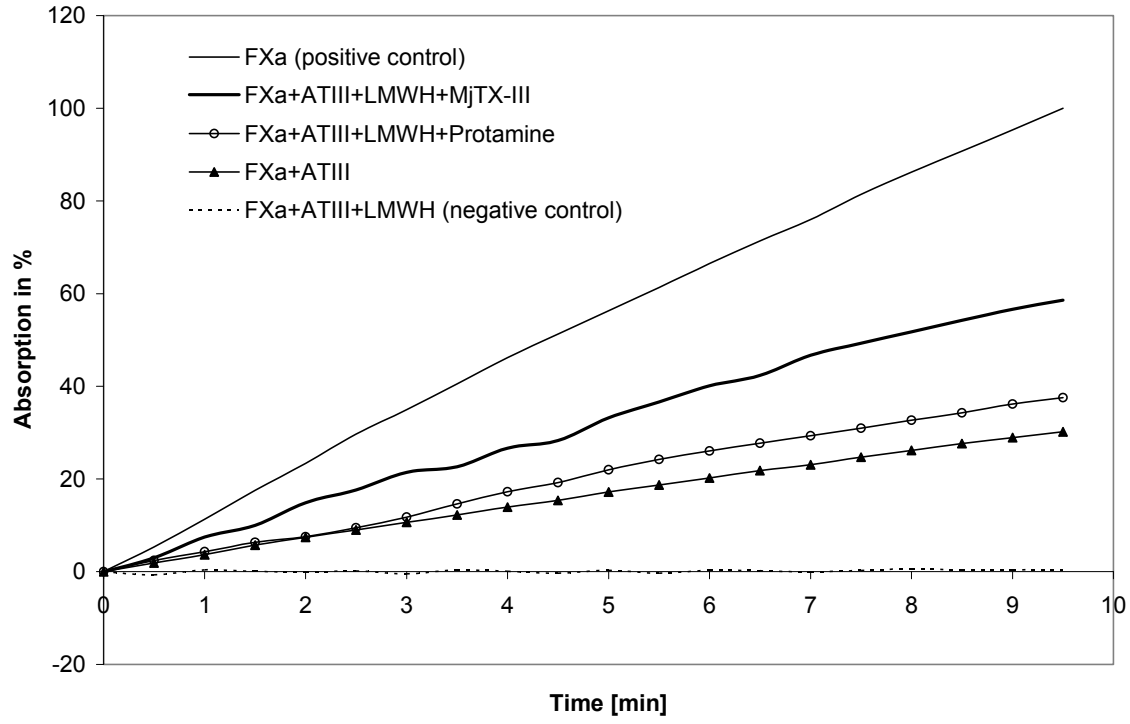



**Figure 5.** Chromogenic measurements using Pefachrome® FXa, inhibition of 0.5 U/ml of UFH (diagram A) and 0.5 U/ml of LMWH by MjTX-III used in the concentration of 0.13 mg/ml (diagram B). FXa alone was used as a positive control (100% activity, 100% absorption), FXa pre-incubated with ATIII and UFH/LMWH, as a negative control (0% activity, 0% absorption). The activity of MjTX-III was compared with protamine hydrochloride.

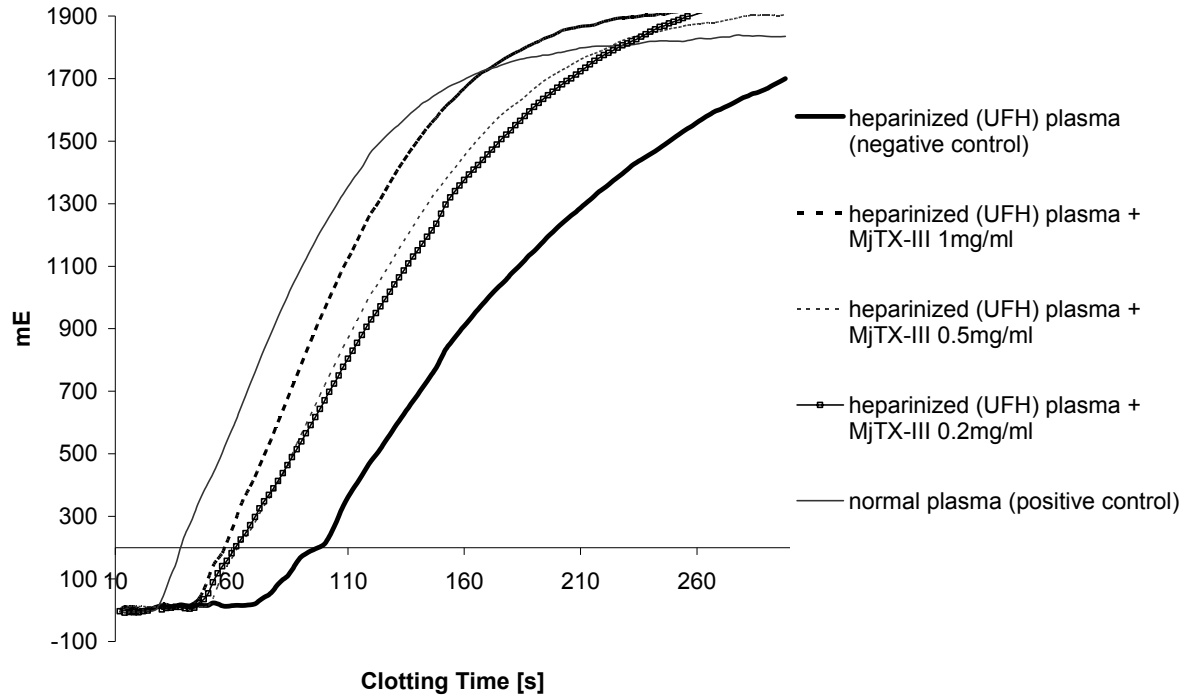
To this end, chromogenic measurements were performed using a chromogenic substrate for FXa activity, in an isolated, non-physiological system, where the enzyme recognizes and selectively cleaves its substrate releasing a chromogenic group, which is detectable at a specific wavelength. FXa was used as positive control (100% of FXa activity) and FXa preincubated with antithrombin III (ATIII) and UFH or LMWH as a negative control (0% of FXa activity). FXa preincubated with ATIII was used to show the function of UFH and LMWH in complete inhibition of FXa activity and thus their anticoagulant significance. Heparin catalyzes the interaction between ATIII and the coagulation factors, accelerating the complex formation and inhibition by 1000-fold. As seen in Figure 6, heparin-binding as well as heparin-neutralizing properties of MjTX-III could be confirmed for both UFH and LMWH, when various preincubation times of MjTX-III and heparin were applied (data not shown).

MjTX-III was found to neutralize anti-FXa activity of UFH to the same extent as protamine hydrochloride (Figure 5A) in its therapeutically recommended dose (1U of protamine neutralizes 1U of UFH). MjTX-III showed, however, increased inhibitory properties against LMWH when compared to protamine hydrochloride (Figure 5B).

***BCS-Test system and PiCT® test***

The study of UFH and LMWH neutralization was performed in a more physiological test system applying human plasma. Plasma clotting was triggered using the PiCT® test, which is an assay specifically designed for the determination of anticoagulant activity based on FXa and/or FIIa inhibition, thus especially suitable for investigating the properties of different heparins and their antidots.

**A.**




**B.**

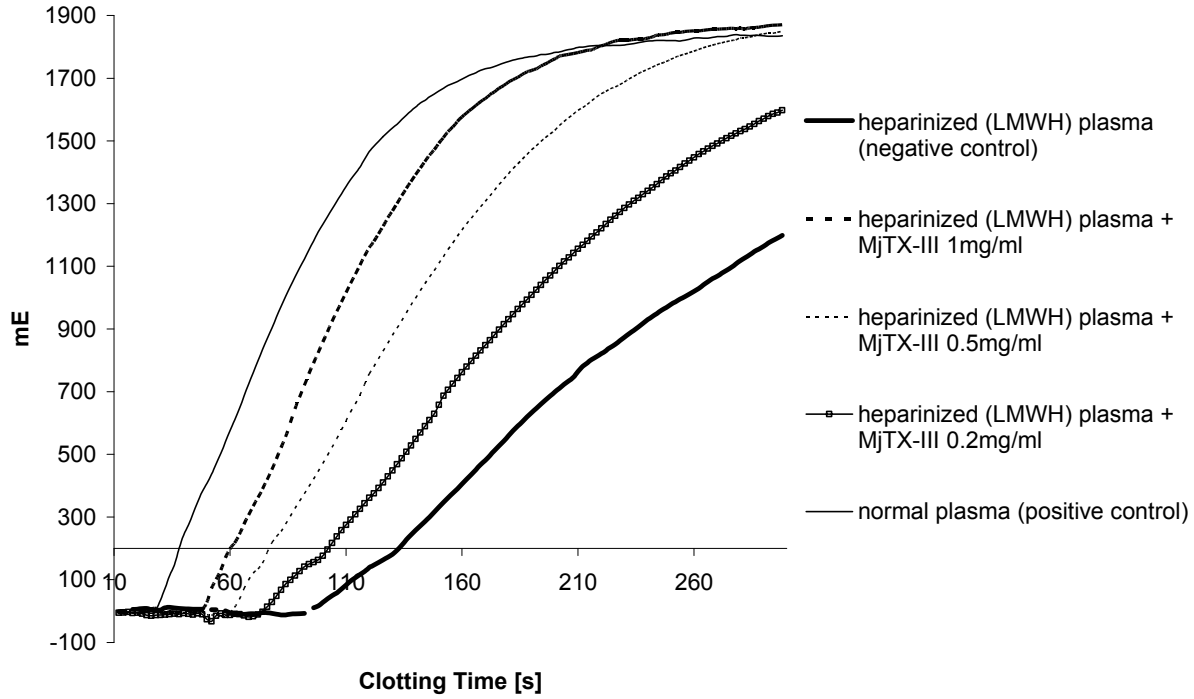



**Figure 6.** BCS-measurements: PiCT® assay performed in plasma spiked with 0.5 U/ml of UFH (diagram A) or 0.5 U/ml of LMWH (diagram B). MjTX-III was used in three concentrations: 1 mg/ml, 0.5 mg/ml and 0.2 mg/ml. Measurements were also performed in normal plasma as positive control.

Consequently, heparinized plasmas were applied for the PiCT® measurements. For the first experiments plasma spiked with 0.5 U/ml of UFH was used (figure 6A). Three different concentrations of MjTX-III (0.2 mg/ml, 0.5 mg/ml and 1 mg/ml) were added into the plasma samples. Measurements in normal and in heparinized plasma without addition of any MjTX-III sample were performed as positive and negative controls, respectively, resulting in a CT of 33.1 s in normal and 81.6 s in heparinized plasma. The neutralization of the anticoagulant action of UFH was clearly measured, however no significant differences between the three concentrations of MjTX-III could be observed (57.1 s for the concentration of 0.2 mg/ml, 56.9 s for 0.5 mg/ml and 50.9 s for 1 mg/ml). Plasma spiked with 0.5 U/ml of LMWH was utilized and again three concentrations of MjTX-III tested (figure 6B). Normal and heparinized plasmas without addition of MjTX-III were used as positive and negative controls, respectively resulting in a CT of 33.1 s in normal and 107.7 s in heparinized plasma. The inhibition of LMWH by MjTX-III was also clearly detectable in a protein concentration-dependent manner (85.2 s for the concentration of 0.2 mg/ml, 67.1 s for 0.5 mg/ml and 54.4 s for 1 mg/ml). Finally, the PiCT® experiment was performed in normal plasma (figure 7), confirming the clotting time prolongation already observed in the ROTEM® system and the aPTT screen. This suggests that MjTX-III probably binds not only to heparin but also to a component of the prothrombinase complex, inhibiting the clotting process.

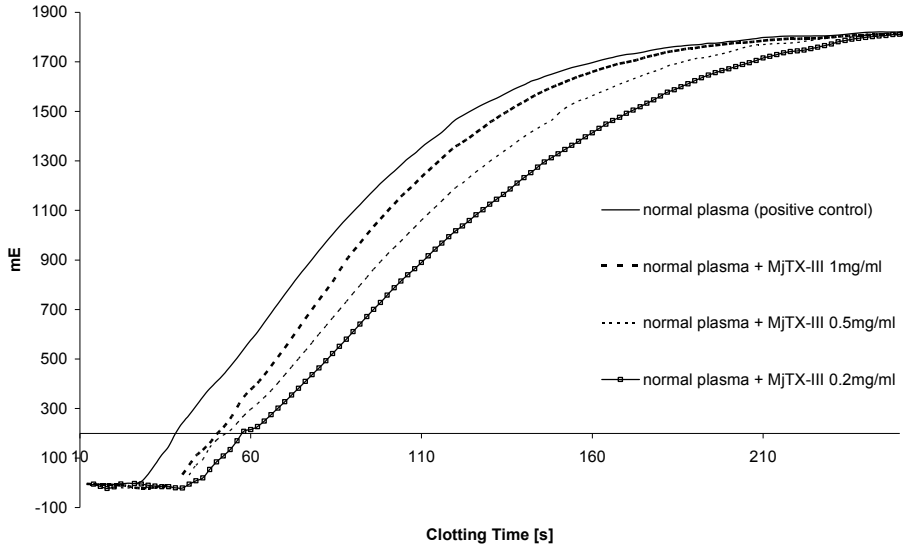



**Figure 7.** BCS-screening: PiCT® assay performed in normal plasma. MjTX-III used in three concentrations: 1 mg/ml, 0.5 mg/ml and 0.2 mg/ml.

***Ball coagulometer and PiCT® test***

The results obtained in various PiCT® measurements on the BCS system were verified utilizing a ball coagulometer (Table 1). The CTs obtained confirmed that MjTX III causes prolongation of the clotting process in normal plasma, but neutralizes the anticoagulant action of both heparins in a concentration-dependent manner. The CT prolongation in normal plasma was not concentration-dependent.

**Table 1.** Results of the PiCT® assay performed in normal and heparanized (0.5 U/ml) plasma, using ball coagulometer KC4A micro.

| | **PiCT (-)** **CT [s]** | | **PiCT (UFH)** **CT [s]** | | **PiCT (LMWH)** **CT [s]** | |
|---|---|---|---|---|---|---|
| **Buffer** | **35.4** | **36.2** | **126.6** | **126.7** | **101.2** | **99.8** |
| **MjTX-III (1mg/ml)** | **54.1** | **53.7** | **61.5** | **62.6** | **64.2** | **64.0** |
| **MjTX-III (0.5mg/ml)** | 61.4 | 61.0 | 72.2 | 72.0 | 75.9 | 75.8 |

## 6.4.3 Protein characterization

The reduced and alkylated MjTX-III displayed a mass of 14649.0 Da (mass difference after alkylation: 814 Da = 14 x 57 Da + 16), revealing that 14 cysteines were alkylated and that one oxidation occurred (+16). The alkylated protein was digested using three proteases (trypsin, *S. aureus* protease V8 and chymotrypsin) giving about fifty fractions after RP-HPLC separation of the digest (data not shown). These fractions were analyzed by MALDI-TOF-MS and the obtained masses were submitted to Mascot Peptide Mass Fingerprint searches, giving high homologies with already described K49-$PLA_2$ from other snake species. About 20 fractions were then judiciously selected for MS/MS sequencing according to their predicted position in order to ensure the full sequence characterization. Figure 8 presents the MS/MS fragmentation spectrum of the parent ion at *m/z* 949.43 which corresponds to the multiply-charged species $[M+3]^{3+}$.

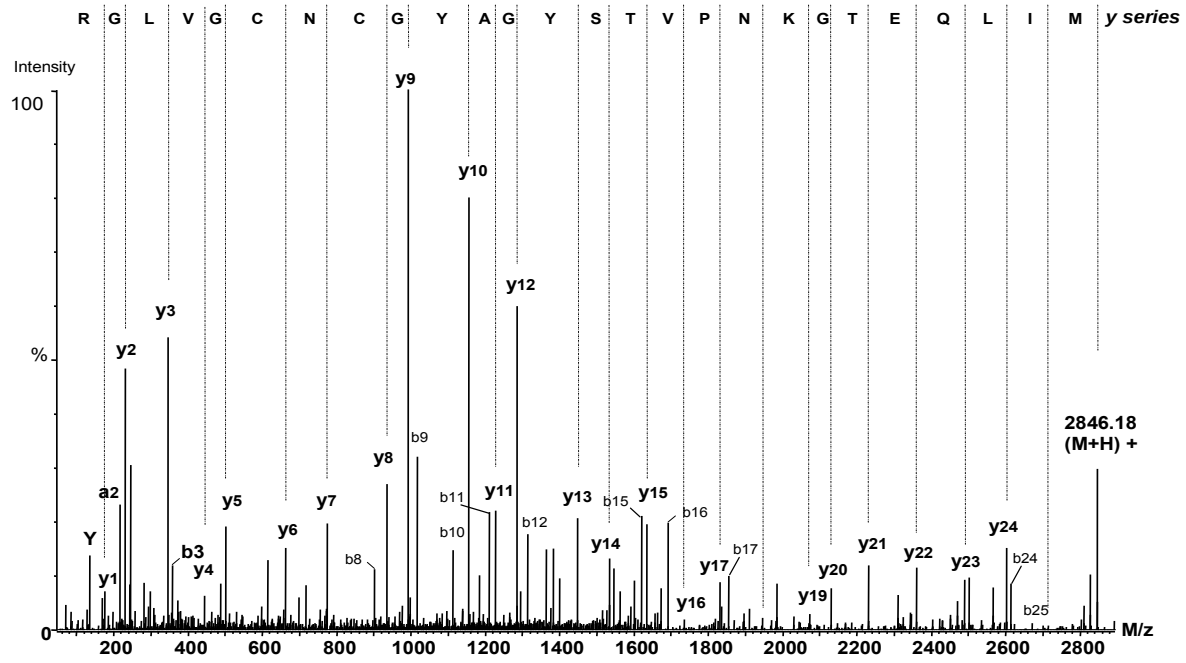



**Figure 8.** ESI-MS/MS spectrum of the triply charged signal at m/z 949.43, corresponding to the molecular mass 2845.29 Da. The multiple charged spectrum was deconvoluted into a singly charged axis using the MaxEnt3 routine from MassLynx 3.5 to facilitate *de novo* sequence analysis. In the fragmentation profile of one of the peptides obtained after enzymatic digestion of MjTX-III the *b*- and *y*-fragment ions are indicated together with the deduced sequence shown on the top of the MS/MS profile.

Sequence analysis of this peptide attributed most of the abundant signals to the *y* ion series and allowed the determination of the amino acid sequence. The sequence M(I/L)(I/L)QETG(K/Q)NPVTSYGAYGCNCGV(I/L)GR corresponds to the particular peptide M8-R33 of K49-$PLA_2$. Observation of *d* and *w* fragments for I and L attribution were previously described using an ESI-QTOF mass spectrometer, a high cone voltage fragmentation and low-energy CID MS/MS (de Souza *et al.*, 2004). Nevertheless, no discrimination between the isobaric residues pairs I/L and K/Q could be obtained in with the applied experimental conditions. For this reason, Edman degradation was performed on selected digest peptides, permitting the removal of all remaining sequence ambiguities. The discrimination between K and Q could be easily done since trypsin digestion facilitated location of all N-terminal K residues of digested peptides. Only two K/Q ambiguities at positions 15 and 69 had

to be resolved. Indeed, the tryptic fragments M8-R33 and Y64-K71 could be isolated, showing miss-cleavage sites at residues K15 and K69. The +16 Da mass difference previously observed after reduction and alkylation was attributed to the oxidation of a carbamidomethylated cysteine, since the Met residue of the M8-R33 fragment was shown to be reduced (-CH2CH2SCH3). Alkylation blocks the thiol but the sulphoxide -S(O)CH2CONH2 can still be formed. Indeed, oxidation may occur with air and trace amount of iodine contained in iodoacetamide. The full sequences of MjTX-III and MjTX-IV were successfully obtained after ESI-MS/MS analysis of about 20 fractions, fragmentation of 75 parent ions and interpretation of about 40 MS/MS spectra for each protein. The full sequences obtained are displayed in Table 2 together with the alignment of K49 $PLA_2$ from various snake venoms especially from *Bothrops* genus. The calculated masses fit exactly with the measured masses for both myotoxines. The two new isoforms of *B. moojeni* myotoxines share a high homology with Lys49 $PLA_2$ myotoxins from other *Bothrops* venoms (78%- 98% of identities with myotoxins shown in Table 2).

**Table 2.** Alignment of the amino acid sequences of two novel K49-$PLA_2$s characterized from the venom of *Bothrops moojeni* with some previously known myotoxins.

| Name | Sequence | Ref. |
|---|---|---|
| | 1 11 21 31 41 51 | |
| *B. moojeni* GF10-71(MjTX-III) | S **L** V E **L** G K M **I** **L** Q E T G **K** N P V T S Y G A Y G C N C G V **L** G R G K P K D A T D R C C Y V H K C C Y K K **L** T D C N P K | This work |
| *B. moojeni* GF12-84 (MjTX-IV) | S **L** V E **L** G K M **I** **L** Q E T G **K** N P **L** T S Y G A Y G C N C G V G G R G K P K D A T D R C C Y V H K C C Y K K M T D C D P K | This work |
| *B. moojeni* MjTX-I | S L V E L G K M I L Q E T G K N P A K S Y G A Y G C N C G V L G R G K P K D A T D R C C Y V H K C C Y K K L T N C D P K | 1 |
| *B. moojeni* MjTX-II | S L F E L G K M I L Q E T G K N P A K S Y G V Y G C N C G V G G R G K P K D A T D R C C Y V H K C C Y K K L T G C D P K | 2 |
| *B. asper* myotoxin II | S L F E L G K M I L Q E T G K N P A K S Y G A Y G C N C G V L G R G K P K D A T D R C C Y V H K C C Y K K L T G C N P K | 3 |
| *B. asper* myotoxin V | S L V E L G K M I L Q E T G K N P V T S Y G A Y G C N C G V L G R G K P K D A T D R C C Y V H K C C Y K K L T G C N P K | 4 |
| *B. pirajai* PrTX-II | S L F E L G K M I L Q E T G K N P A K S Y G A Y G C N C G V L G R G K P K D A T D R C C Y V H K C C Y K K L T G C N P K | 5 |
| *B. atrox* myotoxin I | S L V E L G K M I L Q E T G K N P L T S Y G A Y G C N C G V G G R G K P K D A T D R C C Y V H K C C Y K K M T D C D P K | 6 |
| *B. jararacussu* BthTx-I | S L F E L G K M I L Q E T G K N P A K S H G A Y G C N C G V L G R G K P K D A T D R C C Y V H K C C Y K K L T G C D P K | 7 |
| *A. p. piscivorus* AppK49 | S V L E L G K M I L Q E T G K N A I T S Y G S Y G C N C G W G H R G Q P K D A T D R C C F V H K C C Y K K L T D C N H K | 8 |
| *T. gramineus* $PLA_2$ V | S V I E L G K M I F Q E T G K N P A T S Y G L Y G C N C G P G G R R K P K D A T D R C C Y V H K C C Y K K L T D C D P I | 9 |
| | 61 71 81 91 101 111 121 | |
| *B. moojeni* GF10-71 | K D R Y S Y S W **K** D K T **I** V C G E N N S C **L** K E **L** C E C D K A V A **I** C **L** R E N **L** D T Y N K K Y K N N Y **L** K P F C K K A D P C | This work |
| *B. moojeni* GF12-84 | K D R Y S Y S W **K** D K T **I** V C G E N N S C **L** K E **L** C E C D K A V A **I** C **L** R E N **L** D T Y N K K Y K N N Y **L** K P F C K K A D P C | This work |
| *B. moojeni* MjTX-I | K D R Y S Y D W K N K T I V C G E E N P C L K Q L C E C D K A V A I C L R E N K G T Y N K K R D V Y L K P F C D K G R D C | 1 |
| *B. moojeni* MjTX-II | K D R Y S Y S W K D K T I V C G E N N S C L K E L C E C D K A V A I C L R E N L D T Y N K K Y R Y N Y L K P F C K K A D P C | 2 |
| *B. asper* myotoxin II | K D R Y S Y S W K D K T I V C G E N N S C L K E L C E C D K A V A I C L R E N L N T Y N K K Y R Y Y L K P L C K K A D A C | 3 |
| *B. asper* myotoxin V | K D R Y S Y S W K D K T I V C G E N N S C L K E L C E C D K A V A I C L R K N L D T Y N K K Y K N N Y L K P F C K K A D P C | 4 |
| *B. pirajai* PrTX-II | K D R Y S Y S W K D K T I V C G E N N P C L K E L C E C D K A V A I C L R E N L G T Y N K K Y R Y H L K P F C K K A D D C | 5 |
| *B. atrox* myotoxin I | K D R Y S Y S W K D K T I V C G E K N S C L K E L C E C D K A V A I C L R E N L D T Y N K K Y K N N Y L K P F C K K A D A C | 6 |
| *B. jararacussu* BthTx-I | K D R Y S Y S W K D K T I V C G E N N P C L K E L C E C D K A V A I C L R E N L G T Y N K K Y R Y H L K P F C K K A D A C | 7 |
| *A. p. piscivorus* AppK49 | T D R Y S Y S W K N K A I I C E E K N P C L K E M C E C D K A V A I C L R E N L D T Y N K K Y K A Y F K L K C K K P D T C | 8 |
| *T. gramineus* $PLA_2$ | K D R Y S Y S W V N K A I V C G E D N P C L K E M C E C D K A V A I C F R E N L D T Y D K K K K I N L K L F C K K T S E Q C | 9 |

Positions in grey indicate strictly conserved residues. Residues in bold have been unveiled by Edman degradation. References: (1) (Soares, Andriao-Escarso *et al.* 2000); (2) (Soares, Rodrigues *et al.* 1998); (3) (Francis, Gutierrez *et al.* 1991); (4) (Pescatori, Grasso *et al.* 1998 ; Q9PVE3) (5) (Toyama, Soares *et al.* 2000); (6) (Nunez, Arce *et al.* 2004); (7) (Cintra, Marangoni *et al.* 1993); (8) (Maraganore and Heinrikson 1986); (9) (Nakai, Nakashima *et al.* 1995). Note that Q9PVE3, submitted to GenBank by Pescatori, Grasso *et al.* (1998), is here referred to as *B. asper* myotoxin V (instead of III as in the submission), to avoid confusion with the *B. asper* myotoxin III previously characterized by Kaiser *et al.* (1990) (Nunez, Arce *et al.* 2004).

### *In vitro toxicity*

The *in vitro* toxicity of MjTX-III was determined using normal human fibroblasts (NHF) incubated with the fraction in the presence or absence of UFH (figure 9).

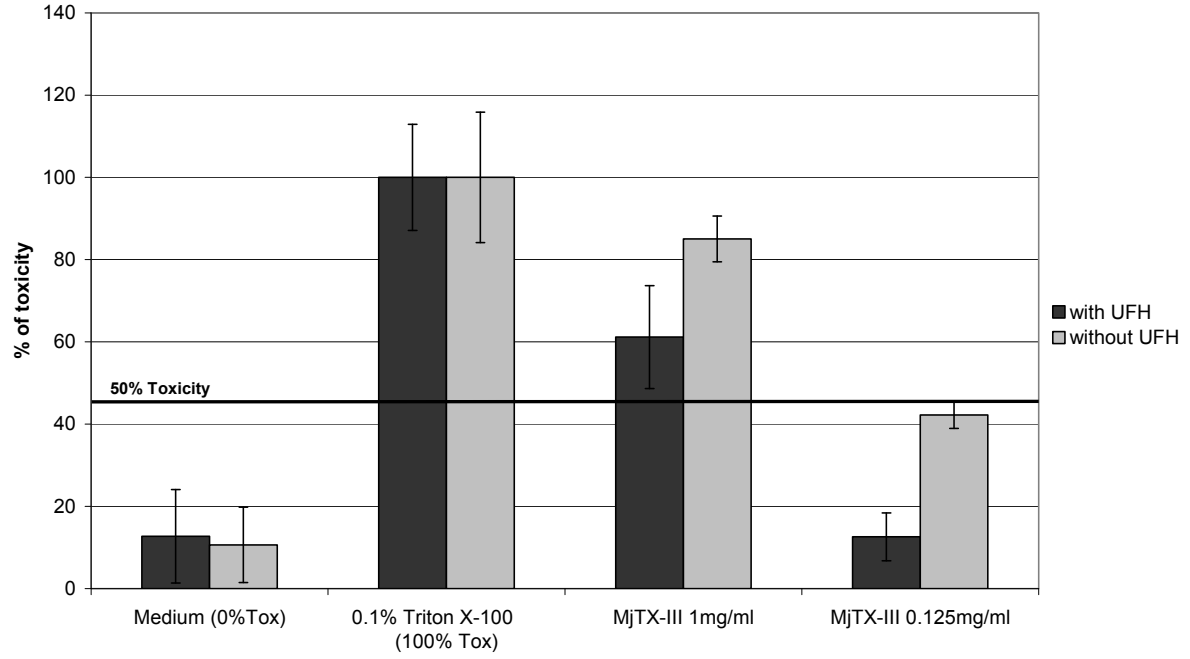



***Figure 9.*** Cytotoxicity of MjTX-III, measurements with and without UFH (1 U/ml); LDH assay; after 3 h of incubation at 37°C. LDH release is expressed as a percentage, considering the activity of cells treated with 0.1% Triton X-100 as 100%.

UFH was used in order to prove its ability to establish an inhibitory interaction with the possibly toxic MjTX-III. Such interactions between heparin and Lys49 $PLA_2$ myotoxins have already been reported. In our study, MjTX-III showed significant toxic effects against the NHF cells which could be considerably reduced by the presence of UFH.

## 6.5 Discussion and conclusions

The crude venom of *Bothrops moojeni* snake has been fractionated, analyzed by complementary MS-techniques and screened for diverse bioactivities. Special attention was paid to activities observed in the field of hemostasis and fibrinolysis. Among diverse influences on the blood coagulation system, heparin neutralization gained our particular interest and the fractions showing such properties were further

characterized. This work describes for the first time the screening of two new isoforms of Lys49 $PLA_2$ in blood coagulation assays and their ability to neutralize the anticoagulant properties of UFH and LMWH in an isolated non-physiological system, as well as in blood plasma.

The two novel Lys49 $PLA_2$-like proteins MjTX-III and MjTX-IV were isolated and fully sequenced by tandem mass spectrometry (Table 2). The few remaining sequence ambiguities were solved using the Edman degradation technique. According to literature, they belong to class II $PLA_2$s on the basis of the Cys residue positions and the characteristic C-terminal extension (Soares *et al.*, 2000). Secreted $PLA_2$ type IIA are found in the venoms of vipers, cobras, rattlesnakes and kraits. MjTX-III and MjTX-IV show a high sequence homology with two already described *B. moojeni* myotoxins: MjTX-III shows 85% and 95% homologies with respectively, MjTX-I and MjTX-II; MjTX-IV display 84% and 94% homologies with MjTX-I and MjTX-II respectively. In addition, those two novel myotoxins display striking homologies with myotoxin V isolated from the venom of *B. asper* (98% for MjTX-III and 95% for MjTX-IV). In an isolated, non-physiological system as well as in blood coagulation screening, the two substances showed an inhibition of anticoagulant properties of both UFH and LMWH.

Two specific assays, the aPTT and the PiCT, were used in order to analyze the anticoagulant properties of heparins in plasma and the neutralization thereof. The classical aPTT screening is a functional test for the effects on the intrinsic pathway of blood coagulation. The PiCT® test is a relatively new functional clotting assay (Calatzis *et al.*, 2000) used for the monitoring of all types of heparins, heparinoids and direct thrombin inhibitors such as hirudin. In this test, the determination of anticoagulant activity is based on factor Xa (FXa) and/or factor IIa (FIIa) inhibition. Therefore, it was an ideal tool to monitor the interaction of MjTX-III with different heparins under conditions close to physiological ones.

Apart of the experiments performed in heparinized plasma, properties of MjTX-III were also verified in normal plasma, showing unexpectedly some influences on the normal clotting time. It was surprising as according to Lomonte *et al.* (Lomonte *et al.*, 1990) the two Lys49 $PLA_2$ myotoxins from *Bothrops moojeni* did not have any detectable anticoagulant effect on sheep platelet-poor plasma. Therefore, a clear explanation for the clotting time prolongation caused by MjTX-III remains to be found.

The normal CT prolongation effect of MjTX-III was observed mostly for the intrinsic pathway of blood coagulation (aPTT and in-TEM tests), where the contact activator is a suspension of negatively charged particles such as kaolin or micronized silica, ellagic acid, a polyphenol, or sulfatides combined with kaolin. Therefore, we initially proposed that the myotoxin, rich in basic residues, can bind to these negatively charged surfaces inhibiting the triggering of the normal clotting. Similar prolongation could however also be detected for the extrinsic pathway in the ex-TEM measurements, as well as in the PiCT® screen, where the measured clotting depends only on the prothrombinase complex formation. Therefore, in addition to the interaction with heparins, MjTX-III probably binds to a component of prothrombinase complex slowing down the coagulation process. However, interaction with heparin seems to be stronger, as the inhibition of its anticoagulant action could be evidently observed in heparinized plasma. A similar inhibition of prothrombinase activity was described for the catalytically inactive $sPLA_2$ (Ba IV) from *B. asper* venom and for the basic subunit CB of the crotoxin isolated from *Crotalus* sp. (Mounier *et al.*, 2001). A possible explanation was given by a putative interaction between myotoxins and FXa (Mounier *et al.*, 2000; Mounier *et al.*, 2001). Also Kini (Kini, 2005) reported that the strongly anticoagulant CM-IV, a $PLA_2$ enzyme isolated from the venom of *Naja nigricollis,* inhibited the prothrombinase complex by a nonenzymatic mechanism. CM-IV would compete with FVa for direct binding to FXa, thus blocking the prothrombinase complex formation. Targeting of FXa and inhibition of the prothrombinase complex appears to be a commonly admitted mechanism of $PLA_2$s anticoagulant effect.

Analyzing the biological activities of MjTX-III, our attention was mainly drawn to its interesting anti-heparin properties, especially the inhibition of LMWH. Heparin had already been shown to neutralize the myotoxic (cytolytic) effects of *Bothrops jararacussu* venom and its bothropstoxin on isolated mammalian skeletal muscles (Melo and Suarez-Kurtz, 1988b; Melo and Suarez-Kurtz, 1988a; Melo *et al.*, 1993). Lomonte *et al.* (Lomonte *et al.*, 1994b) further investigated this finding utilizing myotoxin II, a basic Lys49 $PLA_2$ purified from the venom of *B. asper* (Lomonte and Gutierrez, 1989) and myotoxin III (Kaiser *et al.*, 1990), a Asp49 isoform possessing the $PLA_2$ activity. The conclusion drawn from this work was that in cell culture as well as *in vivo* glycosaminoglycans of the heparin/heparan sulfate family blocked the cytolytic action of myotoxin II. In addition, it was found that the interaction of myotoxin

III with heparins significantly reduced its myotoxic effect without inhibiting its enzymatic activity in an indirect hemolysis assay (Lomonte *et al.*, 1994b). The interactions of heparin with $PLA_2$s of different origins have been described to affect in some cases the enzymatic activity of $PLA_2$, but not in the others (Lomonte *et al.*, 1994a; Lomonte *et al.*, 1994b). For example, the enzymatic activity of porcine pancreatic $PLA_2$ could be inhibited by heparin binding to the N-terminus of the protein (Diccianni *et al.*, 1990). This effect was observed on micellar phospholipids substrates but not on monomeric phospholipids (Diccianni *et al.*, 1991). As the 26 N-terminal residues are known to act as the recognition site of this $PLA_2$, heparin binding to that site leads to conformational changes and blocks the access to the micellar substrates. Nevertheless, heparin binding does not destroy the enzyme's catalytic activity, which could be observed by cleavage of monomeric phospholipids. Similar observations were described by Dua and Cho (Dua and Cho, 1994) for human secretory class II $PLA_2$ and related cationic $PLA_2$s from the venoms of *Agkistrodon halays blomhoffii* and *Agkistrodon piscivorus piscivorus*. Heparin neither blocked nor bound to the active site of these $PLA_2$s, as the enzymes were still able to hydrolyze water-soluble monomeric substrates. Bugs and coworkers (Bugs *et al.*, 2005) studied the Lys49 bothropstoxin-I (BthTx-I) and suggested that heparin binds to any basic residues of the BthTx-I through strong electrostatic interactions between the negatively charged heparin molecules and any positively charged regions on the protein surfaces. The enzyme interaction with heparin was shown to be exclusively dependent on size (number of disaccharide repeats) and charges (Bugs *et al.*, 2005).

Regarding the heparin binding site, Lomonte *et al.* (Lomonte *et al.*, 1994a; Lomonte *et al.*, 1994b) showed that it consists of residues 105-118 (115-129 in the numbering system of (Renetseder *et al.*, 1985)) in the C-terminal loop of the Lys49 myotoxin II from *B. asper*. The synthetic peptide representing the sequence 105-118 appeared to play a central role in toxicity causing a clear cytolytic action which could be however completely abolished by preincubation with heparin. Therefore, this characteristic sequence in the C-terminal region of myotoxin II was found to display both heparin-binding and cytolytic activities as an integral part of the protein as well as of a derived synthetic peptide. Dynamic simulations performed for peptide 105-118 showed that the original backbone conformation of the synthetic peptide was conserved (Lomonte *et al.*, 1994a), probably explaining the maintenance of its toxic properties.

Bugs and coworkers (Bugs *et al.*, 2005) showed by means of size exclusion chromatography and dynamic light scattering that the number of heparin molecules bound per protein increased up to the limit of available positive charges, resulting in the neutralization of all protein charges and forming relatively large heparin-protein aggregates. This could be an explanation for no differences observed for three concentrations of MjTX-III used in our experiments for neutralization of the anticoagulant action of UFH. Also the level of heparin binding to myotoxin II was shown to depend on its saccharide length (Lomonte *et al.*, 1994a) and hexasaccharide was shown to be the smallest myotoxin II-binding saccharide (Lomonte *et al.*, 2003a), as the molecular dimensions of the segment 105-118 and the heparin hexasaccharides are roughly comparable. Therefore, there is a very low probability that MjTX-III could be used to neutralize the anticoagulant action of pentasaccharide (Arixtra®), which is a new efficient antithrombotic agent binding specifically to FXa.

Summarizing the above findings, it was not surprising that the two novel Lys49 $PLA_2$s isolated from the venom of *Bothrops moojeni* were able to bind heparin, especially as their C-terminal sequences show high homology with other known $PLA_2$s from different snake species. As seen in Table 2, at least 5 positively charged residues in the toxic C-terminal region are conserved in several myotoxic $PLA_2$s from Crotalid snakes and responsible for heparin-binding properties (Lomonte *et al.*, 1994a). Those residues are also conserved in MjTX-III and MjTX-IV.

In our work, the two novel myotoxins MjTX-III and MjTX-IV were tested in blood coagulation assays and found to neutralize the anticoagulant properties of UFH and LMWH in both isolated non-physiological system and blood plasma. Interestingly, in both systems better neutralization results were obtained for LMWH then for UFH. The inhibition of anticoagulant properties of LMWH was clearly dependent on the concentration of myotoxin, which was not the case for UHF, at least for the concentrations applied in our experiments. A possible explanation can be that heparins of different sizes cause similar structural interactions, but depending on the size of heparin complex, the specific interaction is dominated by the other positive region on the protein surface, in our case, long chains of UFH bind in different way to the protein than the short LMWH chains. The smaller size of LMWH permits possibly its more intimate accommodation on the protein and thus better neutralization (Bugs *et al.*, 2005).

The interaction between LMWH and myotoxin is already known from literature, however, described in another context. In a cytotoxicity study described by Calil-Elias *et al.* (Calil-Elias *et al.*, 2002), LMWH was shown to preserve more muscle fibers than UFH. This protective effect could be explained by the fact that LMWH had a smaller size and therefore could diffuse more quickly across the capillary endothelium. Within the muscle tissue LMWH could get more easily in contact with the venom, thus neutralizing its effects.

Chromogenic measurements performed in our study showed that MjTX-III can bind and neutralize LMWH to a higher extend than protamine hydrochloride, the known heparin antidote. This lead to the suggestion of a possible use of the enzymatically inactive Lys49 $PLA_2$ or their heparin-binding fragments, previously deprived of toxic properties (Stábeli *et al.*, 2006), as diagnostic or pharmaceutical tools to reverse the anticoagulant activity of heparins, especially of LMWH. This idea was supported by the study of Lomonte *et al.* (Lomonte *et al.*, 1994a) who compared interactions of *B. asper* myotoxin II and platelet factor 4 with heparin, obtaining similar results with both substances. The use of recombinant PF4 (rPF4) to reverse the anticoagulant activity of heparin has been proposed several years ago. Complete neutralization of heparin was achieved with twice the concentration of rPF4 compared with protamine. Although rPF4 was initially evaluated as a clinical alternative to protamine, it is currently not being developed for general clinical applications (Mixon and Dehmer, 2004).

The use of protamine sulfate or hydrochloride is nowadays a typical strategy for reversing the anticoagulant effects of heparin and related compounds. Nevertheless, protamine has many shortcomings and side effects and in addition, its potential to reverse smaller glycosaminoglycans such as LMWH is limited. Thus, the interaction of MjTX-III with LMWH was found to be interesting as there is no clinically used antidote against LMWH and a development of such, e.g. from an active heparin-binding fragment of a bigger protein, would be desirable.

Heparins and heparin related compounds are abundantly used in medicine in order to form an immediately acting inhibition of coagulation. This is useful to prevent or treat thromboembolic disorders or to perform interventions where blood is coming in contact with artificial surfaces inducing blood clotting (catheters, stents, cardiopulmonary bypass devices). Antagonizing the anticoagulant effect of heparins

or heparin-related compounds is also desired in many other diagnostic and medical cases, for example to analyze *in vitro* blood clotting in a patient treated with heparins, for antagonization of bleeding in a patient receiving heparins at the end of procedures that require an anticoagulation of the patient, e.g. at the end of heart surgery. A reduction of the anticoagulation decreases the risk of bleeding and necessity of blood transfusion.

It is important to emphasize that although the 105-118 fragment is know to represent the toxic part of the Lys49 myotoxins, it was shown that the toxic actions of these proteins (Figure 9) cannot always be reproduced by their free synthetic peptides 105-118 (Lomonte *et al.*, 2003b). The goal of our further studies is to synthesize peptides derived from the 105-118 sequence of the C-terminal loop of novel *B. moojeni* MjTX-III, to check their cytotoxic activity *in vitro*, to confirm their ability to bind and neutralize heparin, and to prove their influences on the blood coagulation system. Such compounds, if deprived of any toxic *in vitro* and *in vivo* properties, could then be further developed as a pharmaceutical tool to reverse the anticoagulant activity of LMWH.

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# 7 A protease with activity similar to blood coagulation factor VIIa, isolated from the venom of *Bothrops moojeni* snake

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## 7.1 Abstract

Venoms of the snakes of *Bothrops* species are known to contain various components interacting with blood coagulation. In the present study, we describe the purification and characterization of a *B. moojeni* protein showing properties similar to the coagulation factor VIIa. The applied purification techniques include size exclusion and hydrophobic interaction chromatography. For the functional characterization of the protein revealing a mass of about 30 kDa in gel electrophoresis, different coagulation and amidolytic tests were conducted. The protein clotted normal, FVII-, FIX- and FXII- but not FX-deficient plasma after addition of $CaCl_2$ and shortened the modified activated partial thromboplastin time (aPTT) and the prothrombin time (PT). In addition, it also cleaved chromogenic substrates of urokinase, plasmin, thrombin, kallikrein, FVIIa and activated protein C (APC). Nevertheless, fibrinolytic and APC-like activities could be excluded by means of a fibrin plate experiment and a FVa inactivation assay, respectively.

In conclusion, the newly described protein is procoagulant and acts independently of the contact phase and FIX but dependent on FX. According to its substrate specificity, the activity of the new protein resembles most closely the action of coagulation FVIIa. Such activity has not yet been reported for any snake venom.

**Abbreviations:** APC activated protein C; aPTT activated partial thromboplastin time; CT clotting time; CV column volume; GF gel filtration; HIC hydrophobic interaction chromatography; MW molecular weight; PLs phospholipids; PT prothrombin time; RT room temperature; rFVIIa recombinant factor VIIa; rTF recombinant tissue factor; RVV-V factor V activating enzyme from Russell's viper venom; TDT thrombin dynamics test

## 7.2 Introduction

Snake venoms are complex mixtures of biologically active proteins and peptides. Although snake-bites can be fatal, snake venoms are valuable biological sources of potential diagnostic and therapeutic value [1].

The venoms of *Viperidae* and *Crotalidae* but also *Elapidae* snakes contain a large variety of components involved in haemostasis. They can mimic, activate or deactivate almost every factor involved in coagulation or fibrinolysis [1-3]. Several of these proteins are applied in diagnosis and treatment of haemostatic disorders [1, 4].

Various procoagulant proteins from snake venoms have been isolated and characterized, all being proteases that activate the zymogen of specific coagulation factors or the protein cofactor, factor V (FV) [5].

Procoagulant snake venom proteases can be distinguished according to the level they influence in the coagulation cascade, such as FV activation [e.g. FV activating enzyme from *Daboia russelli* (RVV-V)], FX activation [e.g. FX activating enzyme from *Daboia russelli* (RVV-X)], prothrombin activation (e.g. ecarin from *Echis carinatus*) or direct fibrinogen-clotting (thrombin-like enzymes, e.g. batroxobin from *Bothrops atrox*). Several representatives of these proteases are used either therapeutically (e.g. batroxobin for defibrinogenation of patients) or diagnostically (e.g. ecarin in the ecarin clotting time assay). Regarding the diversity of the active components found in crude snake venoms, it is not surprising that more than 50 years after the introduction of Reptilase® as a haemostatic drug, new proteases are still being discovered and characterized.

In this paper, we describe the isolation and preliminary characterization of a previously unknown protease from the venom of *Bothrops moojeni* snakes. The protease shows properties similar to those of the activated blood coagulation factor VII (FVIIa). To our knowledge, such activity for a component of snake venom is being reported for the first time.

## 7.3 Matherials and methods

***Crude venom***

*Bothrops moojeni* crude venom was collected, pooled and dried at Pentapharm do Brasil. It was stored in the lyophilized form and reconstituted in deionized water.

***Gel filtration (GF)***

Crude, reconstituted venom (0.2 mg/ml) was separated into gel filtration fractions GF 1 to 16 on two in-line XK 26, HiLoad™ 26/60, Superdex™ 75 (GF Healthcare; Uppsala, Sweden) columns [bed height 60 cm, column volume (CV) 320 ml]. For the protein elution, a buffer of 50 mM ammonium acetate and 150 mM sodium chloride, pH 7.5 was used at a flow rate of 2.2 ml/min (24.9 cm/h) and the absorbance was monitored at 280 nm. Aliquots of 200 mg of crude venom were centrifuged (10 min at 3200 rpm) and loaded onto the system resulting in a reproducible separation. To ensure good reproducibility, the columns were regenerated every five runs using 0.5 M NaOH. The collected fractions (6 ml) were appropriately pooled into fractions GF 1 to 16 and stored at -80°C, until they were used for further separation steps and various measurements.

***Hydrophobic interaction chromatography (HIC)***

Fraction GF 6 was further purified on the Phenyl Sepharose HP (GF Healthcare; Uppsala, Sweden) column 10/15 (bed height 97mm, CV 11 ml). For the elution of the proteins, buffer A (50 mM sodium phosphate, 1M ammonium sulfate, pH 7.0) and buffer B (50 mM sodium phosphate, pH 7.0) were used at a flow rate of 2.9 ml/min (222 cm/h), according to the following gradient: 100% buffer A for 50 CV, 11% buffer B for 30 CV and 100% buffer B for 15 CV. Aliquots of 8.9 mg protein (fraction GF 6) were loaded onto the system and the absorbance was monitored at 215 nm.

***Desalting***

Desalting was performed by ultrafiltration (Millipore; Billerica, MA, USA) using Ultracel regenerated cellulose membranes (Ultracel PL-10, 10,000 NMWL; Millipore; Billerica, MA, USA).

### *SDS-PAGE*

The venom fraction GF 6 and its HIC sub-fractions (fractions GF 6A, GF 6B and GF 6C) were submitted to SDS-PAGE (Novex® 4-20% Tris-Glycine Gel 1mm x 10 well from Invitrogen, Basel) in order to determine their purity and the apparent molecular weights (MW) of their components. For the SDS-PAGE under non-reducing conditions, Novex® See-Blue Pre-Stained Standard (Invitrogen, Basel) and purified batroxobin were used as references.

### *Assay panel for the functional characterization of venom fractions*

Several functional assays were performed in order to elucidate the effects of the venom fraction GF 6 and its sub-fractions GF 6A, 6B and 6C. Assays, applied instruments and reagents are summarized in Table 1.

*Assays A and $A_1$*: Clotting experiments were performed with normal (A) or single factor deficient ($A_1$) plasma. Clotting was triggered by addition of the venom fractions and the recalcification of the plasma.

*Assays B and C*: Modified activated partial thromboplastin time (aPTT) and prothrombin time (PT) assays (with less agonist concentration compared to standard aPTT / PT in order to attain higher sensitivity) were performed for the global assessment of the effects of venom fractions on intrinsic (assay B) and extrinsic (assay C) coagulation pathways.

*Assays D and E*: In addition to the optical detection of fibrin clotting (assays B and C), thrombin generation was determined using a fibrin polymerization inhibitor and a chromogenic substrate for thrombin (based on the commercially available thrombin generation assay Pefakit® TDT®). Thrombin generation was detected for the intrinsic coagulation pathway (in-TDT®, assay D) applying the modified aPTT assay, as well as for the extrinsic pathway (ex-TDT®, assay E) using the modified PT assay.

*Assay F*: The amidolytic action of the venom fraction on different chromogenic substrates was assessed.

*Assay G*: The functional FVa inactivation assay was performed based on a commercially available APC resistance assay (Pefakit® APC-R®), following the manufacturer's instructions.

**Table 1.** Assay panel for the functional characterisation of venom fraction

| Assay | | Platelet poor plasma or buffer [1] | Venom Fraction or buffer [1] (control) | Reagent 1 | Incu-bation | Reagent 2 | Detection | Instrument |
|---|---|---|---|---|---|---|---|---|
| **A & $A_1$** | Plasma clotting assay | 50 µl normal plasma (A) or factor deficient plasma ($A_1$)[6] | 50 µl | - | 180 sec | 50 µl 25 mM $CaCl_2$ solution | Clotting onset (mechanical) | KC4 coagulometer [11] |
| **B** | Modified aPTT | 50 µl pooled normal platelet poor plasma (ISTH/SSC) | 25 µl | 25 µl aPTT activator (HemosIL [8]) | | 50 µl 25 mM $CaCl_2$ solution | For 180 sec at 660 nm | BCS Behring Coagulation System (random-access analyser) [10] |
| **C** | Modified PT | | | 25 µl rTF (Recombiplastin [8]) | | | | |
| **D** | Modified aPTT with amidolytic detection of thrombin generation [3] | | | 25 µl aPTT activator (HemosIL [8]) | | 50 µl 25 mM $CaCl_2$ solution + fibrin polymerisation inhibitor + chromogenic substrate [2] | For 180 sec at 405 nm | |
| **E** | Modified PT with amidolytic detection of thrombin generation [3] | | | 25 µl rTF (Recombiplastin [8]) | | | | |
| **F** | Chromogenic substrate assays | 170 µl buffer [1] | 20 µl | 20 µl chromogenic substrate solution [5] | - | 50 µl 25 mM $CaCl_2$ solution | 20 cycles over 10 min at 405 nm | Tecan multiwell reader [9] |
| **G** | FVa inactivation assay [4] | | 12 µl | 38 µl RVV-V solution [+] 20 µl FV-inactivated plasma [7] | | 50 µl FVa-dependent prothrombin activator (Noscarin) [7] | Clotting onset (mechanical) | KC4 coagulometer [11] |

[1] 50 mM HEPES buffer pH 7.5
[2] based on the commercially available reagent Pefakit® TDT® (Pentapharm Ltd.; Basel, CH)
[3] from the Pefakit® TDT® reagent
[4] based on commercially available reagent Pefakit® APC-R (Pentapharm Ltd.; Basel, CH)
[5] Pefachrome® FXIIa, FXa, FIXa, FVIIa, TH, PK, uPa, PCa, PL (all by Pentapharm, Basel)
[6] deficient plasmas for FXI, FIX, FX, FVII (all by Instrumentation Laboratory, Milano, IT), normal plasma (ISTH/SSC) served as control
[7] from the Pefakit® APC-R reagent
[8] by instrumentation Laboratory, Milano, IT
[9] by Tecan, Groedig, Salzburg, D
[10] by Dade-Behring, Marburg, D
[11] by Trinity Biotech, Lemgo, D

***Fibrin plate***

Potential fibrinolytic activity was evaluated by means of a fibrin plate assay performed as follows: 22 mg of 0.4% bovine fibrinogen (Enzyme Research Lab; Swansea, UK) were dissolved in 3 ml of deionized water and fibrin formation was triggered by addition of 75 μl thrombin solution (25 U/ml in 0.9% NaCl). After 30 min incubation, 20 μl of samples and controls were applied onto the fibrin plate and incubated for 20 h at RT.

## 7.4 Results

The crude venom of *B. moojeni* snake was separated using gel filtration (GF) chromatography, as shown in Figure 1A. Fraction GF 6 was chosen for further investigations, as preliminary experiments indicated the presence of a component with properties similar to activated protein C (referred as F 41 in the previous study [6]). Hydrophobic interaction chromatography (HIC) performed with GF 6 revealed four fractions GF 6A to 6D (Figure 1B).

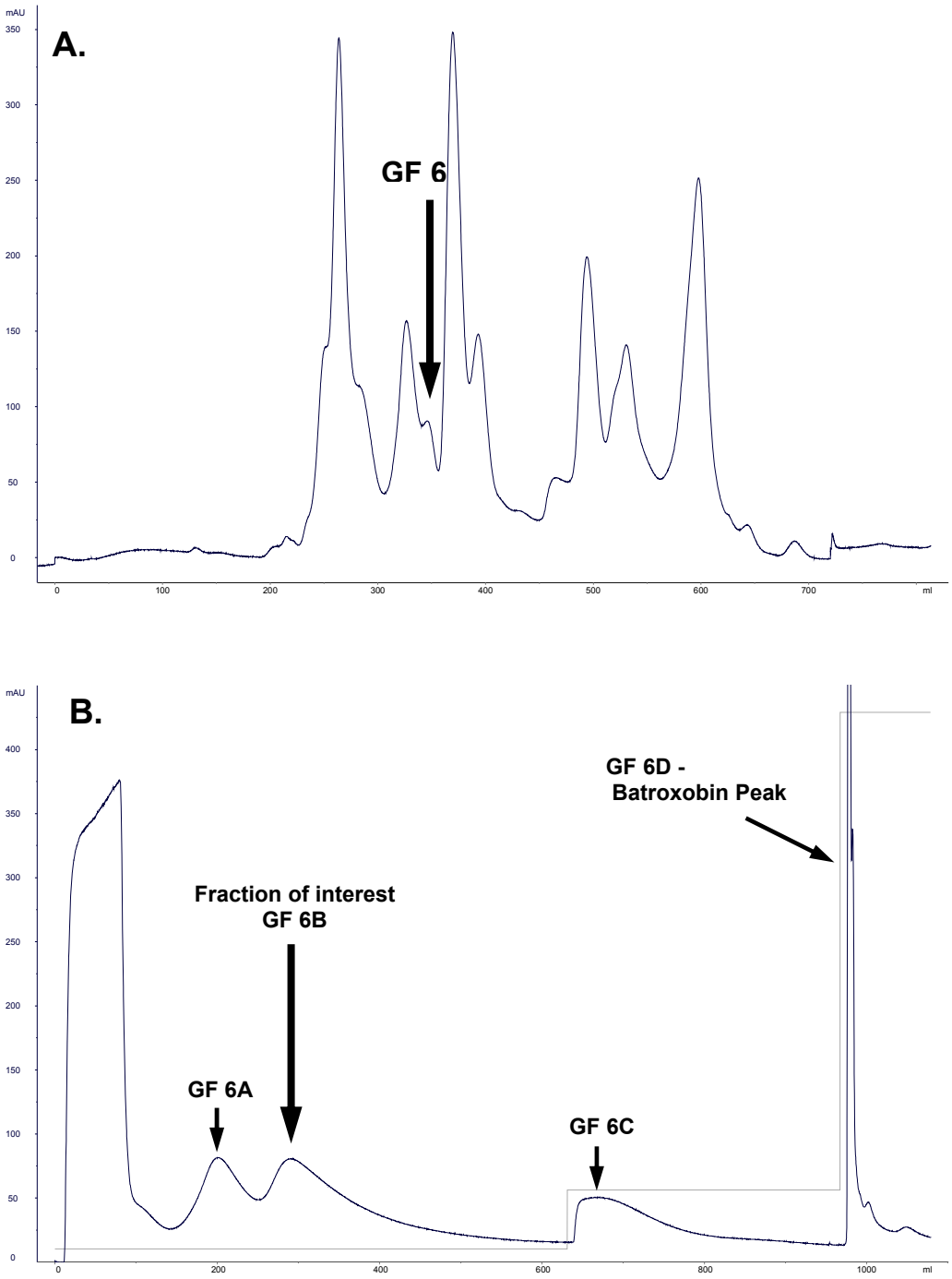


**Figure 1 A.** Gel filtration profile of the crude venom of the B. moojeni snake; fraction of interest GF 6 indicated by an arrow. **B.** Fraction GF 6, further purification profile, HIC Phenyl Sepharose HP column.

The crude fraction GF 6, used as a reference in SDS-PAGE (Figure 2, row 5), showed high complexity. The particular components could be, however, further purified applying the HIC technique.

Traces of thrombin-like enzyme batroxobin present in fraction GF 5 as major component, were also detected in fraction GF 6 by the clotting assays A (data not shown), as well as by SDS-PAGE (Figure 2, row 5). Using the HIC method, batroxobin (fraction GF 6D) could be successfully separated from other components (Figure 2, row 6).

Fraction GF 6A (Figure 2, row 1) revealed two bands between 30 and 36 kDa on the SDS-PAGE. These bands were not present in any other HIC fraction. The component showing properties similar to activated protein C (data not shown) could be identified in fraction GF 6B. This fraction (Figure 2, rows 2 and 3) appeared as a pure protein showing a single band at about 30 kDa. Another component, clearly present in the fraction GF 6C (Figure 2, row 4) appeared at about 47 kDa.

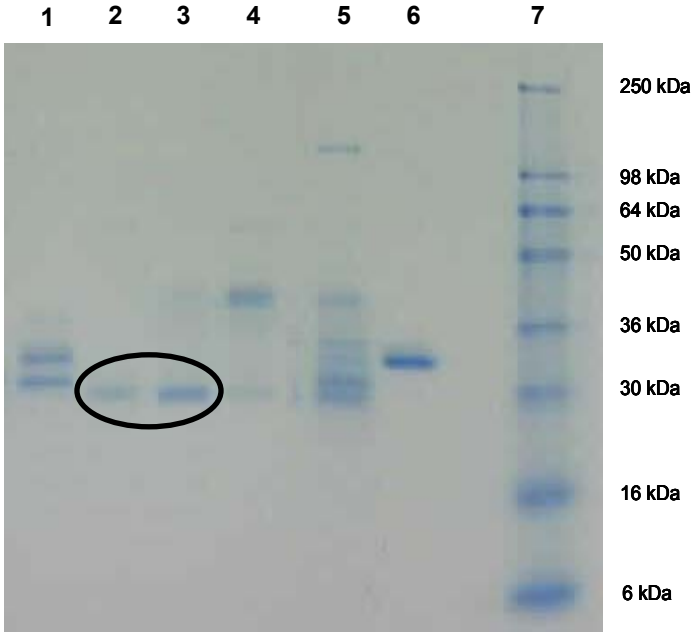


**Figure 2.** SDS-PAGE gel performed with crude fraction GF 6 and its subfractions: 1: GF 6A, 2 and 3: GF 6B, 4: GF 6C, 5: Crude fraction GF 6, 6: GF 6D - batroxobin, 7: Seeblue Standard.

After desalting, fraction GF 6B was investigated in the functional assay panel (Table 1). Addition of fraction GF 6B resulted in the clotting of normal plasma within 69.6 s after the addition of $CaCl_2$, while a control experiment with the addition of buffer instead of fraction GF 6B did not show any clotting within 200 s. This indicates a procoagulant activity of fraction GF 6B which was also confirmed by a shortening of the modified aPTT (from 37.7 s to 33.8 s, assay B, Table 1) and PT (from 51.1 s to 36.8 s, assay C Table 1). Using the amidolytic detection of thrombin generation (assays D and E, Table 1) it could be shown that the addition of fraction GF 6B to the modified aPTT and PT tests, results in an acceleration of the onset of thrombin generation but not in an increased amount of thrombin formed (Figures 3A-B).

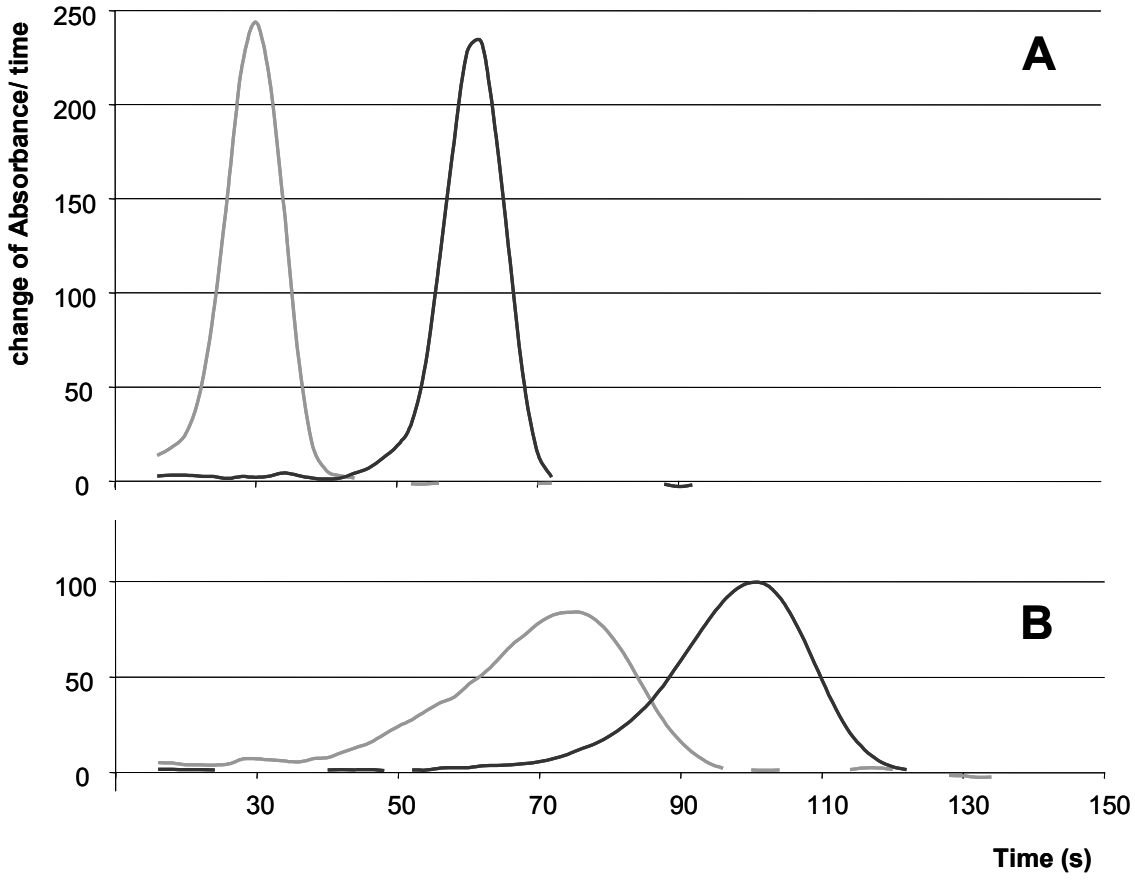


**Figure 3.** Blood coagulation assays performed on Behring Coagulation System (BCS); black - control, grey – fraction GF 6B: panel A: modified aPTT with amidolytic detection of thrombin generation (in-TDT® test); panel B: modified PT with amidolytic detection of thrombin generation (ex-TDT® test).

In the following approach the procoagulant activity of GF 6B was tested with normal four factor-deficient plasmas (assay A1, Table 1). The obtained results are shown in Table 2.

**Table 2.** Screening of the procoagulant activity of fraction GF 6B in normal and immunodepleted plasmas.

| Plasma | Assay | Clotting time (s) | |
|---|---|---|---|
| | | Control | Fraction GF 6B |
| **Normal plasma** | A | > 200 | 75.3 |
| **FVII-deficient plasma** | $A_1$ | > 200 | 98.4 |
| **FIX-deficient plasma** | $A_1$ | > 200 | 89.5 |
| **FX-deficient plasma** | $A_1$ | > 200 | > 200 |
| **FXI-deficient plasma** | $A_1$ | > 200 | 86.9 |

After addition of fraction GF 6B, in all but FX-deficient plasma, similar effects were measured. Clot formation was detected between 75 s and 98 s after the coagulation was triggered with $CaCl_2$. The shortest CT of 75 s was measured in normal plasma. No clotting was observed (CT>200 s) for fraction GF 6B in the FX-deficient plasma, neither for the control measurements with any of the applied plasmas. As a result, possible kallikrein- and FX-like activities of fraction GF 6B could be excluded as FXII-deficient plasma did and FX-deficient plasma did not clot.

**Table 3.** Screening of the activity of fraction GF 6B towards different chromogenic substrates, assay F. $\Delta E_{405}$ - absorbance increase measured at 405 nm; HHT – cyclohexyltyrosine, CHA – β-cyclohexylalanine, CHG – cyclohexylglycine, pNa – p-nitroaniline, Pyr – L-pyroglutamic acid, Abu – L-α-aminobutyric acid.

| Chromogenic Substrate | $\Delta E_{405}$/10min | |
|---|---|---|
| | Control | Fraction B |
| **Pefachrome®FXIIa (H-D-HHT-Gly-Arg-pNA)** | 0.00 | 0.04 |
| **Pefachrome®FXa ($CH_3OCO$-D-CHA-Gly-Arg-pNA)** | 0.00 | 0.09 |
| **Pefachrome®FIXa ($CH_3SO_2$-D-CHG-Gly-Arg-pNA)** | 0.00 | 0.06 |
| **Pefachrome®FVIIa ($CH_3SO_2$-D-CHA-Abu-Arg-pNA)** | 0.01 | 0.64 |
| **Pefachrome®PL (H-D-Ala-HHT-Lys-pNA)** | 0.00 | 0.39 |
| **Pefachrome®TH (H-D-CHG-Ala-pNA)** | 0.01 | 0.40 |
| **Pefachrome®uPa ($CH_3SO_2$-D-HHT-Gly-Arg-pNA)** | 0.00 | 0.18 |
| **Pefachrome®PK (H-D-Abu-CHA-Arg-pNA)** | 0.01 | 1.59 |
| **Pefachrome®PCa (Pyr-CHG-Arg-pNA)** | 0.00 | 1.59 |

Table 3 shows the increase of optical density due to cleavage of chromogenic substrates by GF 6B with different specificities (assay F, Table 1). No cleavage of the chromogenic substrates for FXIIa, FXa and FIXa was detected. Pefachrome® FVIIa, PL (for plasmin), TH (for thrombin) and uPa (for urokinase) were cleaved with a moderate absorbance increase, whereas for Pefachrome® PK (for plasma kallikrein) and PCa (for APC) a strong raise of the absorbance was detected (Table 3).

An activity similar to the action of APC, which was suggested by the previous study [6] and the cleavage of Pefachrome® PCa (Table 3), could be excluded by the procoagulant properties of GF 6B. The lack of the APC-like activity could be also confirmed by means of the FVa inactivation assay (assay G, Table 1). Identical clotting times for control and fraction GF 6B (approx. 29 s) were determined, indicating no cleavage of FVa.

A possible plasmin- or urokinase-like activity of fraction GF 6B could be excluded using standard fibrin plate methodology, as no fibrinolysis could be observed (data not shown).

## 7.5 Discussion

In this communication, the purification and characterisation of a procoagulant protease from the venom of *Bothrops moojeni* snake, showing blood coagulation FVIIa-like properties, is described. According to our knowledge, a protease with such activity profile has not been described before in any snake venom.

The functional properties of the corresponding venom fractions were characterized according the assay panel presented in Table 1. First, the addition of fraction GF 6B to normal plasma and immunodepleted plasmas with deficiencies in factors FVII, FIX and FXI induced clotting, while control experiments without the addition of fraction GF 6B did not. Therefore, the action of the protease was clearly procoagulant. Nevertheless, fraction GF 6B did not clot FX deficient plasma and thus, a procoagulant activity based on the cleavage of fibrinogen (thrombin-like activity) or prothrombin (prothrombinase activity) can be excluded. In addition, because fraction GF 6B was able to trigger clot formation in FVII, FXI and FXI deficient plasmas, activities equivalent to tissue factor (TF), kallikrein, FXIIa, FXIa can be excluded. Such properties would result in an activation upstream of the missing factor and would therefore not induce sample clotting (TF would not clot FVII deficient plasma, FXIIa or kallikrein would not clot FXI or FIX deficient plasma). Finally, an activity based on the equivalence of fraction GF 6B to TF, being not a serine protease, would not have been detected as proteolytic activity in assay F (Table 1).

In addition, an efficient ($\Delta E_{405}$/10min of 1.59, for comparison see Table 3) cleavage of the APC chromogenic substrate (Pefachrome® PCa) was found. Nevertheless, the assumed APC-like activity of the fraction GF 6B, resulting in an anticoagulant effect, was definitely excluded using the FVa-inactivation assay (Table 1, assay G).

A complete cleavage of the chromogenic substrate for plasmin (Pefachrome® PL) was detected as well. However, fibrinolytic activity could be excluded by means of a fibrin plate assay. Again, a plasmin-like activity would not have resulted in the procoagulant actiivity found in the clotting experiments.

The results above leave FVIIa- and FIXa-like properties as the remaining possible explanations of the action of the new protease from *B. moojeni* venom. Regarding the lack of activity of fraction GF 6B against the FIXa chromogenic substrate and the degradation of the chromogenic substrate Pefachrome®FVIIa (Table 3), we conclude that FVIIa-like activity is the most likely reason of the procoagulant effects triggered by the new protease.

A FVIIa-like activity has not been previously reported for any snake venom protease, although different venoms were examined for their ability to convert single-chain FVII into two-chain FVIIa [7]. The closest related activities described are the FVII-activating effect of oscutarin, a prothrombin activator from the venom of *Oxyuranus scutellatus* [5, 7] and the FVIIa inhibiting action of the hemextin AB complex from the venom of *Hemachatus haemachatus* [8-10].

FVIIa plays a central role in the initiation of the coagulation cascade via the extrinsic pathway, and has also a pivotal function in the “cell-based model of haemostasis” [11]. Therefore, we suppose that this new protease from *B. moojeni* venom could probably find a therapeutic application as a FVIIa-like procoagulant drug.

Recombinant FVIIa (rFVIIa) is used as an effective therapy for patients with inhibitors against FVIII or FIX as well as in individuals with inherited platelet function disorders [12]. The ability of rFVIIa to enhance thrombin generation on the surface of activated platelets makes it a potential haemostatic agent in any situation with a need for the formation of a tight haemostatic plug, resistant to premature lysis i.e. in severe hemophilia and in nonhemophilia patients with profuse, heavy bleeding [13, 14]. Therefore a new haemostatic agent derived from snake venom and resembling the blood coagulation FVIIa could be of therapeutic interest.

The present study was limited by the fact that only a small amount of the venom protein was available. More detailed examinations would be necessary for the final characterization of this novel protease.

In conclusion, a previously unknown procoagulant protease from the venom of *B. moojeni* is described. Its molecular weight is in the range of 30 kDa and its functional properties most likely resemble the action of coagulation FVIIa.

*The present study was performed in the framework of a diploma thesis at the Zürcher Hochschule in Winterthur, Switzerland.*

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# 8 Platelet-active substances in the venom of *Bothrops moojeni* snake – a novel evaluation method using whole blood aggregometry

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## 8.1 Abstract

The objective of the present study was an investigation of the crude *Bothrops moojeni* venom, aiming at the identification of new compounds with platelet-activating or -inhibiting activity. The venom was separated by gel filtration chromatography into 18 fractions, which were tested by means of whole blood aggregometry for their activities affecting the aggregation of blood platelets. In order to eliminate interferences caused by prothrombin activators or thrombin like-enzymes, which are frequently present in snake venoms, a test method for screening protein mixtures was developed. To avoid clotting of the blood samples, the thrombin inhibitor hirudin and the synthetic inhibitor of fibrin polymerization Pefabloc® FG were applied.

In the present study, a platelet aggregation activator with an activity resembling thrombocytin from *B. atrox* was identified in one of the examined venom fractions. In addition, a platelet antagonist – most likely a disintegrin – with broad inhibitory activity against aggregation triggered by collagen, adenosine diphosphate and thrombin receptor activating peptide, was identified.

**Abbreviations:** ACN acetonitrile; ADP adenosine diphosphate; AU aggregation unit; COL collagen; FPI fibrin polymerization inhibitor; GF gel filtration; HIC hydrophobic interaction chromatography; LAAO L-amino acid oxidase; MALDI-TOF matrix assisted laser desorption ionisation – time of flight; MW molecular weight; MS mass spectrometry; MSP 1 and 2 basic serine proteases from the venom of *Bothrops moojeni*; PAR protease-activated receptor; PA-BJ platelet-aggregating enzyme from the venom of *B. jararaca*; $PLA_2$ phospholipase $A_2$; PRP platelet-rich plasma; PTA prothrombin activator; RT room temperature; SPE solid phase extraction; TFA trifluoroacetic acid; TLE thrombin-like enzyme; TRAP-6 thrombin receptor activating peptide; vWF von Willebrand factor

## 8.2 Introduction

Snake venoms are complex mixtures of biologically active proteins and peptides, which can affect blood coagulation and platelet functions in various ways [1-11]. There are venom proteins that can mimic, activate or deactivate almost every factor involved in coagulation or fibrinolysis [1, 8, 12]. In addition, several venom-derived factors that either activate [e.g. botrocetin, convulxin, cerastocytin, thrombocytin, $PLA_2$s, L-amino acid oxidases (LAAOs)] or inhibit (e.g. echicetin, disintergrins, jararhagin, $PLA_2$s, 5'-nucleotidases) blood platelet aggregation have been described. They show high structural and functional similarity to different natural ligands of platelet adhesion receptors [13] and thus affect platelet functions by different mechanisms, such as: (i) binding or degradation of vWF or platelet receptors caused by e.g. C-type lectins (botrocetin, bitiscetin, echicetin, convulxin), disintegrins (batroxostatin, jarastatin, echistatin) or metalloproteinases (crotalin, kaouthiagin); (ii) activation of protease-activated receptors, by thrombin-like enzymes (TLEs), such as cerastocytin, thrombocytin, PA-BJ (platelet-aggregating enzyme from the venom of *B. jararaca*), jararhagin and alborhagin; (iii) modulation of adenosine diphosphate (ADP) release (some L-amino acid oxidases (LAAOs) e.g. from the venom of *Echis colorata* and 5'-nucleotidases e.g. from the venom of *Trimeresurus gamineus*); (iv) modulation of tromboxane $A_2$ formation ($PLA_2$s, e.g. from the venom of *Ophiophagus hannah*) and finally (v) some LAAOs (e.g. from the venoms of *Bothrops alternatus, B. moojeni* or *Trimeresurus jerdonii*) activate platelets via $H_2O_2$ production [1, 8, 12].

Our objective was an in-depth investigation of the crude *B. moojeni* venom [14], aiming at the identification of new bioactive compounds which may be applicable in the field of thrombosis and haemostasis for diagnostic or therapeutic purposes.

As already shown by Francischetti et al. [9], *B. moojeni* venom possesses a moderate platelet pro-aggregatory activity and a low inhibitory effect (not exceeding 30% inhibition) on platelet aggregation triggered by collagen or thrombin. In addition, it contains two basic serine proteases, MSP 1 and MSP 2, which activate platelet aggregation and increase the aggregation induced by ADP [15]. MSP 1 resembles thrombocytin, the platelet-aggregating enzyme from *B. atrox* venom. Both, MSP 1 and thrombocytin are basic glycoproteins with similar molecular weight with reactivity

on washed platelets and platelet-rich plasma (PRP). Their aggregating activity is not inhibited by hirudin. In addition, they potentiate the ADP-induced platelet aggregation releasing low concentrations of constituents acting synergistically with the granule substances released by ADP, leading to an irreversible aggregation reaction [15]. In comparison to MSP 1 and thrombocytin, a 20-fold higher concentration of MSP 2 is required to trigger analogous platelet aggregation effects. Both, MSP 1 and MSP 2, were also demonstrated to unspecifically degrade all fibrinogen chains and only the α-chain and α-polymer of a fibrin clot [15].

Recently, Stábeli *et al.* [16] investigated the biochemical and functional properties of LAAO from the venom of *B. moojeni* (BmooLAAO-I) and reported that BmooLAAO-I induces dose-dependent platelet aggregation in PRP.

Based on literature reports on *B. moojeni* venom [9, 14-19], platelet active substances as well as agonists of plasmatic coagulation factors had to be expected in the venom fractions prepared in the present study. In order to detect direct effects of the venom components on platelets, we therefore faced a number of obstacles. Prothrombin-activating enzymes can trigger platelet aggregation by initiating thrombin formation, while thrombin-like enzymes may disturb aggregation assays by splicing fibrinogen to fibrin resulting in gelation of the sample. In addition, most of the investigations studying platelet modulation by snake venoms have been conducted with washed platelets [9, 20] or PRP [15, 21-24], i.e. conditions that do not represent the physiological environment of platelet aggregation.

In the present study, a novel whole blood aggregometry method was applied [25-27]. For that purpose, the standard performance of Multiplate® analysis described by Tóth *et al.* [25] was adapted in order to eliminate interference of prothrombin-activating enzymes or thrombin-like enzymes in the examined venom fractions. Briefly, instead of saline used in the standard procedure, a buffer containing recombinant hirudin and a fibrin polymerisation inhibitor (FPI) was added to citrated whole blood. By this approach, known as well as novel platelet-active substances could be identified in the *B. moojeni* venom.

## 8.3 Methods

***Crude venom***

*Bothrops moojeni* crude venom was collected, pooled and dried at Pentapharm do Brasil. It was stored in the desiccated form and reconstituted in deionized water (250 mg/ml).

***Gel filtration (GF)***

Crude, reconstituted venom (250 mg/ml) was separated into 18 gel filtration fractions (fractions GF 1 to GF 18) using two in-line Superdex-75 XK 26 columns (GE Healthcare). Aliquots of 300 mg venom were loaded for each run and the proteins were eluted using a 50 mM ammonium acetate, 150 mM sodium chloride, pH 7.5 buffer at a flow rate of 2.2 ml/min (24.9 cm/h). The absorbance was monitored at 280 nm. To ensure good reproducibility of the process, the columns were regenerated every five runs using 0.5 M NaOH. Collected venom fractions were appropriately pooled into GF 1 to GF 18 and stored at -80°C until further measurements and separation steps were performed.

***Hydrophobic interaction chromatography (HIC)***

Fraction GF 6 was further separated on a 1 ml Resource$^{TM}$ PHE (Phenyl) column (GE Healthcare) using an Äkta Explorer liquid chromatography system (GE Healthcare). The fraction was diluted 1 : 2 in 3 M ammonium sulfate before injection onto the column. For the protein elution, buffer A (50 mM phosphate, 1.5 M ammonium sulfate, pH 7.2) and buffer B (50 mM phosphate, pH 7.2) were used. Elution was performed at a flow rate of 1 ml/min (187 cm/h) using a gradient of 0-100% buffer B over 90 min. The absorbance was monitored at 280 nm. Obtained fractions were called Botmo GF 6/1 to GF 6/44.

***Clotting assay using ball coagulometer KC4 A micro (batroxobin detection)***

Ball coagulometer KC4 A micro (Heinrich Amelung GmbH, Lemgo, Germany) was used for the identification of fractions containing batroxobin. Coagulation in 50 µl of normal plasma (ISTH/SSC plasma) was triggered by adding 50 µl of venom sub-fractions.

***Desalting and concentration***

The high molecular weight venom fractions (GF 2 to GF 8) were desalted by means of gel filtration using a HiTrap desalting column Sephadex™ G-25 Superfine (GE Healthcare) and 50 mM phosphate buffer, pH 7.2.

The low molecular weight venom fractions (GF 9 to GF 17) were submitted to solid phase extraction (SPE) by acidification with 0.1% trifluoroacetic acid (TFA) and loading on Sep-Pak $C_{18}$ classic cartridge (Waters™). Solvation, equilibration, sample application and elution were performed according to manufacturer's instructions. In each case, the fraction eluted with 60% aq. acetonitrile (ACN) and thus enriched in peptides and proteins was then freeze-dried under vacuum in a SC210A Speed Vac™ Plus from Savant™. The desalted fractions were stored at -20°C until they were used for further experiments.

***SDS-PAGE***

The venom fraction GF 6 and its HIC sub-fractions GF 6/15 and GF 6/35 to GF 6/39 were submitted to SDS-PAGE (Novex® 4-20% Tris-Glycine Gel 1mm x 10 well from Invitrogen, Basel, Switzerland) in order to determine their purity and the apparent molecular weight of their components. For the SDS-PAGE under non-reducing conditions, Novex® See-Blue Pre-Stained Standard (Invitrogen, Basel, Switzerland) was used as reference.

***RP-HPLC***

The desalted and concentrated fraction GF 10 was reconstitute in 0.1% TFA and processed by RP-HPLC on a Waters Alliance 2695 System using a semi-preparative HPLC column (Vydac #218TP510 protein & peptide $C_{18}$, 10 mm x 250 mm). Proteins and peptides were eluted using a 2-50% gradient of 90% ACN in aq. TFA (0.1%) at a flow rate of 3 ml/min. The absorbance was monitored at 225 nm and the sub-fractions were collected manually. The sub-fractions GF 10/1 to GF 10/95 were stored at -20°C until further measurements were performed.

***Preparation of blood***

Blood was collected from healthy volunteers using a Multifly® Set and plastic S-Monovette® (Sarstedt AG, Nümbrecht, Germany) containing 1/10 volume sodium citrate (final concentration in blood 0.106 mM). All volunteers denied taking any

medication affecting platelet function for two weeks preceding the study. Aggregation measurements were performed between 0.5 to 4 h after venipuncture. The blood was kept in closed polyethylene tubes at RT during the experiments.

***Whole blood aggregation***

Whole blood aggregation was determined by impedance aggregometry using the Multiplate® analyzer (Dynabyte Medical, Munich, Germany) as described by Tóth *et al.* [25]. The device has five channels for parallel tests, and a disposable test cell with duplicate impedance sensors. The impedance change determined by each sensor is recorded independently. During the analysis, the sample-reagent mixture is stirred using a polytetrafluoroethylene-coated magnetic stirrer. Briefly, 300 µl of pre-heated (37°C) saline or buffer (20 mM Tris-HCl, pH 7.4) containing recombinant hirudin (Refludan, Pharmion, 10 µg/ml) and a fibrin polymerisation inhibitor (FPI, Gly-Pro-Arg-Pro, Pefabloc® FG, Pentapharm, Basel, Switzerland; 1.6 mg/ml) were placed into the test cells, before 300 µl of citrated whole blood were added. After 3 min incubation and stirring at 37°C, the measurement was started by adding 20 µl of venom fraction alone (to study platelet activation) or 20 µl of venom fraction in addition to 20 µl of a platelet agonist (to study platelet inhibition). Collagen (COL; 0.1 mg/ml), adenosine diphosphate (ADP; 0.2 mM) or thrombin receptor activating peptide (TRAP-6; 1 mM) were used for activation (all by Dynabyte, Munich, Germany). The impedance change by the adhesion and aggregation of platelets on the electrodes was continuously detected over a period of 6 min and expressed in arbitrary aggregation units (AU). Ecarin (26 U/ml) and batroxobin (5 U/ml), both form Pentapharm, Basel, Switzerland, were used for validation experiments.

## 8.4 Results

***Fractionation of the B. moojeni venom***

The crude venom of *B. moojeni* was separated by means of gel filtration chromatography into 18 fractions (GF 1 to 18). The UV chromatogram is displayed in figure 1A showing good separation of the venom components according to their molecular weights (MW). Concerning the MALDI-TOF MS analyses (data not shown), venom fractions GF 1 to 18 were divided into high (GF 1 to 8) and low (GF 9 to 18) MW fractions.

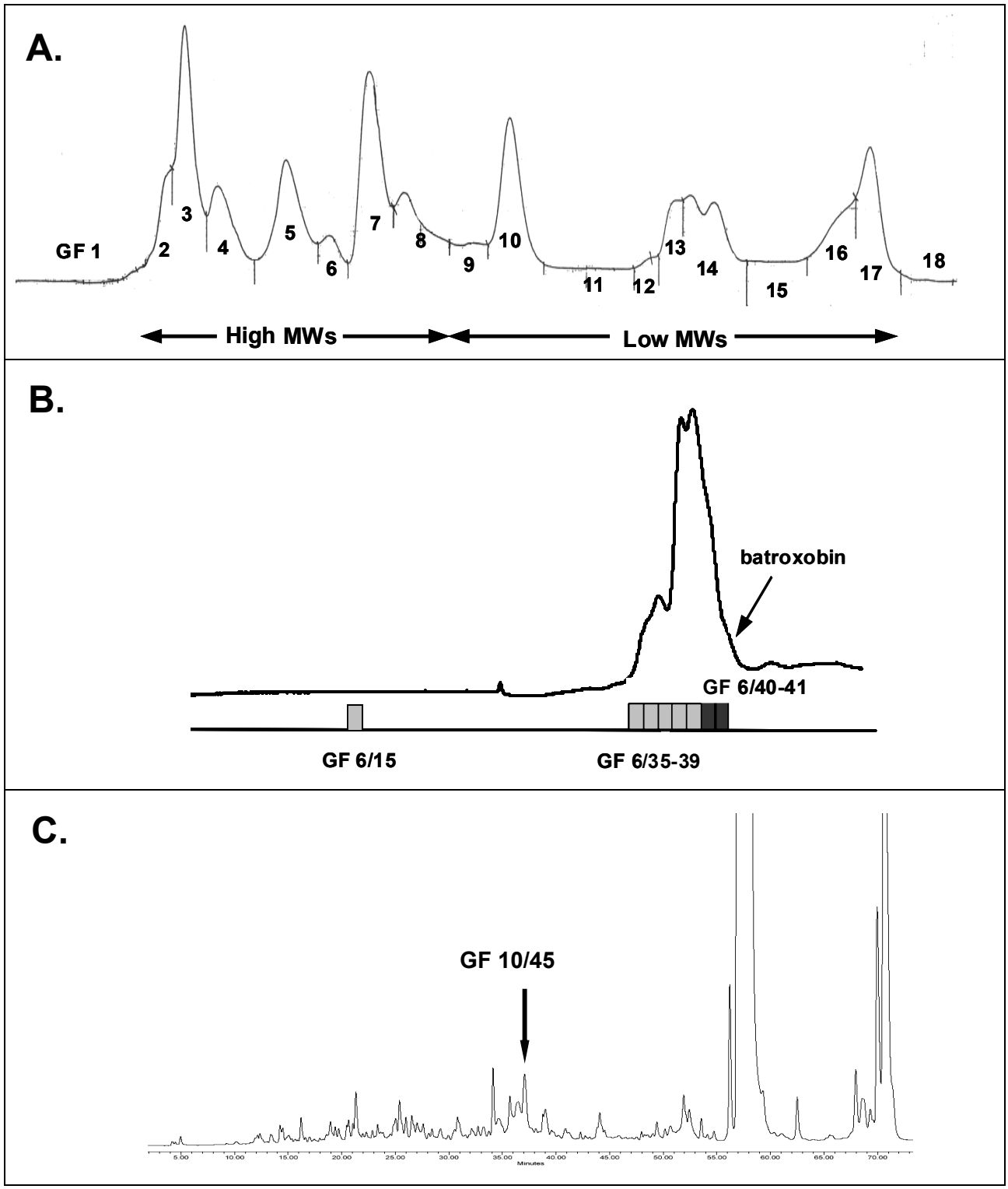


**Figure 1.** Fractionation of the *B. moojeni* venom; **A.** Gel filtration profile of the crude venom (300 mg protein loaded). **B.** Sub-fractionation of fraction GF 6 on HIC Resource™ PHE column, sub-fractions GF 6/40 and GF 6/41 containing batroxobin as indicated by an arrow. **C.** RP-HPLC profile of semi-preparative separation of the GF 10 fraction.

According to the chromatogram in Figure 1A, the venom fractions GF 1 and GF 18 do contain only minor amounts of protein and were therefore not further processed. All other fractions, GF 2 to GF 17, were desalted prior to any further measurement or purification step. Since fraction GF 6 activated platelet aggregation (shown below in Figure 4), it was additionally separated using hydrophobic interaction chromatography (HIC) (Figure 1B).

In addition, some of the low MW GF fractions were further processed by RP-HPLC, among them fraction GF 10 (Figure 1C), which was also shown to inhibit platelet aggregation (shown below in Figure 5).

The sub-fractions obtained after the HIC separation of Botmo GF 6 were screened for their activities on blood platelets and some of them, as well as the crude fraction GF 6, were analysed by SDS-PAGE (Figure 2). Crude fraction GF 6, applied in the SDS-PAGE in two different concentrations, revealed the presence of at least four components (Figure 2, rows 7 and 8). Concerning their apparent MW, proteins present in the sub-fractions GF 6/37 to GF 6/39 were compared to *Bothrops* proteases known from literature to activate platelet aggregation [15, 28, 29].

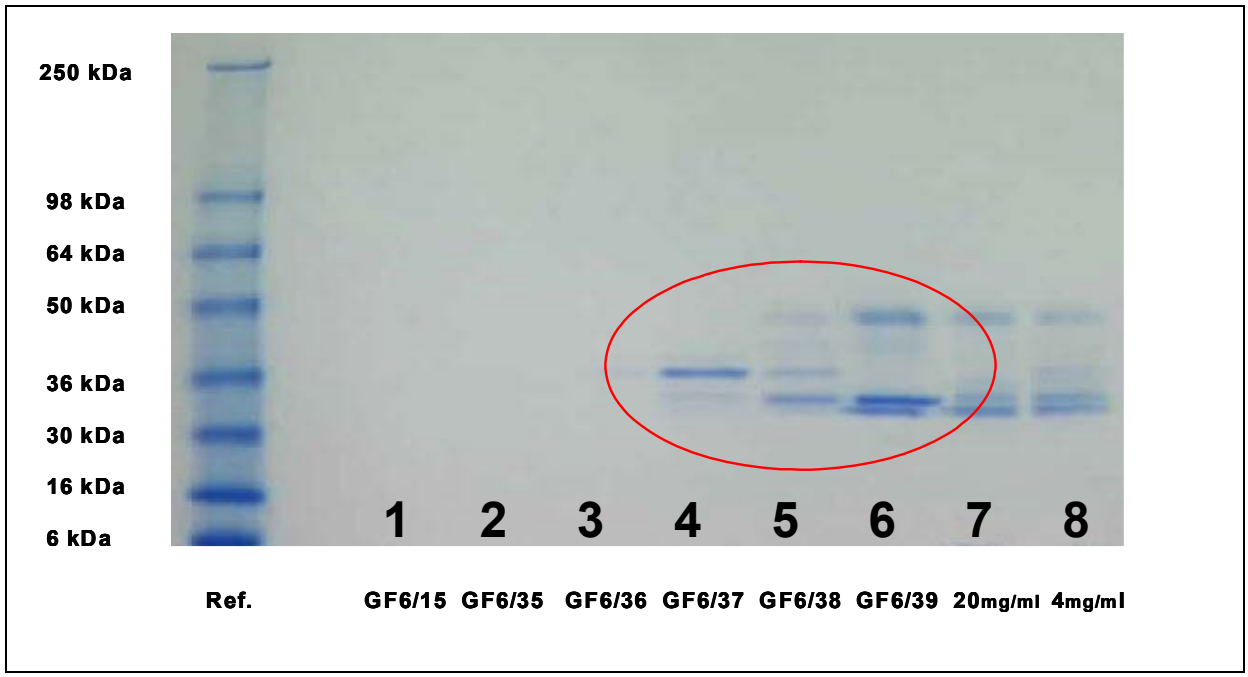


**Figure 2.** Selected HIC sub-fractions of GF 6 (GF 6/15 and GF 6/35 to GF6/39) were submitted to SDS-PAGE. Crude fraction GF 6 is shown in rows 7 and 8 (20 and 4 mg/ml, respectively).

In the sub-fraction GF 6/15 (Figure 2, row 1), eluting just at the beginning of HIC purification (Figure 1 B), as well as in the sub-fractions GF 6/35 and GF 6/36 (Figure 2, rows 2 and 3) no protein bands were detected. In the sub-fraction GF 6/37 (Figure 2, row 4) one major protein band was present at about 36 kDa. This band was also detectable in only the sub-fraction GF 6/38 (Figure 2, row 5), however with much lower intensity. Furthermore, two new bands between 30 and 36 kDa were present in the venom sub-fractions GF 6/38 and 6/39 (Figure 2, rows 5 and 6), suggesting the presence of components similar to *B. moojeni* MSP 1, which was shown to possess two protein bands between 32.5 and 35 kDa [15]. Nevertheless, sub-fraction GF 6/39 had no activating properties on platelet aggregation (shown below in Figure 4B), indicating that the activating property is related to a protein of different molecular size and, consequently, not MSP 1. Furthermore, neither GF 6/37 nor GF 6/38 revealed any protein band at 30 kDa, indicating that there is no protein similar to PA-BJ from the venom of *B. jararaca* (apparent MW of 30 kDa [29]). Finally, for both sub-fractions GF 6/37 and 6/38 protein bands were visible at 36 kDa, suggesting the presence of a protein resembling thrombocytin isolated from the *B. atrox* venom (apparent MW of 36 kDa [28]).

### *Whole blood aggregometry*

As already shown in our previous work [14], the *B. moojeni* venom fractions GF 3 and GF 5 contain an ecarin-like prothrombin activator (PTA) and the thrombin-like enzyme (TLE) batroxobin, respectively. Both substances trigger blood clotting and therefore entirely disturb the performance of aggregation measurements.

Figure 3 shows the effect of the PTA (ecarin) and TLE (batroxobin) on whole blood aggregation with and without the addition of hirudin and fibrin polymerisation inhibitor (FPI).

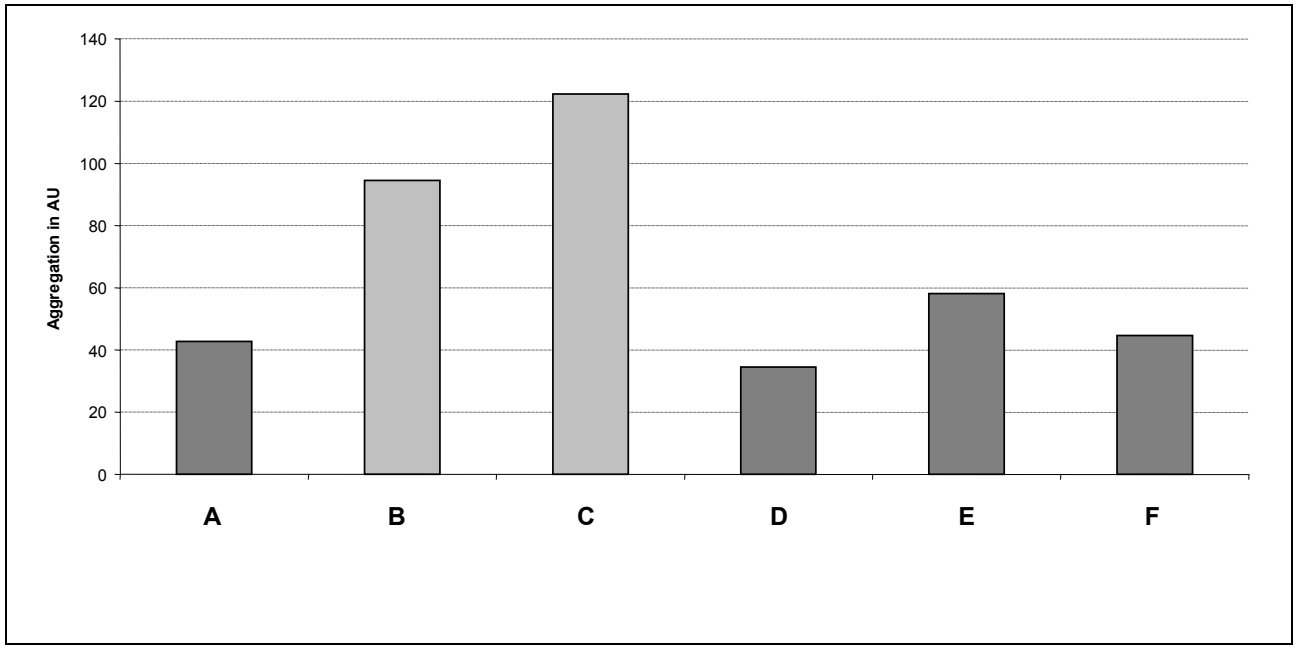


**Figure 3.** Influences of TLE and PTA on platelet aggregation, measurements performed in whole blood (mean values of 4 samples are shown): **A**: spontaneous aggregation, **B**: addition of PTA (ecarin), **C**: addition of TLE (batroxobin), **D**: addition of PTA with buffer containing hirudin/FPI, **E**: addition of TLE with buffer containing hirudin/FPI, **F**: control with buffer containing hirudin/FPI.

Both ecarin and batroxobin resulted in significantly enhanced platelet aggregation compared to the control measurement (Figure 3 A, B and C). The ecarin mediated aggregation (Figure 3 B) is most likely due to the PAR receptor mediated platelet activation by the generated thrombin. Batroxobin (Figure 3 C) clotted the sample, which most likely resulted in an accumulation of fibrin and platelets on the impedance sensors. Both effects could be eliminated by the addition of hirudin and FPI to the

reaction mixture (Figure 3, D and E). Finally, as shown by bar F, buffer containing hirudin/FPI has no major effect on platelet aggregation. Therefore, all the following experiments were performed using the pre-heated buffer containing recombinant hirudin and FPI, instead of pre-heated saline.

***Screening for platelet aggregation activators in the venom fractions GF 2 to 17***

In order to screen for platelet activators fractions GF 2 to 17 were added to the blood samples without the addition of any platelet agonist.

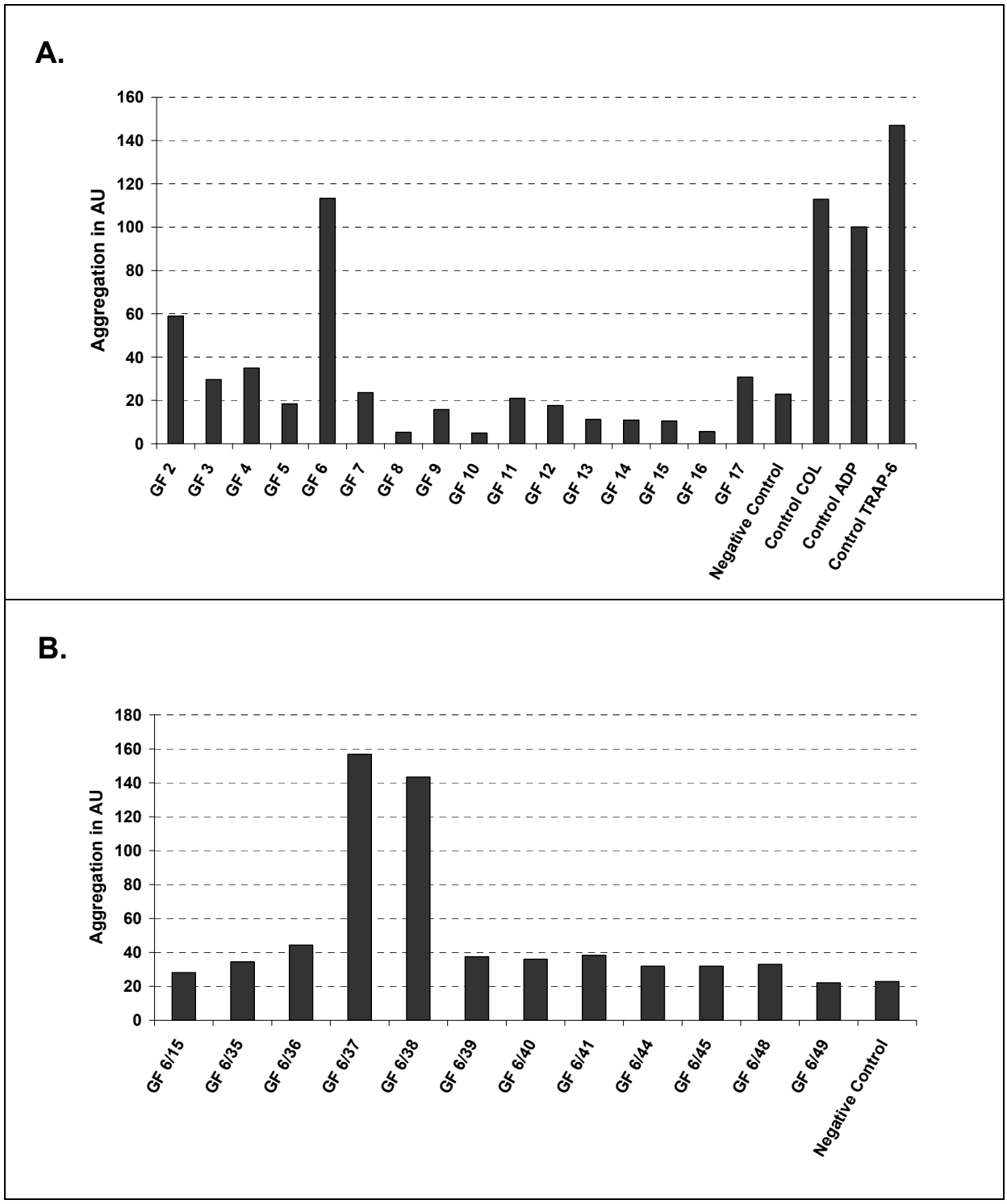


**Figure 4 A.** Activation of platelet aggregation by fractions GF 2 to GF 17, maximum aggregation obtained during the measurement time (5 min). The measurement of spontaneous aggregation was performed as negative control. As positive controls, platelets were activated by collagen (COL; 100 µg/ml), adenosine diphosphate (ADP; 0.2 mM) and thrombin receptor activating peptide (TRAP-6; 1mM). **B.** Activation of platelet aggregation by selected sub-fractions of GF 6 after the HIC purification.

In Figure 4A, the maximum aggregation obtained for the fractions GF 2 to GF 17 is compared to the spontaneous aggregation (negative control) and to aggregation caused by known agonists COL, ADP and TRAP-6 (positive controls). The most pronounced platelet activation was found in the fractions GF 2 and GF 6, while only minor activities were detected for GF 3, GF 4 and GF 17. Finally, no activating potential was found in the fractions GF 5 and GF 7 to GF 16.

As the highest activity was detected in fraction GF 6, it was further purified by HIC (Figure 1B). Batroxobin (GF 6/40 and GF 6/41) was identified by means of clotting time measurements performed in plasma on ball coagulometer KC4 A micro and was clearly separated from the platelet activating substance present in the sub-fractions GF 6/37 and GF 6/38 (Figure 4B).

In an additional experiment, using a chromogenic substrate for thrombin (Pefachrome® TH, Pentapharm, Basel, Switzerland), the active component present in sub-fractions GF 6/37 and 6/38 could be identified as a serine protease (data not shown).

In addition to the strong platelet activating potential, sub-fraction GF 6/37 significantly prolonged the clotting of fibrinogen triggered by thrombin (control: 25.3 sec, with the addition of GF 6/37 sub-fraction: 99.9 sec). However, in the fibrin plate experiments (data not shown), no fibrinolysis was found using both sub-fractions GF 6/37 and 6/38.

***Screening for platelet aggregation inhibitors in venom fractions GF 2 to 16***

In order to study platelet aggregation inhibition, venom fractions GF 2 to GF 16 were added to the blood samples activated by the known platelet agonists COL, ADP and TRAP-6. As shown in Figure 5A, fractions GF 9, GF 10 and GF 11 exhibited a substantial inhibitory potential for all three examined agonists. The most active fraction GF 10 was further separated by means of semi-preparative RP-HPLC (Figure 1C). For the sub-fraction GF 10/45 platelet aggregation inhibition could be demonstrated (Figure 5B).

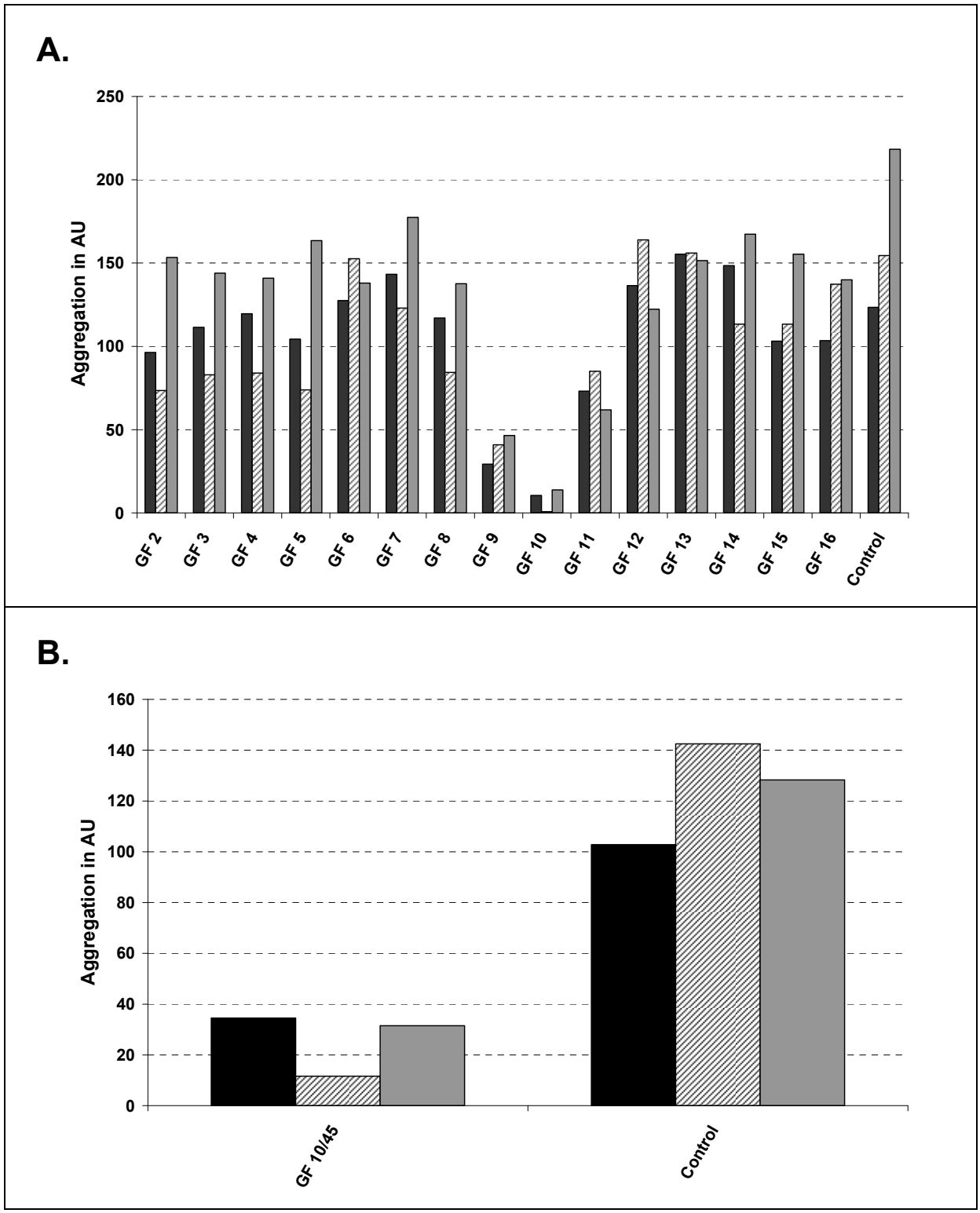


**Figure 5.** Influences of the fractions GF 2 to 16 on platelet aggregation triggered by COL – black, ADP – dashed and TRAP – 6 grey bars. **B.** Influences of the sub-fraction GF 10/45 on platelet aggregation triggered by: COL – black, ADP – dashed and TRAP-6 – grey bars. Blood samples without addition of any venom fraction were measured as control.

## 8.5 Discussion and conclusions

For the screening of different substances isolated from the venom of *B. moojeni* snake a new whole blood aggregometry method was used, employing different platelet aggregation activators. Whole blood aggregometry was selected for activity measurements due to several reasons: (i) The low sample volume and high throughput of the method allowed a differentiated assessment of platelet function; (ii) The analysis of whole blood resembles the physiological environment more than PRP or washed platelets, as it contains nearly all coagulation factors, which could interact *in vivo* with the active substance added to the sample; and, finally; (iii) Alterations of blood platelets during preparation of PRP can be avoided.

Tests were performed on a novel whole blood aggregometer, the Multiplate® analyzer. In order to overcome interferences by prothrombin-activating and thrombin-like enzymes (previously identified in venom fractions GF 3 and GF 5, respectively [14]), the standard whole blood aggregation measurement procedure described by Tóth *et al.* [25] was modified. Recombinant hirudin (specific inhibitor of generated thrombin) and the fibrin polymerization inhibitor Pefabloc® FG (to avoid fibrin gelation) were applied.

By examining the *B. moojeni* venom fractions, a significant platelet activating potential was found in fraction GF 6. Applying further purification steps, traces of batroxobin were successfully removed and the platelet activating component could be concentrated in the two neighbouring sub-fraction GF 6/37 and GF 6/38. These were further characterized by SDS-PAGE and aggregation assays and compared to known platelet aggregators from other *Bothrops* venoms, e.g. to MSP 1 known to be a proteolytic serine protease with α-fibrinogenase-like activities, i.e. weak fibrinolytic and fibrinogenolytic properties [15, 30]. Based on additional experiment, using a chromogenic substrate for thrombin (data not shown), both venom sub-fractions appeared to contain a serine protease that prolonged thrombin clotting time, but showed no fibrinolytic activity. The lack of the fibrinolytic effect could be due to the low protein concentration in the tested fractions or due to a very weak activity of the component of interest. On the other hand, fraction GF 6 did not enhance ADP-induced platelet aggregation. Since this property was clearly assigned for MSP 1 [15,

29], we concluded that the active component of the fraction GF 6 is not related to MSP 1.

Furthermore, the platelet aggregating component of sub-fraction GF 6/37 was sensitive against heparin inhibition (data not shown). According to literature [21], such inhibition was reported for two snake venom serine proteases showing platelet aggregation activating properties: thrombocytin from *B. atrox* and PA-BJ from *B. jararaca*.

Regarding the protein bands present in the SDS-PAGE gel, the main component of sub-fraction GF 6/37 having an apparent MW of 36 kDa resembles thrombocytin. However, further characterisation of the sub-fractions GF 6/37 and 6/38 is necessary. At this point we can only conclude that the agonist present in GF 6/37 and 6/38 is most likely a so far not reported *B. moojeni* protein closely related to *B. atrox* thrombocytin.

Neither MSP 1 nor MSP 2 could be identified in our study, which is in contrast to the findings of Serrano *et al.* [15]. The reasons are probably the different experimental conditions: PRP measurements in the study of Serrano *et al.* vs. whole blood aggregometry in the present study. In addition, we could not observe the effects described by Stábeli *et al.* [16] with BmooLAAO-I. Among the 18 GF fractions obtained in this study, we assume the presence of a LAAO in fraction GF 3, concerning its MW and the yellow colour, which is caused by the presence of LAAOs possessing the flavin adenine dinucleotide (FAD) in their structures [31]. Nevertheless, fraction GF 3 triggered only a weak platelet aggregation (Figure 4A). This could, on the other hand, be caused by a low concentration of LAAO. The dose-dependency of aggregation activation by LAAO has already been mentioned by Stábeli *et al.* [16]. The fraction GF 5 did not cause any platelet aggregation, confirming that batroxobin, previously found in this fraction, does not affect the integrity and functions of the blood platelets [32].

Regarding platelet aggregation inhibitors, the active fraction GF 10 was further processed by RP-HPLC. A platelet antagonist could be identified in sub-fraction GF 10/45 inhibiting aggregation triggered by all three known activators (COL, ADP, TRAP-6) and revealed a mass of about 7.5 kDa by MALDI-TOF MS measurements (data not shown). Both findings are in accordance with disintegrin characteristics.

Disintegrins are potent inhibitors of platelet aggregation induced by ADP, collagen, and thrombin and are well known for *Bothrops* snakes (e.g. batroxostatin from *B. atrox* [33], jarastatin from *B. jararaca* [34], bothrasperin from *B. asper* [22]). However, no disintegrin in *B. moojeni* venom has been described so far. Further investigations of the sub-fraction GF 10/45 in respect to the exact structure of the newly found platelet inhibitor could be of interest.

In conclusion, the preliminary functional investigation of *B. moojeni* venom in respect to platelet-active substances revealed one hitherto undescribed platelet agonist in fraction GF 6 and one platelet antagonist in fraction GF 10. In contrast to previous reports, we could not identify the fibrinolytic activity of MSP 1 and MSP 2.

The developed methodology for the whole blood aggregation with the addition of thrombin inhibitor and an inhibitor of fibrin polymerization might be of interest also for the investigation of other snake venoms and substance mixtures of different origins, e.g. plant extracts, possibly influencing the clotting system.

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# 9 Schlangengifte

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***For Hämostaseologie 2nd Edition***

## 9.1 Einführung

Schlangengifte sind eine reichhaltige Quelle biologisch aktiver Proteine, potenziell einsetzbar in Diagnostik und Therapie. Die Wirkstoffe können verschiedenen Proteinfamilien zugeordnet werden (Serinproteasen, Metalloproteinasen, Typ C-Lectine, Disintegrine Phospholipasen). Diese beeinflussen die selektive Wirkung der Venome auf die verschiedene Gerinnungsfaktoren, Blutzellen und Gewebe. So kann die Wirkung der Gerinnungsfaktoren imitiert oder gehemmt werden. Viele Wirkstoffe haben bereits Einzug in Diagnostik und Therapie von Gerinnungsstörungen gehalten. Gemäss ihrer Wirksamkeit werden sie in Gruppen klassifiziert: gerinnungsaktivierende Faktoren (FV-, FX-Aktivatoren, thrombinartige Enzyme, Prothrombinaktivatoren), Antikoagulantien (FIX-/FX-bindende Proteine, Protein C-Aktivatoren, Thrombininhibitoren, Typ $A_2$-Phospholipasen), Thrombozytenaktivatoren (Typ C-Lektine, Proteinasen) und -inhibitoren (Metalloproteinasen, Disintegrine, Typ C-Lektine), Fibrinolyse-beeinflussende Wirkstoffe (Plasminogenaktivatoren, Fibrinolytika), Hämorrhagien auslösende Wirkstoffe (Metalloproteinasen) (Lu *et al.* 2005; Marsh u. Williams 2005; Koh *et al.* 2006).

## 9.2 Substanzklassen und Präparate

Thrombinartige-Enzyme (SVTLE, snake venom thrombin-like enzymes) sind die in medizinischen Anwendungen am häufigsten genutzten Schlangengiftkomponenten. **Batroxobin**, ein Enzym zur Defibrinogenierung, wird gewonnen aus dem Gift der Brasilianischen Steppenlanzenotter (*Bothrops moojeni*) und beispielsweise unter dem Handelsnamen **Defibrase®** vertrieben. Ein Wirkstoffgemisch aus **Batroxobin** und einer Komponente mit thromboplastinartigen Eigenschaften, gewonnen aus dem Gift der Gemeinen Lanzenotter (*Bothrops atrox*), wird zur Blutungsstillung verwendet und als **Hemocoagulase** z.B. unter dem Handelsnamen **Reptilase®** geführt. Die Präparate werden hauptsächlich in den asiatischen Märkten therapeutisch verwendet: Defibrase® und Reptilase® in Japan und China, letztere darüber hinaus in Korea und Indien. Aus dem Gift der Malayischen Mokassinschlange (*Agkistrodon rhodostoma*) wird ein weiteres SVTLE isoliert. Das den Wirkstoff **Ancrod** enthaltene Präparat wurde in der Vergangenheit unter dem Handelsnamen **Arvin®** und

neuerdings unter **Viprinex®** vertrieben. **Viprinex®** wird in einer Reihe aussereuropäischer Länder verwendet. In den USA befindet sich das Präparat in Phase III Studien.

Rekombinant hergestellt wird der Wirkstoff **Alfimeprase**. Es handelt sich um eine verkürzte Form der **Fibrolase**, eine direkt fibrinolytisch und fibrinogenolytisch wirkende Zink-Metalloproteinase, ursprünglich gewonnen aus dem Gift des Nordamerikanischen Kupferkopfs (*Agkistrodon contortrix contortrix*). Phase I und II Studien bei Patienten mit arteriellen und venösen Thrombosen bewiesen die Wirksamkeit und eine gute Verträglichkeit (Deitcher *et al.* 2006). Derzeit befindet sich das Präparat in den Phase III Studien.

Basierend auf der Struktur der Thrombozyten-inhibierenden **Disintegrine** wurden synthetische Derivate mit einer sehr viel höheren Affinität zum thrombozytären Glykoprotein IIb-IIIa-Rezeptor entwickelt. In die Klinik eingeführt wurden **Eptifibatid** (**Integrilin®**)und **Tirofiban** (**Aggrastat®**). **Eptifibatid**, ein zyklisches Heptapeptid mit einer pharmakologisch aktiven Gruppe (KGD), leitet sich von der Struktur des **Barbourin**, einem Glykoprotein IIb-IIIa-Inhibitor aus der Dunklen Zwergklapperschlange (*Sisturus miliarus barbouri*) ab (Scarborough *et al.* 1991; Scarborough *et al.* 1993; Phillips u. Scarborough 1997). **Tirofiban** imitiert die adhäsive Aminisäuresequenz RGD und ist abgeleitet von der Schlangengiftkomponente **Echistatin**, isoliert aus dem Gift der Sandrasselotter (*Echis carinatus*) (Hantgan *et al.* 2004). Beide Wirkstoffe wurden für die Therapie des akuten Koronarsyndroms und der Prävention von Thromboembolien bei perkutanen Koronarinterventionen zugelassen (Koh *et al.* 2006). Auf die Thrombozytenhemmer wird in Kapitel (…) näher eingegangen.

## 9.3 Wirkmechanismus

Im Unterschied zu Thrombin, das sowohl Fibrinopeptid A wie auch B (FPA, FPB) vom Fibrinogen abspaltet und gleichzeitig FXIII aktiviert, spalten die meisten SVTLE lediglich FPA ab (nur sehr wenige auch FPB). Dadurch entstehen Fibrinfäden, aber kein dreidimensionales Fibrinnetzwerk. Nach Applikation von **Batroxobin** wird die Spaltung der Bindung Aα16Arg-17Gly im Plasmafibrinogen katalysiert und führt zur Abspaltung von FPA und zur Entstehung von monomeren Fibrin I (des-A-Fibrin).

Darüberhinaus wird die Freisetzung von tPA aus dem Endothel stimuliert. Fibrin I wird durch das entstehende Plasmin zu Fibrinspaltprodukten (FSP) abgebaut. Über diesen Mechanismus erfolgt eine dosisabhängige Senkung der Fibrinogenkonzentration im Plasma und Reduktion der Viskosität des Blutes. Im Gegensatz zu thrombingebildetem Fibrin II (des-A-des-B-Fibrin), das in verdünnter Essigsäure nicht löslich ist, geht batroxobingebildetes Fibrin I nach Zusatz von Essigsäure sofort in Lösung und geliert bei Neutralisierung der Säure wieder. Dieses Verhalten von Fibrin I wird zur Herstellung eines chirurgischen Gewebeklebers und Hämostyptikums (Vivostat®) unter Verwendung von Eigenblut, genutzt. Im Unterschied zu Thrombin aktivieren die SVTLE weder andere Gerinnungsfaktoren, noch aktivieren sie Thrombozyten. Sie werden nicht durch Thrombininhibitoren wie AT und Hirudin gehemmt. Obwohl sie in ihrer Funktion dem Thrombin ähnlich sind, unterscheiden sie sich grundlegend von diesem in Struktur und Funktion (Stocker 1990; Stocker 1999; Kini 2006).

Die Wirkung von **Ancrod** ist weitgehend analog zu der von Batroxobin mit dem Unterschied dass Plasminogen nicht durch Ancrod aktiviert und somit bereits gebildetes Fibrin nicht abgebaut wird. Daher kann es auch postoperativ angewendet werden (Quelle: www.rxmed.com).

**Hemocoagulase** führt bei lokaler topischer Applikation im Falle von Hautverletzungen dosisabhängig zur Fibrinbildung und nachfolgend zur Blutungsstillung. Die Injektion einer relativ geringen Dosis führt über einen längeren Zeitraum zur Bildung von Fibrin I und einer erhöhten Gerinnbarkeit des Blutes (Stocker 1990).

Abgeleitet von der Fibrolase wirkt **Alfimeprase** direkt fibrinogenolytisch auf die Fibrinogen Aα Kette. Im Gegensatz zu den plasminogenartigen, thrombolytischen Substanzen sind Fibrolase und Alfimeprase nicht abhängig vom endogenen Fibrinolysesystem. Alfimeprase wird durch die Bindung an α2-Makroglobulin und Ausbildung nicht redissoziierbarer makromolekularer Komplexe neutralisiert. *In vivo* wird im Vergleich zu Plasmin oder Urokinase ein Gerinnsel mit bis zu einer sechsfach höheren Geschwindigkeit aufgelöst (Toombs 2001; Swenson *et al.* 2004; Deitcher *et al.* 2006).

## 9.4 Indikationen

Durch die Senkung der Fibrinogen- und Antiplasminkonzentration im Blut, das Auslösen einer fibrinolytischen Reaktion und Reduktion der Blutviskosität dienen sowohl eine Defibrase- wie auch Ancrodbehandlung der allgemeinen Thromboseprophylaxe, einer Verstärkung der physiologischen und medikamentös unterstützten Fibrinolyse, sowie einer verbesserten Mikrozirkulation.

**Defibrase®** wird eingesetzt bei tiefer Beinvenenthrombose, Lungenembolien, zur Thromboseprophylaxe bei Hochrisikopatienten, Zentralvenenthrombosen, Arteriosclerosis obliterans, Thrombangiitis obliterans, diabetischen Mikroangiopathien, Erfrierungen, Thromboseprophylaxe nach fibrinolytischer Therapie, Raynaud-Syndrom, paVK, Sklerodermie und dem Vibrationsyndrom (Arzneimittel Kompendium der Schweiz 1991).

**Viprinex®** wird eingesetzt zur Prävention und Behandlung tiefer Beinvenenthrombosen, Zentralvenenthrombosen, thrombotisch verursachtem Priapismus, thrombotisch verursachter pulmonaler Hypertonie, Embolien bei künstlichen Herzklappen, Rezidiven nach Thrombolysetherapie, bei gefässchirurgischen Eingriffen sowie zur Behandlung von Durchblutungsstörungen (u.a. bei Thrombophlebitis,Thrombangiitis obliterans, diabetischen Mikroangiophathien und Morbus Raynaud). Randomisierte Studien haben die Wirksamkeit von Ancrod in der Therapie des akuten ischämischen Hirninfarkts gezeigt (Hennerici *et al.* 2006).

**Reptilase®** wird eingesetzt bei prä- und postoperativer Blutungsneigung, bei Tonsillektomie, Ophthalmologie, Odontologie, bei Epistaxis, spontanen oder traumatisch bedingten Blutungen und hämorrhagischen Diathesen. Für lokale Anwendungen wird Reptilase auf die Wundfläche getragen, wie z.B. bei nachblutenden Zahnextraktionen (Arzneimittel Kompendium der Schweiz 1991).

Basierend auf der direkten thrombolytischen Aktivität von **Alfimeprase** und der nur geringen haemorrhagischen Aktivität und Neutralisierung durch Bindung an α2-Makroglobolin besteht ein gutes Sicherheitsprofil im Vergleich zu anderen therapeutisch verwendeten Plasminogenaktivatoren. Alfimeprase wurde als Therapeutikum für periphere arterielle Verschlüsse und Katheterthrombosierungen

entwickelt. Potenzielle weitere Anwendungsbereiche sind arterielle und venöse Gefässverschlüsse (Deitcher *et al.* 2006).

**Dosierung und Applikation**

Präparate zur Defibrinogenierung werden basierend auf der Plasmafibrinogenkonzentration dosiert. Die einzustellende Zielkonzentration für **Defibrase®** (s.c. oder i.v.) wird mit 70 ± 30 mg/dl (Arzneimittel Kompendium der Schweiz 1991) und für **Viprinex®** (s.c. oder i.v.) mit 20 - 70 mg/dl (Quelle: www.rxmed.com) angegeben. **Defibrase®:** Therapie (i.v.): Initialdosis 20 oder 10 BU/d (BU, Batroxobin Units) in mind. 100 ml NaCl, nachfolgend 5 BU/d über 1 h in 250 ml NaCl, Zeitraum von 1 - 6 Wochen (gemäss Packungsbeileger). **Viprinex®:** Therapie (s.c.): 70 U/d während der ersten 4 d Behandlung, nachfolgend 70 - 140 U/d. Bei Erreichen der gewünschten Fibrinogenkonzentration Fortführung der Therapie mit 2 - 3 mal wöchentlich 210 - 280 U. Prophylaxe (s.c.): nach Chirugie 4U/ kg Körpergewicht (KG), während der darauffolgenden 4 Tage 1 U/ kg KG. Prophylaxe (i.v.): Initialdosis über 12 Stunden 1 - 2 U/ kg KG in 250 - 500 ml NaCl- oder 5% Dextroselösung, danach 0.5 - 1 U/ kg KG in 250 - 500 ml NaCl- oder 5% Dextroselösung (Quelle: www.rxmed.com).

Gemäss Packungsbeileger wird bei **Reptilase®** wird eine Dosierung von 1 - 2 KU (KU, Klobusitzky-Unit), bei Kindern 0,3 – 1 KU (i.v. oder i.m.) empfohlen, in Notfällen 1 KU i.v., falls notwendig wiederholt 1 KU i.v. Reptilase® präoperativ: entweder eine Stunde vor Beginn 1 KU i.m. oder 15 min vorher 1 KU i.v. Reptilase® postoperativ: Empfehlung von 1 KU/d i.m. über einen Zeitraum von 3 d. Eine topische oder s.c. Anwendung des Präparats ist gleichfalls möglich. Eine Therapiebeobachtung mittels Bestimmung der Blutungszeit wird empfohlen.

Die maximal zu verabreichende Dosierung von **Alfimeprase** richtet sich nach der α2-Makroglobulin-Konzentration im Plasma und seiner Bindungskapazität für Alfimeprase. Dosierungen bis zu 0.6 mg/kg KG wurden in den Phase I und II Studien verwendet. Die Applikation erfolgte i.a. und/oder direkt in das vorliegende Gerinnsel (Deitcher *et al.* 2006).

## 9.5 Nebenwirkungen

**Defibrase®** wird allgemein gut vertragen. Beschriebene Nebenwirkungen sind allergische Reaktionen, Kopfschmerzen sowie Schläfrigkeit. In seltenen Fällen kann es zu Fieberreaktionen, Ekchymosen, Hämatomen und Blutungen aus der Punktionsstelle kommen. Nach 4 - 6 Behandlungswochen kann sich eine immunologische Resistenz gegen Defibrase entwickeln, die einen Anstieg des Plasmafibrinogenspiegels trotz fortgeführter Therapie auslöst. Die Therapie ist ggf. mit Ancrod weiterzuführen (Arzneimittel Kompendium der Schweiz 1991; Bell 1997).

Die spezifischere Wirkung von **Viprinex®** verringert das Blutungsrisiko, es wurden jedoch Blutungen auch unter Ancrod beschrieben. Bei s.c. Gabe können ggf. auftretende lokale oder systemische allergische Reaktionen mit Antihistaminen behandelt werden (Quelle: www.rxmed.com).

**Reptilase®** wird allgemein gut vertragen. Bislang wurde nur über geringfügige Nebenwirkungen berichtet (Arzneimittel Kompendium der Schweiz 1991).

Die Gefahr von systemischen Blutungen wird durch die inhibitorischen Eigenschaften des α2-Makroglobulin auf **Alfimeprase** reduziert. Während der klinischen Prüfung konnte lediglich über zwei Fälle mit Nebenwirkungen berichtet werden, zum einen trat eine milde Hautrötung auf, in dem zweiten Fall traten leichte Kopfschmerzen auf (Deitcher *et al.* 2006).

## 9.6 Labordiagnostik

Schlangengiftwirkstoffe kommen neben der therapeutischen Anwendung in der *in vitro* Diagnostik zur Anwendung (Marsh 2001; Marsh u. Williams 2005; Schoni 2005).

SVTLE werden für die Analyse des Fibrinogens (z.B. Dysfibrinogenämien) und dessen Polymerisation (Fibrinpolymerisationhemmung durch FSPs) eingesetzt (z.B. Reptilase®-Zeit). Diagnostisch macht man sich zu Nutze, dass die Reptilase-Zeit im Gegensatz zur Thrombinzeit nicht durch Heparin- oder direkte Thrombininhibitoren gehemmt wird.

Prothrombinaktivatoren (PTA) werden eingesetzt zur Durchführung der Ecarin-Clotting Time (ECT), zum funktionellen Nachweis einer Faktor V Leiden-Mutation

(FVL) und zur Untersuchung des Prothrombins (quantitative und qualitative Mängel). Bekannte Wirkstoffe sind Ecarin (Sandrasselotter, *Echis carinatus*), Noscarin (Tigerotter, *Notechis scutatus*), Oscutarin (Taipan, *Oxyuranus scutellatus*) und Textarin (Gewöhnliche Braunschlange, *Pseudonaja textilis*).

Aktivatoren des FX und FV (RVV-X, RVV-V, Russell's viper venom, isoliert aus dem Gift der Kettenviper *Daboia-russelli*) und des Protein C (Protac®, isoliert aus dem Gift des Nordamerikanischen Kupferkopfs *Agkistrodon contortrix contortrix*) werden in Testen zur Untersuchung des Protein C-Systems (v.a. Protac®), der Lupus-Antikoagulantien (v.a. RVV-V und RVV-X, aber auch Textarin) sowie im Antikoagulantien-Monitoring (PiCT®: Kombination von FXa und RVV-V) eingesetzt.

Botrocetin®, ein Wirkstoff isoliert aus dem Gift der Jararaca-Lanzenotter (*Bothrops jararaca*), aggregiert von Willebrand Faktor (vWF) -abhängig die Thrombozyten und wird eingesetzt zur Untersuchung von vWF-assozierten Defekten wie z.B. Bernard-Soulier Syndrom. Die Verwendung von aus Schlangengiften isolierten Disintegrinen zur Untersuchung von Thrombozytenglykoproteinen (zB GPIIb/IIIa, GPIb) wurde beschrieben, ebenso die Verwendung von C-Typ Lektinen zur Modulierung der Thrombozytenfunktion. Bekannte C-Typ Lektine sind Echicetin (Sandrasselotter, *Echis carinatus*) und Convulxin (Südamerikanische Klapperschlange, *Crotalus durissus terrificus*).

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# 10 Summary and outlook

## 10.1 Snake venoms and haemostasis

Snake venoms contain a large number of biologically active compounds. Some of them are quite common and similar to one another within each family of snakes, while others are specific for each species and may have quite unique biological features, e.g. compounds exerting varied activities on the haemostatic and fibrinolytic system. These components are often very specific in their mode of action, i.e. they activate or deactivate different mechanisms of both systems. Some purified snake venom proteins with known activity have become valuable tools as diagnostics and are used for pharmaceutical applications or preparative procedures in the field of haemostaseology, neurobiology and complement research.

Early studies in the 1930s on the venom of South-American Lancehead snakes of the *Bothrops* genus have led to the discovery of compounds like batroxobin and botrocetin which show high activity in haemostasis. Batroxobin (trade names Reptilase® and Defibrase®) is still widely used for pharmaceutical and diagnostic applications. However, most of the *Bothrops moojeni* venom material utilized for its isolation is not further processed, although it still contains a large number of compounds not or at least not fully characterized.

## 10.2 "*Bothrops moojeni* venom proteomics" project (Botmo Project)

In order to close this gap, the "*Bothrops moojeni* venom proteomics" project and this thesis (the *Botmo Thesis*) were focused on the crude venom of *B. moojeni* snake using various protein purification methods, mass spectrometry and biocomputing, as well as newly developed screening tools for activities in the fields haemostasis and fibrinolysis. So far, several unknown activities have been identified, however, many active compounds still wait to be discovered and further characterized.

In a first purification step of the crude *B. moojeni* venom, a size exclusion chromatography (gel filtration; GF) was applied resulting in 18 GF fractions (for details see chapter 3). Some of them were further separated using different

chromatographic methods and afterwards submitted to two different approaches: “function to structure study”, resulting in data presented in the framework of the present *Botmo Thesis* (chapters 3 and 6-8) and “structure to function approach”, shown with an example of chapter 5 and summarized below (complete data not shown).

As mentioned in chapter 3, the preliminary random MALDI-TOF-MS analyses of fractions obtained in different purification steps revealed the presence of about 1’200 molecular masses in the range of 500 to 10’000 Da. The measured molecular masses represent the highest number of compounds ever detected in crude snake venom. The actual number of different venom components is, however, probably smaller, as the same peptide chain might lead to several signals when it undergoes modifications or fragmentations. Due to the large amount of the crude *B. moojeni* venom available as source material for this project, also minor components could be detected in the separation processes.

## 10.3 The “structure to function approach”

Random ESI-MS/MS *de novo* sequencing of peptides from the complex HPLC fractions was performed by the Atheris-Team resulting in:

- 7 sequences (4 novel) of bradykinin-potentiating peptides (BPPs),
- a histidin-rich peptide (66% of His),
- a fragment of small myotoxin [41% identity with the *N*-terminal part of myotoxin A from *Crotalus viridis* (Swiss-Prot Accession number AN: P01476) and crotamines from *C. durissus* (Swiss-Prot Accession number AN: P01475 / P24334] and
- new sequences with no homologies found in databases.

Additionally, 11 peptides (120 amino acids, 25% sequence coverage) were found to correspond to the L-amino acid oxidase (Swiss-Prot Accession number AN: Q6TGQ8) and at least three different metalloproteinases were predicted in the *B. moojeni* venom, concerning the found similarities:

- 5 peptides corresponding to (13% sequence coverage, 87% identity with the precursor) Jararhagin (152-355) / Disintegrin (356-447) from *B. jararaca* (Swiss-Prot Accession number AN: P30431),
- 2 peptides (10% sequence coverage) corresponding to *B. jararacussu* metalloprotease (Swiss-Prot Accession number AN: Q7T1T3), and
- 5 peptides (9% sequence coverage) corresponding to *B. insularis* Insularinase (Swiss-Prot Accession number AN: Q5XUW8).

The "structure to function" approach showed that 40% of the revealed sequences correspond to degradation fragments from metalloproteinases and L-amino acid oxidase, suggesting that the venom underwent proteolytic action, either naturally, during collection and storage, or induced by the fractionation steps. These findings indicate that some activities found especially in the low molecular weight fractions, might result not from the entire proteins but from their peptidic degradation fragments still maintaining the active sides. Such compounds, actually not identified in the *B. moojeni* venom so far, would be of special interest. Their low molecular sizes would allow simple synthetic approaches for the preparation of large amounts of pure material needed for further investigations.

Further, applying a novel approach described in chapter 5 and summarized below, a total of 15 new BPP sequences have been identified based on their specific structural determinants.

## 10.4 The "function to structure study"

Special blood coagulation screening-platform consisting of 14 assays has been developed for the needs of the "function to structure" approach. The tool was designed for the detection and evaluation of any kinds of effect on distinct parts and functions of the blood coagulation system, such as: (i) the intrinsic pathway, (ii) the extrinsic pathway, (iii) fibrinolysis, (iv) the APC system, (v) the antithrombin function, (vi) the prothrombinase complex, (vii) blood coagulation activation, and (viii) thrombin dynamics in the intrinsic or extrinsic pathway. The screening was performed on an automated Coagulation Test System BCS (Behring Coagulation System).

## 10.5 Novel active ingredients studied in the framework of Botmo Thesis

- Phospholipase showing heparin-inhibiting properties

In addition to proteins and peptides already characterized in the *B. moojeni* venom, two novel phospholipases $A_2$ ($PLA_2$) have been isolated and fully sequenced in this *Botmo Thesis*. Both of these belong to the enzymatically non-active Lys49 $PLA_2$ variants. They consist of 122 amino acids and share a characteristic sequence in the *C*-terminal region. This sequence, composed of clusters of basic and hydrophobic amino acids, is known from the literature to interact with heparin. So far, it has been reported that heparin could interact with basic $PLA_2$s and was used *in vivo* and *in vitro* to neutralize different toxic effects caused by some snake venoms and their $PLA_2$s (for details see chapter 6).

The two novel $PLA_2$ variants (MjTX-III and MjTX-IV) were isolated using size exclusion chromatography and RP-HPLC, fully sequenced by ESI-MS/MS techniques and characterized by means of coagulation assays performed in heparanized plasma. They interact *in vitro* with unfractionated heparin (UFH) and low molecular weight heparin (LMWH), neutralizing their anticoagulant properties. Although it is well known that $PLA_2$s from snake venoms influence the blood coagulation system, the use of those substances to antagonize the anticoagulant effect of heparin *in vivo* or *in vitro* has never been proposed until now. Discovery of a LMWH inhibitor is of medical interest as, until now, there is no clinically available antidote.

- Blood coagulation FVIIa-like enzyme

A procoagulant protein with a molecular weight of about 30 kDa, acting independently of the contact phase and FIX, but dependent on FX (for details see chapter 7) was identified. Taking into account the cleavage of a chromogenic substrate with high sensitivity for FVIIa, the activity of the new protein resembled most closely the action of coagulation FVIIa. Such activity has not been reported for any snake venom so far and further investigations thereof would be of medical interest.

- Platelet activators and inhibitors

Another aspect of haemostasis, also investigated using the *B. moojeni* venom GF fractions, is the aggregation of blood platelets (for details see chapter 8). For this approach, whole blood aggregometry was applied and a special test method for screening of protein mixtures was developed. In order to eliminate interferences caused by prothrombin activators or thrombin like-enzymes, both present in the *B. moojeni* venom (fractions GF 3 and GF 5, respectively), inhibitors of thrombin and fibrin polymerisation were used. The preliminary analyses performed with all *B. moojeni* venom GF fractions revealed the presence of a platelet aggregation activator resembling the activity of thrombocytin (from *B. atrox* venom) and also a platelet antagonist with broad inhibitory activity, which is most likely a disintegrin.

## 10.6 *Bothrops moojeni* venom as a model substance for the development of analytical screening tools

*B. moojeni* crude venom and some of its fractions were also used as model substances for the development of different screening tools applying MS technology. The complexity of the crude venom and its GF fractions may provide a good model for selectivity assessment of the developed methodologies.

Using the *B. moojeni* crude venom an original method was established for the discovery of BPPs in natural extracts. It is based on the BPP-structural determinants (a predominant signal at *m/z* 213.1 and additionally characteristic signals at *m/z* 226.1 and 240.1 in ESI-MS/MS analysis) and applies liquid chromatography coupled to ESI-MS/MS operated in precursor ion scan mode (for details see chapter 5). Subsequently, MALDI-TOF-MS analysis and LIFT-TOF/TOF-MS experiments were performed on the *B. moojeni* pre-treated venom showing the complementarity of the used techniques and revealing the presence of 20 peptides belonging to the BPP-like family. A total of 15 new sequences have been identified by this approach.

*B. moojeni* venom fractions were also submitted into a multiplex ESI-MS based assay developed as a tool for screening of proteolytic activities in protein mixtures and used for the simultaneous determination of five different substrate/product pairs (for details see chapter 2). Activity patterns of each fraction were generated and used to sort the

fractions into three different categories of proteolytic activity, confirming the abundant presence of proteases in the venom. Further, a similar ESI-MS based approach was developed for the determination of acetylcholinesterase inhibiting activity in complex protein mixtures (for details see chapter 4). *B. moojeni* venom fractions were used in this assay as model substances; two of them revealing the presence of acetylcholinesterase inhibiting properties. To our knowledge, it was the first time such properties were described in the venom of *B. moojeni* snake.

## 10.7 Botmo Thesis - Conclusions

From the above results obtained in the *Botmo Thesis*, it can be concluded that *B. moojeni* venom is an extremely complex mixture and still remains a "source of undiscovered potential". Thus, its further separation and additional investigations of its properties would be desired within the "*Bothrops moojeni* venom proteomics" project. Heparin-neutralizing properties of the synthetic peptides derived from the MjTX-III should be clarified, as well as the activity features and structural determinants of the FVIIa-like protein and the substances active on blood platelets. Finally, the research should also be expanded to other than the haemostasis fields of application *i.e.* activity on ion channels, cell penetration, antifungal, antibacterial, anti-parasitic or antiviral activities, other enzymatic activities and inhibition thereof.

# Appendix

*Bothrops moojeni*

# *Bothrops moojeni* Proteomics – a Second Look at the Venom


**A.M. Perchuc[1,2], L. Menin[3], R. Stöcklin[3], B.Bühler[1] and R. Schöni[1]**

**[1] Pentapharm Ltd., Basel, Switzerland**

**[2]Institute of Molecular Pharmacy, University of Basel, Switzerland**

**[3] Atheris Laboratories, Geneva, Switzerland**


## Introduction

Early studies in the 1930s on the venom of South –American Lancehead snakes of the *Bothrops* genus have led to the discovery of compounds very active in haemostasis like batroxobin and botrocetin. Batroxobin under its trade name Reptilase® is still widely used in pharmaceutical and diagnostic applications. Most of the *Bothrops moojeni* venom material needed for its production is not further processed. But like all snake venoms it contains still a large number of biologically active compounds not yet discovered or fully described.

The scope of our investigations is to have a deeper look at the crude venom of *B. moojeni* using state-of-the-art proteomics and peptidomics methods. The techniques used up to now have included chromatography, mass spectrometry and biocomputing, as well as newly developed bioassays screening for activities in the different fields of application. Our goal is to analyze the crude venom by several transversal techniques in order to have a full overview of its major components that can be divided in four classes:

- Proteins of more than 10 kDa, usually with enzymatic activities;
- Mini-proteins between 1 and 10 kDa with disulfide bonds, and typically toxins possibly acting as ion channel modulators or specifically binding to receptors or other targets;
- Linear peptides between 200 and 5'000 Da with no disulfide bonds, possibly acting as enzyme inhibitors, neuropeptides, etc.;
- Others, mainly small compounds of non peptidic structure.

Using this approach we want to identify and describe new bioactive compounds, which might be used as active pharmaceutical ingredients (APIs) or diagnostic tools. We focus on the field of haemostasis and fibrinolysis, but look as well to other fields of application like enzymatic activity, enzyme inhibitors, activity on ion channels, binding to different receptors or other targets.

## *B. moojeni* Crude Venom – the Whole Picture

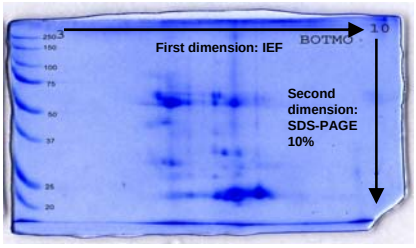


***Fig.1*** B.moojeni 150 mg of crude venom, 2D-SDS-PAGE analytical scale

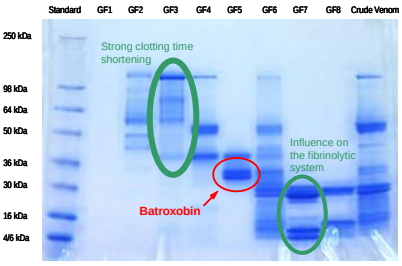


***Fig.2*** B.moojeni gel filtration fractions 1-8, SDS-PAGE analytical scale

## Purification steps

### ➢ Step I – Gel Filtration (GF)

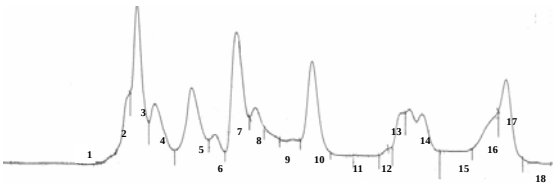


***Fig.3*** Gel filtration chromatogram obtained with 300 mg of crude *B. moojeni* venom. 5g of *B.moojeni* crude venom have been separated in 20 individual runs. The reproducibility of all performed experiments was very good, so that pooling into 18 distinct GF-fractions collected from run to run was successfully performed.

### ➢ Step II– HPLC

Six gel filtration fractions were selected for further purification and analysis. Three of them are shown below:

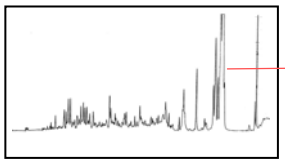

***Fig.4a*** HPLC profile of B.moojeni GF fraction 9.

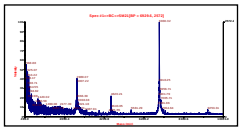

***Fig.4b*** MALDI-TOF-MS of Botmo GF9-HPLC 114, measured MW in $PLA_2$ range; activity confirmed in $PLA_2$ assay.

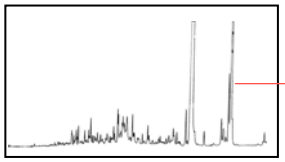

***Fig.5a*** HPLC profile of B.moojeni GF fraction 10.

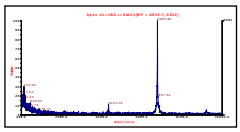

***Fig.5b*** MALDI-TOF-MS of Botmo GF10-HPLC 85, measured MW in $PLA_2$ range; activity confirmed in $PLA_2$ assay.

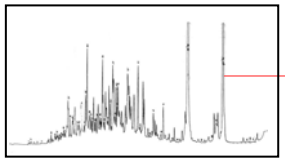

***Fig.6a*** HPLC profile of B.moojeni GF fraction 12.

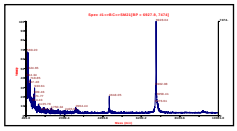

***Fig.6b*** MALDI-TOF-MS of Botmo GF12-HPLC 96, measured MW in $PLA_2$ range; activity confirmed in $PLA_2$ assay.

- From each HPLC separation about 100 sub-fractions were obtained;
- About 200 of them were analysed by MALDI-TOF-MS using α-cyano-4-hydroxycinnamic acid (4-HCCA) matrix, resulting in about 1200 measured molecular masses in the range of 500 to 10'000 Da;
- Also the minor compounds present in the venom could be detected, since a large amount of crude venom was used as source material for the separation steps;
- The amount of measured molecular masses indicates the highest number of compounds ever detected in a raw snake venom. But although the number of observed masses is impressive, it is probably slightly overestimated, as some of them can correspond to the same peptide chain, which underwent different modifications.

## Overview of the Activities on Blood Coagulation System Found in the Preliminary Screening

All gel filtration fractions, as well as 600 HPLC sub-fractions were already preliminary screened for their possible activities influencing the blood coagulation system. The activities found so far in the common bio-assays are listed below:

- Strong clotting time shortening by the Batroxobin containing fraction (GF5);
- Strong clotting time shortening by a non-Batroxobin fraction (GF3);
- Influence on the fibrinolytic system (GF7);
- Influence on the protein C pathway;
- Clotting time prolongation or no clot formation by the fractions containing $PLA_2$ activity ($PLA_2$ activity confirmed in the $PLA_2$ assay);
- Modification of heparin action on the blood coagulation system;
- Modification of $PLA_2$ action on the blood coagulation system.

## Conclusions

Out of 18 GF fractions originally prepared all have now been checked in the preliminary screening for their activities on blood coagulation system. Three of them, further separated by HLPC, have been analysed in a broad screening attempt verifying the blood coagulation effects. Besides the known activities several new and interesting ones have been found. They have to be studied and confirmed in supplementary more specific bioassays. Their structures will be further characterized using proteomics and biochemical techniques, such as reduction and alkylation, digestion and Edman sequencing, as well as MS/MS *de novo* sequencing. Other fractions need additional separation and purification steps before further screening.

In several cooperation projects some of the fractions are also investigated for their possible effects not connected with blood coagulation.


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15th European Symposium on Animal, Plant and Microbial Toxins
Brdo Estate (Kranj), Slovenia
June 19-23, 2004

*Bothrops moojeni*

# *Bothrops moojeni* Venom - a resource of undiscovered potential


**A.M. Perchuc[1,2], L. Menin[3], R. Stöcklin[3], B.Bühler[1] and R. Schöni[1]**

**[1] Pentapharm Ltd., Basel, Switzerland**

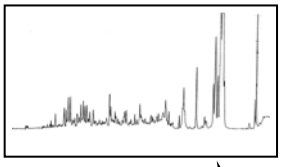
**[2]Institute of Molecular Pharmacy, University of Basel, Switzerland**

**[3] Atheris Laboratories, Geneva, Switzerland**

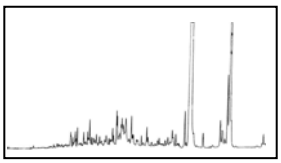

## Introduction

Snake venoms contain a large number of biologically active compounds. Some of them are quite common and similar to one another within each family of snakes. Others are specific for each species analyzed and may have unique features to be used in either pharmaceutical or diagnostic applications.
Early studies in the 1930s on the venom of South –American Lancehead snakes of the *Bothrops* genus have led to the discovery of compounds very active in haemostasis like batroxobin and botrocetin. Batroxobin under its trade name Reptilase® is still widely used in pharmaceutical and diagnostic applications. But most of the *Bothrops moojeni* venom material needed for its production is not further processed, although it contains a huge amount of compounds still not fully characterized and described.
In this cooperation project we are having a deeper look into the crude venom of *B. moojeni* using state-of-the-art proteomics and peptidomics 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 chromatography, mass spectrometry, and bio-computing. The bioassays used are focused on enzymatic and other activities in the field of haemostasis and fibrinolysis. But our studies expand also to other activities like enzyme inhibitors, ion channel activation or blocking, or binding to different receptors or other targets.
The crude venom is being analyzed by several transversal techniques in order to have a full overview of its major components. They can be classified as proteins (most likely with enzymatic activities), mini-proteins (most likely toxins possibly acting as ion channels modulators or specifically binding to receptors or other targets), linear peptides (possibly acting as enzyme inhibitors or neuropeptides) and other, mainly small compounds of non- peptide structure.
Using this approach several promising new activities have been found. They still have to be analyzed and studied in view of their possible role in pharmaceutical and diagnostic applications.

## *B. moojeni* Venom – the Whole Picture



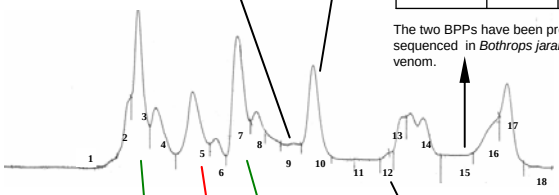
HPLC profile of B.moojeni GF 9.



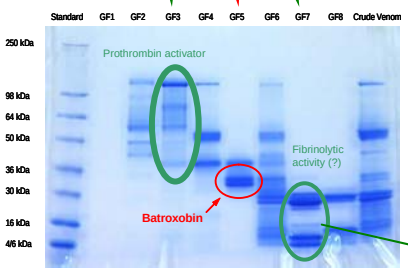
HPLC profile of B.moojeni GF 10.

| Sequence | MS/MS Measured Mass (Da) | Calculated Mass (Da) |
|---|---|---|
| QPWPRPGPELPP | 1369.72 | 1369.71 |
| ZSWPPGNIPP | 1075.55 | 1074.51 |

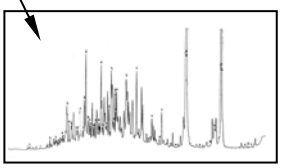
The two BPPs have been previously sequenced in *Bothrops jararaca* snake venom.



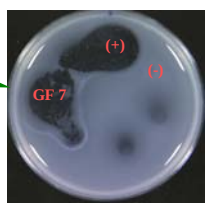

Gel filtration chromatogram obtained with 300 mg of crude *B. moojeni* venom.




B.moojeni gel filtration fractions 1-8, SDS-PAGE analytical scale

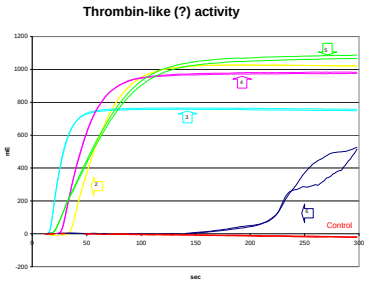


HPLC profile of B.moojeni GF 12.




Fibrin plate.

## ➢ Clotting properties: Batroxobin/Prothrombin Activator

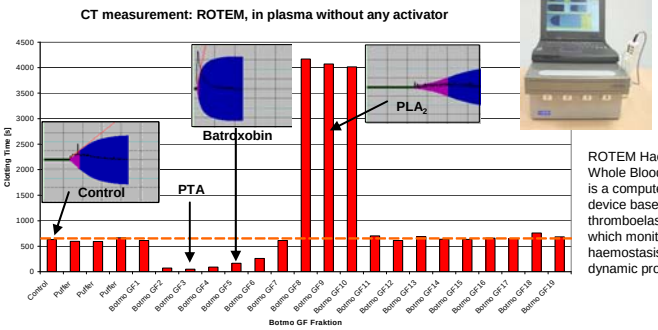



It was not possible to differentiate between the thrombin-like (Batroxobin) and prothrombin activating properties when the experiments were performed in plasma. Therefore additional measurements of fibrinogen clotting time were performed on appropriate Botmo GF fractions:

| Fraction | Fibrinogen Clotting Time (sec) |
|---|---|
| GF3 | >200 |
| GF4 | 87 |
| GF5 | 7 |
| GF6 | 45 |
| GF7 | >200 |

| PTA | CT | | | |
|---|---|---|---|---|
| | Plasma | | Plasma + UFH 0.5U/ml | |
| Ecarin 5U/ml | 26.5 | 25.8 | 26.1 | 25.8 |
| GF3 1:100 | 20.8 | 20.8 | 21.4 | 20.8 |

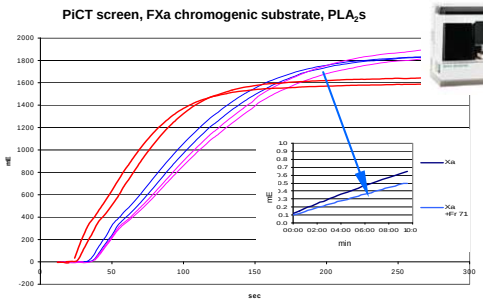
## ➢ Clotting properties: ROTEM System






ROTEM Haemostasis Whole Blood Analyzer is a computerized device based thromboelastometry, which monitors haemostasis as a whole dynamic process.

## ➢ Influences on the prothrombinase complex: $PLA_2$ binding to FXa




BCS (Coagulation Test System) was used to establish a multifunctional physiological assay. This procedure allows us to study blood coagulation system influences on the different levels of the coagulation cascade and was used as a preliminary screening for all the fractions obtained in any of the purification steps.

## Conclusions

Out of 18 GF fractions originally prepared all have now been checked in the preliminary screening for their activities on blood coagulation system. Three of them, further separated by HLPC, have been analysed in a broad screening attempt verifying the blood coagulation effects. Besides the known activities several new and interesting ones have been found. They have to be studied and confirmed in supplementary more specific bioassays. Their structures will be further characterized using proteomics and biochemical techniques, such as reduction and alkylation, digestion and Edman sequencing, as well as MS/MS *de novo* sequencing. Other fractions need additional separation and purification steps before further screening.

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NP2D - Natural Peptides to Drug
Zermatt, Switzerland
30th November – 3rd December, 2004

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# The potential of *Bothrops moojeni* venom in the field of hemostasis. Established use and new insights.

**Perchuć A.M.**[1,2], Menin L.[3], Stöcklin R.[3], Bühler B.[1], Schöni R.[1]

[1] *Pentapharm AG, Basel, Switzerland;* [2] *Institute of Molecular Pharmacy, University of Basel, Switzerland;* [3] *Atheris Laboratries, Geneva, Switzerland*

**INTRODUCTION:** Among many diverse proteins contained in snake venoms, there is a number of compounds exerting varied activities on hemostatic and fibrinolytic systems. These components are often very specific in their mode of action activating or inactivating different components of both systems. Some purified snake venom proteins with known activity have become valuable tools as diagnostic reagents, are used for pharmaceutical applications or preparative procedures in the field of hemostaseology, neurobiology and complement research.
Batroxobin, isolated from the venom of South–American Lancehead snakes of the *Bothrops* genus, under its trade name Reptilase® is still widely used in pharmaceutical and diagnostic applications. Though, most of the *Bothrops moojeni* venom material needed for its production is not further processed. It contains, however, a huge amount of active compounds still not fully characterized and described. Thus, the scope of our investigations is to have a second, deeper look at the crude venom of *B. moojeni.* The main purpose of this study is to identify and describe new bioactive compounds to be used as active pharmaceutical or diagnostic ingredients (APIs or ADIs) in the field of hemostasis and fibrinolysis.

**MATERIAL AND METHODS:** Gel filtration 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 gel filtration (GF) fractions on the Superdex-75 column. For preliminary evaluation of the potential of the 18 fractions obtained from gel filtration chromatography, a high throughput screening method was used. The test method consisted of a set of 14 screens of different levels and mechanisms of the blood coagulation system. This screening was performed on an automated Coagulation Test System BCS (Behring Coagulation System). 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. Six GF fractions were selected for further purification by the means of HPLC and further characterization of their active compounds.
Besides the BCS screening, coagulation tests were also performed on a ball coagulometer with mechanical clotting detection and in Rotem® system (thromboelastography), and for further analyses different chromogenic substrates were used.

## *B. moojeni* Venom – the Whole Picture

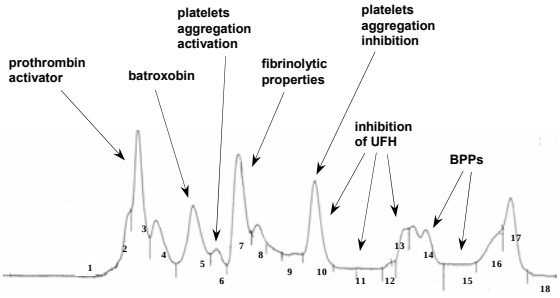


***Fig.1*** Gel filtration chromatogram obtained with 300 mg of crude *B. moojeni* venom. 5g of *B.moojeni* crude venom have been separated in 20 individual runs. The reproducibility of all performed experiments was very good, so that pooling into 18 distinct GF-fractions collected from run to run was successfully performed.

## Identification of prothrombin activator

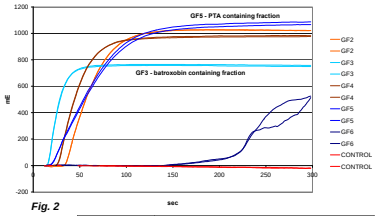


***Fig. 2***

| PTA | CT | | | |
|---|---|---|---|---|
| | Plasma | | Plasma + UFH 0.5U/ml | |
| Ecarin 5U/ml | 26.5 | 25.8 | 26.1 | 25.8 |
| GF3 1:100 | 20.8 | 20.8 | 21.4 | 20.8 |

***Fig.2*** It was not possible to differentiate between the thrombin-like (batroxobin) and prothrombin activating properties when the experiments were performed in plasma. Therefore additional measurements of fibrinogen clotting time were performed on appropriate Botmo GF fractions:

| Fraction | Fibrinogen Clotting Time (sec) |
|---|---|
| GF3 | >200 |
| GF4 | 87 |
| GF5 | 7 |
| GF6 | 45 |
| GF7 | >200 |

**RESULTS:** Out of 18 GF fractions originally prepared by a gel filtration method all have been checked in the preliminary screening for their activities on hemostasis. Six of them (from the low molecular weight range), further separated by HPLC, have been analysed in a broad screening attempt verifying the blood coagulation effects.

Besides the known activities new and interesting ones have been found:
- Strong clotting time shortening by batroxobin containing fraction (GF5);
- Strong clotting time shortening by prothrombin activator containing fraction (GF3);
- Fibrinolytic properties (GF7);
- Clotting time prolongation or no clot formation by the fractions containing $PLA_2$ activity;
- Presence of bradykinin potentiating peptides
- Inhibition of heparin action on the blood coagulation system;
- Activation of platelets aggregation;
- Inhibition of platelets aggregation.

## Inhibition of anticoagulant properties of unfractionated heparin (UFH)

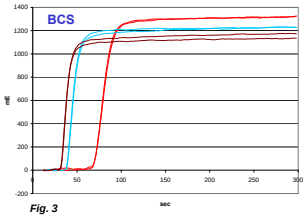


***Fig. 3***

***Fig. 3*** Using the BCS test system, aPTT screen was performed in heparanized plasma (UFH:0.5U/ml). One of the Botmo GF10 subfractions (Botmo GF 10-71) obtained in the HPLC separation showed an ability to neutralize the anticoagulant action of UFH.

While UFH alone prolonged the normal clotting time (CT), when measured in plasma, using HEPES buffer as a control-sample (Control), the Botmo GF 10-71 subfraction, added into the heparinized plasma, was able to bring the CT to close to the value measured in the plasma without UFH addition (compare: Control (-) UFH)

***Fig. 4*** The activity of Botmo GF10-71 subfraction was further studied using chromogenic substrate for FXa. UFH was preincubated for 3 min. with the active fraction and after that time FXa and antithrombin III (ATIII) were added and absorbency measured.
Without addition of Botmo GF10-71 the activity of FXa was inhibited by UFH and ATIII, thus could not be measured. However, Botmo GF10-71 was able to completely neutralize the anti-FXa properties of UFH.

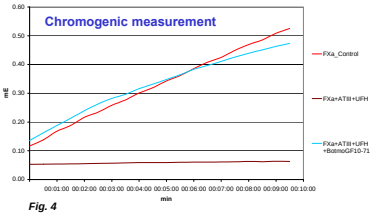


***Fig. 4***

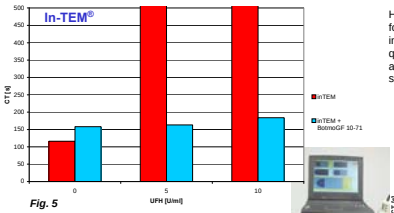


***Fig. 5***

Heparin not only affects the onset time of clotting, i.e. the begin of clot formation, but also the clot formation kinetics. Therefore the effects of heparin inhibition were examined in the Rotem® system, an inventive technique which quantifies continuously the clot formation kinetics. This method was applied and the UFH neutralizing properties of Botmo GF 10-71 subfraction were studied further in the intrinsic and extrinsic pathway of blood coagulation.

***Fig. 5*** Using the in-TEM® test and the heparinized plasma, the activity of Botmo GF 10-71 subfraction was measured in the intrinsic pathway of blood coagulation. However clotting time prolongation could be observed in the measurements performed in plasma without UFH addition, very good neutralization of UFH anticoagulant activity was obtained even for the concentration of 10 U of UFH in 1 ml plasma.

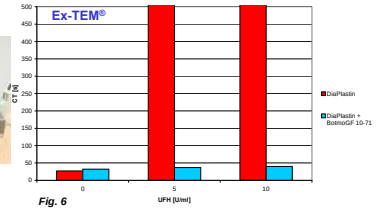


***Fig. 6***

***Fig. 6*** Using the ex-TEM® test and the heparinized plasma, the activity of Botmo GF 10-71 subfraction was studied further in the extrinsic pathway of blood coagulation. A slight clotting time prolongation could be observed in the measurements performed in plasma without UFH addition. Also in the extrinsic pathway very good neutralization of UFH was obtained even for the concentration of 10 U UFH in 1 ml plasma.

**CONCLUSIONS:** Within the framework of our project several interesting activities have been found. The active compounds still need to be identified and further characterized. Their structures need to be confirmed and their possible use in diagnostic and/or 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 before being applied to specific bioassays.

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**50. Jahrestagung der Gesellschaft für Thrombose- und Hämostaseforschung (GTH), 15.-18. Februar 2006, Basel**

P006

# Two novel Lys49 $PLA_2$s from the venom of *Bothrops moojeni* and some synthetic peptides derived from their C-terminus are able to bind and neutralize anticoagulant activities of different heparins.

**Perchuć A.M.**[1], Menin L.[2], Heidl M.[1], Favreau P.[2], Bühler B.[1], Bulet P.[2], Schöni R.[1] and Stöcklin R.[2]

[1] *Pentapharm AG, Basel, Switzerland;* [2] *Atheris Laboratries, Geneva, Switzerland*

## INTRODUCTION:

**Among new peptides and proteins already characterized in the *Bothrops moojeni* venom, two novel phospholipases $A_2$ ($PLA_2$) have been purified and fully sequenced. Both of them belong to the enzymatically non-active Lys49 $PLA_2$ variants. They consist of 122 amino acids and share a characteristic sequence in the C-terminal region composed of clusters of basic and hydrophobic amino acids. This sequence is known from the literature to interact with heparin.**

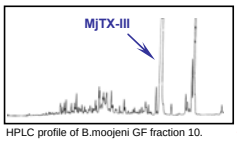
**So far, it has been reported that heparin could interact with basic $PLA_2$s and was used *in vivo* and *in vitro* to neutralize different toxic effects caused by some snake venoms and their $PLA_2$s (Lomonte, Moreno et al. 1994; Lomonte, Tarkowski et al. 1994; Calil-Elias, Martinez et al. 2002; Calil-Elias, Thattassery et al. 2002)**

**The two $PLA_2$ variants were isolated using size exclusion chromatography and RP-HPLC, fully sequenced by ESI-MS/MS techniques and characterized by means of PiCT and aPTT assays performed in heparanized plasma. They interact *in vitro* with unfractionated heparin (UFH) and low molecular weight heparin (LMWH), neutralizing their anticoagulant properties. Although it is well known that $PLA_2$s from snake venoms influence the blood coagulation system, the use of those substances to antagonize the anticoagulant effect of heparin *in vivo* or *in vitro* has never been proposed until now.**

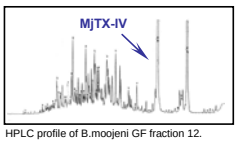
**We synthesized the peptides corresponding to the characteristic sequence positions 105-121 from the C-terminal region of *B. moojeni* $PLA_2$ (MjTX-III). In addition, the natural sequence has been modified in order to create different peptides and optimize their capability to neutralize the anticoagulant properties of LMWH. Since there is no available antidote, we focused our efforts on peptidomimetic and structure-function studies on the putative LMWH neutralizing capacity of these peptides. The possible use of synthetic peptides derived from *B. moojeni* MjTX-III in diagnostic and/or pharmaceutical applications is currently under investigation.**

• **Calil-Elias, S., A. M. Martinez, et al. (2002). "Effect of heparin and antivenom on skeletal muscle damage produced by Bothrops jararacussu venom." Histol Histopathol. 17(2): 463-70.**
• **Calil-Elias, S., E. Thattassery, et al. (2002). "Effect of perimuscular injection of Bothrops jararacussu venom on plasma creatine kinase levels in mice: influence of dose and volume." Braz J Med Biol Res. 35(10): 1233-1235.**
• **Lomonte, B., E. Moreno, et al. (1994). "Neutralizing interaction between heparins and myotoxin II, a lysine 49 phospholipase A2 from Bothrops asper snake venom. Identification of a heparin-binding and cytolytic toxin region by the use of synthetic peptides and molecular modeling." J Biol Chem. 269(47): 29867-29873.**
• **Lomonte, B., A. Tarkowski, et al. (1994). "Neutralization of the cytolytic and myotoxic activities of phospholipases A2 from Bothrops asper snake venom by glycosaminoglycans of the heparin/heparan sulfate family." Biochem Pharmacol. 47(9): 1509-1518.**

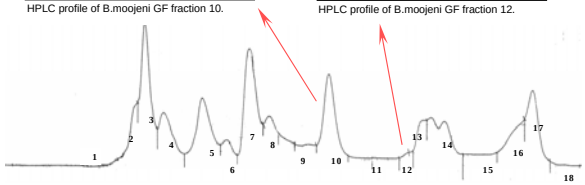
## *B. moojeni* Venom – From function to structure




HPLC profile of B.moojeni GF fraction 10.




HPLC profile of B.moojeni GF fraction 12.




Gel filtration chromatogram obtained with 300 mg of crude *B. moojeni* venom. 5g of *B.moojeni* crude venom have been 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.

| Name | Sequence |
|---|---|
| | 1 11 21 |
| *B. moojeni* MjTX-III | S L V E L G K M I L Q E T G K N P V T S Y G A Y G C N C G V |
| *B. moojeni* MjTX-IV | S L V E L G K M I L Q E T G K N P L T S Y G A Y G C N C G V |
| | 31 41 51 |
| *B. moojeni* MjTX-III | L G R G K P K D A T D R C C Y V H K C C Y K K L T D C N P K |
| *B. moojeni* MjTX-IV | G G R G K P K D A T D R C C Y V H K C C Y K K M T D C D P K |
| | 61 71 81 |
| *B. moojeni* MjTX-III | K D R Y S Y S W K D K T I V C G E N N S C L K E L C E C D K |
| *B. moojeni* MjTX-IV | K D R Y S Y S W K D K T I V C G E N N S C L K E L C E C D K |
| | 91 101 111 121 |
| *B. moojeni* MjTX-III | A V A I C L R E N L D T Y N **K K Y K N N Y L K P F C K K** - A D P C |
| *B. moojeni* MjTX-IV | A V A I C L R E N L D T Y N **K K Y K N N Y L K P F C K K** - A D P C |

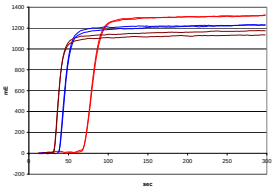
Alignment of the amino acid sequences of two novel K49-$PLA_2$s purified and characterized from the venom of *B. moojeni*

## CONCLUSIONS:

Peptides corresponding to the natural heparin-binding site of Lys49 PLA2 from *B. moojeni (B. moojeni* MjTX-III) as well as other peptides representing diverse modifications of the natural sequence were synthesized and tested for their heparin-neutralizing activity in plasma. The present study was focused on their ability to neutralize LMWH anticoagulation activity in plasma.

The highest activity has been found with a 13 amino acid (AA) peptide closely related to the natural sequence (P02), for a peptide of identical structure but having four Lys replaced by Arg residues (P12), and for a truncated 9 AA peptide (P08) being also the shortest peptide in this series. Based on these findings further derivatives of these lead peptides need to be synthesized. Moreover additional studies are needed to decide whether this principle can successfully be used for diagnostic and/or pharmaceutical applications.

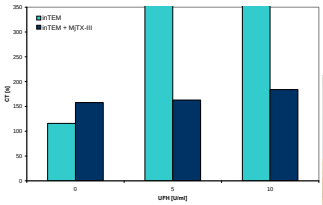
## *B. moojeni* HPLC subrfaction (MjTX-III) – Functional assays: inhibition of anticoagulant activity of UFH

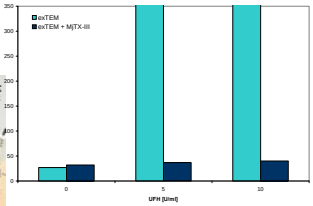



Using a BCS test system, aPTT screen was performed in heparanized (0.5U/ml) plasma. One of the Botmo GF10 HPLC-subfractions characterized later as MjTX-III showed the ability to neutralize anticoagulant activity of UFH.

While UFH alone prolonged the normal clotting time (CT), when measured in plasma, using HEPES buffer as a control-sample (Control), the MjTX-III subfraction, added into the heparinized plasma, was able to bring the CT close to the value measured in the plasma without UFH addition (compare: Control (-) UFH)

The effects of heparin and inhibition thereof were also examined on a ROTEM® system. The UFH neutralizing properties of MjTX-III were studied further by means of this method for their effects on intrinsic and extrinsic pathway of blood coagulation. The obtained CTs were compared:



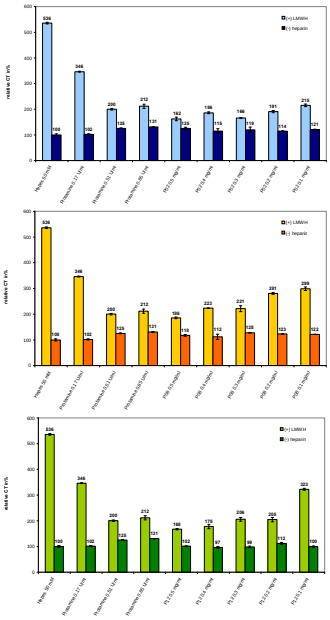




Using the in-TEM® test and heparinized plasma, the influences of MjTX-III on the intrinsic pathway of blood coagulation were measured. However clotting time prolongation could be observed in the measurements performed in plasma without UFH addition, very good neutralization of UFH anticoagulant activity was obtained even for the concentration of 10 U of UFH in 1 ml plasma.

Using the ex-TEM® test and heparinized plasma, the influences of MjTX-III on the extrinsic pathway of blood coagulation were studied. A slight clotting time prolongation could be observed in the measurements performed in plasma without UFH addition. Also in the extrinsic pathway very good neutralization of UFH was obtained even for the concentration of 10 U UFH in 1 ml plasma.

## Synthetic peptide derived from *B. moojeni* MjTX-III and modifications thereof – Inhibition of anticoagulant activity of LMWH

| Peptide Number | Sequence |
|---|---|
| | 105 K K Y K N N Y L K P F C K K A D P 118 |
| P01 | K K Y K N N Y L K P F Abu K K A D P |
| P02 | K K Y K N N Y L K F Abu K K |
| P03 | K Y K N N Y L K P F Abu K |
| P04 | Y K N N Y L K P F Abu K K |
| P05 | K K Y K N N Y K F Abu K K |
| P06 | K K Y K N N K F Abu K K |
| P07 | K K Y K N K F Abu K K |
| P08 | K K Y K K F Abu K K |
| P09 | K R Y K N N Y L K P F Abu R K |
| P10 | R R Y K N N Y L K P F Abu K K |
| P11 | R R Y K N N Y L K F Abu K K |
| P12 | K R Y R N N Y L R F Abu R K |
| P13 | R R Y K N N Y L R F Abu R K |




Synthetic peptide derived from *B. moojeni* MjTX-III (P01) and different modifications thereof (P02-P13):

• replacement of Cys by Abu (L-2-amino-butyric acid) in order to avoid oxidative dimerization,

• omitting of Pro in order to support the formation of an amphiphatic α-helix – structure favorable for heparin-binding,

• shortening of the whole sequence in order to obtain possibly short peptides and lower the costs of the synthesis,

• replacement of different Lys by Arg residues in order to increase the basicity and affinity to negatively charged heparin.

Peptides showing the best neutralization of LMWH (P02, P08 and P12) are highlighted.

BCS-measurements performed using the three peptides are displayed in the diagrams. PiCT® assay was performed in normal plasma and in plasma spiked with 1 U/ml of LMWH. The properties of synthetic peptides P02, P08 and P12 were compared with protamine hydrochloride in normal and heparinized plasma. Relative CT is expressed in % (CT obtained in normal plasma without addition of any substance was considered as 100%).

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**51. Jahrestagung der Gesellschaft für Thrombose- und Hämostaseforschung (GTH), Dresden, 21.-24. Februar 2007**

# Anna Maria Perchuć

## *CURRICULUM VITAE*

## PERSONAL DETAILS


| | |
|---|---|
| **Date of birth:** | 12.02.1977 (Warsaw, Poland) |
| **Nationality:** | Polish |
| **Address:** | Im Margarethenletten 1<br>4053 Basel, Schweiz |
| **Phone:** | + 41 79 230 89 01 |


## EDUCATION

| | |
|---|---|
| **01.2003 - 09.2008** | PhD thesis under the guidance of Prof. B. Ernst (University of Basel), within the "*Bothrops moojeni* Proteomics" project in the Haemostasis and Test Kit Development Group, Pentapharm Ltd., Basel, Switzerland |
| **11.2002** | Completed studies at the Warsaw University of Technology, Warsaw, Poland; Faculty of Chemistry; Degree: MSc., Chemical Engineer; Specialisation: Bio-active Compounds and Cosmetics Technology<br>Awarded for the distinguished Master's Diploma |
| **08.2001 - 08.2002** | Master's Thesis in the Tissue Culture Laboratory; Pentapharm Ltd., Basel, Switzerland; Master's Thesis Subject: The Investigation of Antioxidative Properties of Vitamin C Esters in Cell Cultures.<br>Master's Thesis Advisors: D. Imfeld, PhD (Pentapharm) and J. Arct, PhD (Warsaw University of Technology) |
| **2000 - 2002** | Warsaw University of Technology, Warsaw, Poland<br>Faculty of Chemistry; Specialisation: Bio-active Compounds and Cosmetics Technology |
| **1996 - 2000** | Warsaw University of Technology, Warsaw, Poland<br>Interdepartmental studies at: Faculty of Chemical and Process Engineering, Faculty of Chemistry, Faculty of Environmental Engineering; Specialisation: Biotechnology: |
| **1992 - 1996** | Jaroslaw Dabrowski XXXVII Secondary School, Warsaw, Poland, A-Level exams (1996) |

## ADDITIONAL EXPERIENCES

| | |
|---|---|
| **08.2005 - 12.2006** | Supervision of three diploma thesis completed in the framework of "*Bothrops moojeni* venom proteomics" project:<br>1. "Potential der Komponenten des *Bothrops moojeni* Giftes in Bezug auf Plättchenaktivierung und -inhibition" (Christine Demler, University of Natural Sciences and Technology in Isny, Germany; awarded with Max-Buchner Fachhochschulpreis 2007);<br>2. "Aufreinigung und Charakterisierung eines gerinnungsaktiven Proteins aus dem Gift der südamerikanischen Lanzenotter *Bothrops moojeni*" (Valérie Ritz, Zürcher Hochschule Winterthur, Switzerland);<br>3. "Aufreinigung eines Calcium und Phospholipid abhängigen Prothrombinaktivators aus dem Rohgift der *Pseudonaja textilis*" (Karoline Dietrich, Zürcher Hochschule Winterthur, Switzerland) |
| **09.2005** | Venöse Blutentnahmen Kurs |
| **10.2004** | Strategies and trends in pharmaceutical development and production – course in the framework of continuing education program of the center of pharmaceutical sciences Basel-Zurich |
| **11.2003** | GTH-Intensivkurs für klinische Hämostaseologie, Hannover, Germany |
| **04.2003 - 10.2003** | Atheris Laboratories, Geneva, Switzerland,<br>Protein Purification and Mass Spectrometry training in the framework of my PhD Thesis |
| **08.2001 - 12.2001** | Pentapharm Ltd., Basel, Switzerland,<br>Tissue culture laboratory; research and laboratory work<br>LEONARDO DA VINCI II PROGRAMME; "Industrial Training of University Students for Modern Technology" in the scope of 5$^{th}$ year of my studies |

## LANGUAGES

| | |
|---|---|
| **Polish** | mother tongue |
| **German** | fluent in written and spoken language |
| **English** | fluent in written and spoken language |

## INTERESTS

Climbing, flamenco dance, cinema, literature, theatre, art