

# Production of Monoclonal Antibodies (mAbs) Purified Anti-metalloprotease from the Venom of the Serpent *Bitis arietans*

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**Abstract:** The African snake *Bitis arietans* is of great medical importance and is found in sub-Saharan Africa and in savannas and pastures of Morocco and western Arabia. Contributes complacently in the epidemiology of snakebites in humans and animals. The lack of specific antivenoms aggravates this situation. Identifying toxins, knowing their toxic properties and developing antitoxins are the goals of emerging projects. This project aims at monoclonal anti-metalloprotease (mAbs) from the toxin of *Bitis arietans* venom. mAbs will serve as sources of complementarity determining regions (CDR). The main applied methodologies will not develop the project fit mainly in the purification of metalloprotease and immunization of mice to obtain lymphocytes and replicate it. So far, we have been able to highlight a metalloprotease of interest, which will be properly identified for the production of antibodies, a finding that was confirmed by proteomic and transcriptomic analyzes. The next step will be to immunize mice and validate the antibodies produced.

**Keywords:** *Bitis arietans*; metalloprotease; monoclonal antibodies; complementarity - determining regions

**Key Contribution:** The main advancement of the project so far has been the finding of purified SVMP and the F5-4 fraction, forwarded to continue developing the project. From this, we can also find an SVSP, take advantage of other emerging projects, in addition to standardizing our purification protocols. These findings will contribute to the next experimental steps.

**Citation:** Godoi, K.S.d.; Guidolin, F.R.; Megale, A.A.; Portaro, F.V.C.; Silva, W.D.d. Production of Monoclonal Antibodies (mAbs) Purified Anti-metalloprotease from the Venom of the Serpent *Bitis arietans*. *Proceedings* **2021**, *65*, x. <https://doi.org/10.3390/xxxxx>

Received: 5 November 2020

Accepted: 7 January 2021

Published: 15 January 2021

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## 1. Introduction

Ophidian accidents represent a major public health problem, especially in tropical and subtropical countries in Asia, Africa, Oceania and Latin America [1,2]. An epidemiological analysis of underreported snakebite accidents on data collected from 160 publications from 68 countries worldwide estimates that there are about 1,800,000 accidents with 94,000 deaths per year [1]. Besides deaths, survivors with sequelae, disabilities and lack of antivenoms, these factors are highly reported. In sub-Saharan Africa, there are an estimated 250,000 accidents, with 12,000 deaths and 9,000 complications, of which 95% occur in rural regions [1].

The World Health Organization (WHO) faced the association between the severity of snakebite accidents and the lack of medical care for victims, decided to put the snakebite accident back to the condition of neglected disease [3].

The *Bitis arietans* snake, popularly known as the "puff adder", is one of the snakes of greatest medical importance present in the African continent, which is also distributed in

sub-Saharan Africa and in the savannas and pastures of Morocco and western Arabia as shown in Figure 1.



**Figure 1.** *Bitis arietans* and distribution in sub-Saharan Africa. World Health Organization (2020).

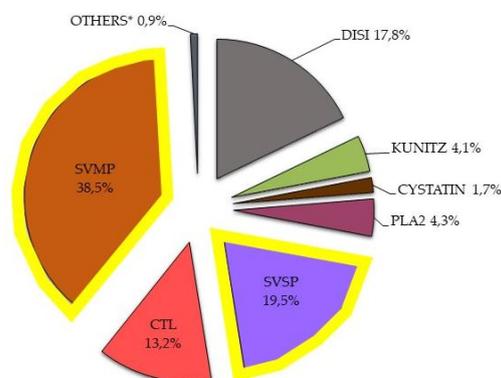
The venom of *Bitis arietans* causes local effects, severe pain [4-7], swelling [7-11], blistering [4-7], ecchymosis [4,6,7], hemorrhage at the bite site [4,6,7], necrosis [4,6,7] and dilation of the lymph nodes in the affected region [4]. These actions fall within the symptomatology in four of the five cardinal signs: edema, heat, redness and pain. Irreversible disabilities and mortality add the fifth sign, loss of function [4]. General parameters of the inflammatory response observed in patients bitten by *Bitis arietans* were experimentally recovered [10].

Antiphidic sera of recurrent use in the immunotherapy of humans bitten by snakes are, in general, produced in horses. The production process must follow the standards established by WHO [11]. In the final product, there is a predominance of fragments F (ab) 2, pH  $7.0 \pm 0.5$ , sterile, without endotoxin or pyrogenic factor, and containing the desired amount of LD<sub>50</sub>/mL. The final product is expensive, an impacting factor for developing countries. These general observations, plus about two decades of involvement in projects on animal venoms and antivenoms stimulated the development of experiments aimed at obtaining antivenoms of high specificity, avidity, affinity, specific activity and neutralizing potency of toxins at low cost, but with high quality. Our project proposal includes these objectives.

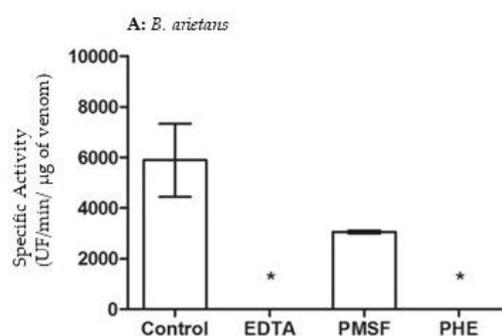
The main strategies used in the development of the project were: #1 use of established knowledge about the molecular and cellular mechanisms involved in the differentiation of B, T lymphocytes and memory plasma cells [12], in addition to carrying out preliminary experiments aimed at identifying points in the appropriate immunization protocols to stimulate memory plasma cells; #2 isolate and characterize relevant toxins, develop anti-toxin mAbs, isolate hypervariable regions of these mAbs, identify and sequence CDRs and model scFv antitoxins.

PhD student Ângela Alice Amadeu Megale in the development of her thesis [10], started to identify parameters identifying the local and systemic inflammation produced by *Bitis arietans* intoxication [13]. Analysis of the venom proteome and transcriptome, which revealed that the venom is made up of proteins belonging to a restricted number of families, of which metalloproteases and serinoproteases are the most abundant as shown in Figure 2. She purified these two proteases from *Bitis arietans* venom, which were identified as rhinocerase, being classified as serinoprotease (SVSP) [14] and metalloprotease (SVMP) [15]. Figure 3 confirms that biological activity tests of this venom carried out by our group demonstrated, based on the selective inhibition with EDTA, PMSF and PHE, the presence of metalloproteases and serinoproteases, corroborating the previous results of the proteome and transcriptome. In the process of purifying the proteases, the author

must identify the fractions of SVSP and SVMP substrates “Fluorescent Resonance Energy Transfer” (FRET), with Abz-RPPGFSPFR-QEDDnp to identify SVSP and SVMP and Abz-FRSSR-QEDDnp that identifies SVSP [16].



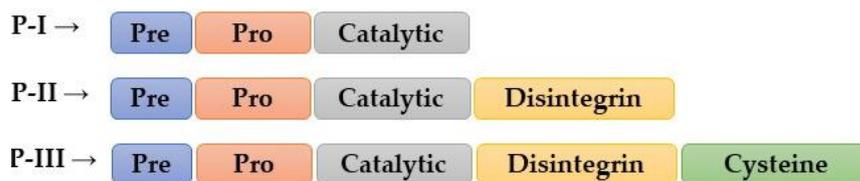
**Figure 2.** Analysis of the *Bitis arietans* snake venom transcriptome and proteome reveal that the venom is made up of a restricted number of protein classes, of which the SVMPs and SVSPs are the most abundant, CTL, PLA<sub>2</sub>, cystatin: cysteine protease inhibitor, Kunitz and DISI SVSP inhibitors.



**Figure 3.** Assays of the biological activity of *Bitis arietans* venom from selective inhibition with EDTA, PMSF and PHE. Tests for the biological activity of the venom confirm the presence of SVMPs and SVSPs, based on the selective inhibition of proteolytic and gelatinolytic activities, respectively, with EDTA and PHE or with PMSF.

SVSPs have a highly conserved catalytic triad. They have a high identity: 51-98% among themselves, 26-33% with human thrombin and 34-40% with human plasma kallikrein. They are generally presented as a single chain with 26-33 kDa, depending on the glycosylation content, which can vary from 0-30%. In general, they affect the coagulation cascade and blood pressure; they are fibrinolytic and cleave the kininogen culminating in the release of vasoactive kinins.

SVMPs, representing almost 40% of this venom, are zinc dependent endopeptidases and phylogenetically related to mammalian metalloproteases and ADAM proteins. They have binding domain to the conserved substrate and variable molecular weight, depending on their composition. They are classified into three classes, according to the composition of their domains. The PI class is the least complex and in its active form it is formed only by the catalytic domain. Class P-II have a disintegrin domain after the catalytic domain. It is important to note that few class P-II SVMPs are found in venoms, as they undergo auto-proteolysis, releasing a catalytic domain and a free disintegrin domain. Class P-III, in addition to the catalytic and disintegrin domains, have a domain rich in cysteine. SVMP are the main components of venoms involved in local and systemic hemorrhage. In general, hemorrhagic or not, they are fibrinolytic and cleave components of the extracellular matrix [17, 18] (Figure 4).



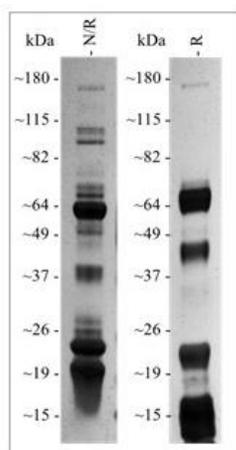
**Figure 4.** Three classes of SVMPs according to the composition of their domains.

The purification of metalloproteases in this project aims to develop specific mAbs for epitopes related to the toxic domains of the snake venom *Bitis arietans*. Preliminary results indicate the relevance of this criterion in the appropriate selection of antitoxin hybridomas.

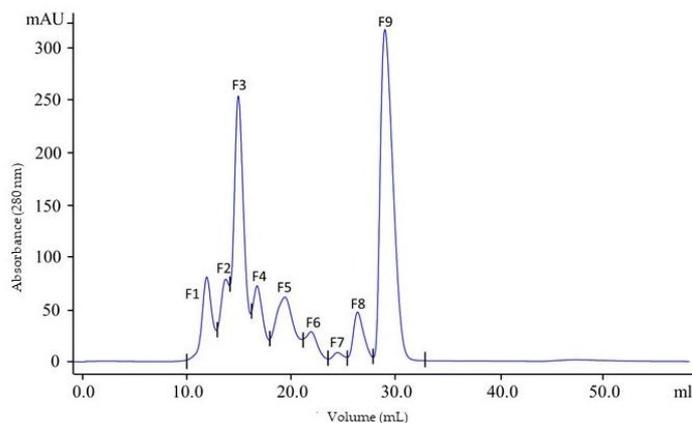
## 2. Results

### 2.1. First chromatography step

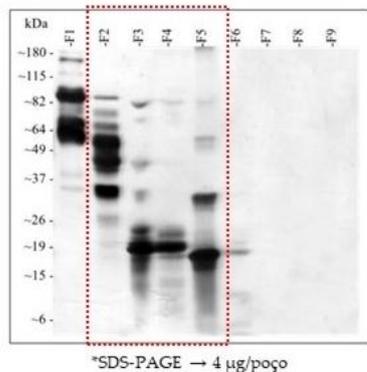
*Bitis arietans* venom (BaV) is composed by a complex mixture of toxins. The analysis of the electrophoretic profile of BaV revealed the presence of proteins with molecular weight from 180 to 6 kDa. (Figure 5). The first step of the BaV fractionation by molecular exclusion chromatography gave rise to 9 subfractions, denominated F1-F9 (Figure 6A), which the electrophoretic profile is observed in SDS gel (Figure 6B). Considering the molecular weight, proteolytic activity and stability of these fractions, fractions 2, 3, 4 and 5 were selected for the inhibition steps.



**Figure 5.** 15 µg of the venom were subjected to gradient gel electrophoresis (8-16%) under non-reducing (N/R) and reducing (R) conditions. The bands were revealed by impregnation with silver nitrate.



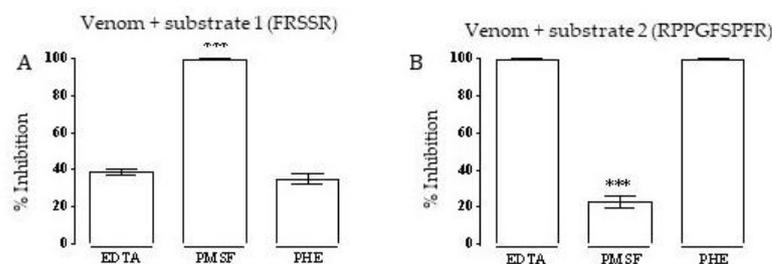
**Figure 6A.** 20 mg of lyophilized venom were subjected to a phase of molecular exclusion chromatography in a Superose 12 HR column, balanced and eluted with ammonium acetate (50 mM) in a climate-controlled environment ( $22 \pm 2^\circ\text{C}$ ). The samples were collected in a flow and 0.4 mL/min. and the protein content was monitored under 280 nm absorbance on the UPC-900 reader.



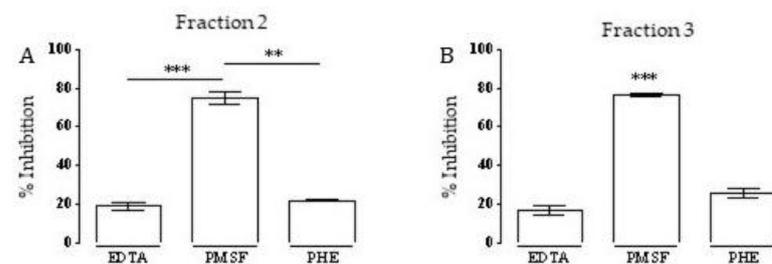
**Figure 6B.** The electrophoretic profile was evaluated on gel with a gradient of 8-16% acrylamide and the bands revealed by impregnation with silver nitrate.

2.2. Inhibition of proteolytic activity of fractions

Using selective inhibitors, the presence of serinoproteases and metalloproteases in BaV were indicated by inhibition with PMSF or with EDTA and phenanthroline, respectively (Figure 7A). In fractions 2 and 3 the majority presence of serinoproteases was revealed (Figure 7B) and in fractions 4 and 5 the majority presence of metalloproteases (Figure 7C). Based on these results and the electrophoretic profile of the fractions, fractions 3 and 5 were selected to obtain a purified SVSP and SVMP.

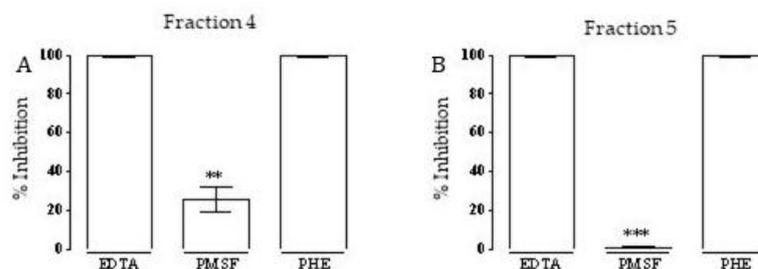


**Figure 7A.** BaV fractions (0.2 - 5ug/well), were incubated with EDTA (100 mM/well), PMSF (2 mM/well) and 1.10-PHE (2 mM/well). After thirty minutes of incubation at room temperature, 10 mM of FRET substrate (Abz-FRSSR-EDDnp (substrate 1) or Abz-RPPGFSPFR-EDDnp (substrate 2)) was added, in a final volume of 100 uL and the proteolytic activity was measured by spectrofluometry for 10 minutes. The substrate was preferably selected from each sample incubated only in PBS as 100% activity. **A)** Venom + substrate 1 (**S1**); **B)** Venom + S2.



**Figure 7B.** BaV fractions (0.2 - 5ug/well), were incubated with EDTA (100 mM/well), PMSF (2 mM/well) and 1.10-PHE (2 mM/well). After thirty minutes of incubation at room temperature, 10

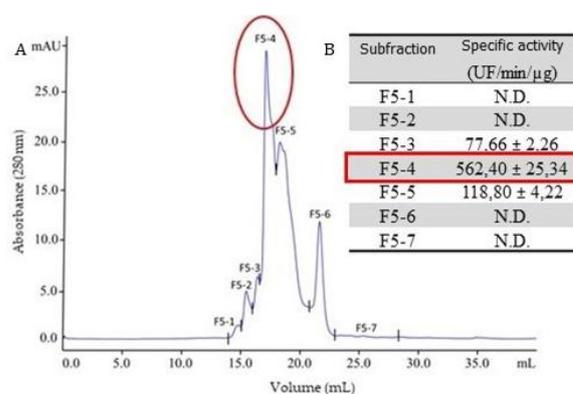
mM of FRET substrate (Abz-FRSSR-EDDnp (substrate 1) or Abz-RPPGFSPFR-EDDnp (substrate 2)) was added, in a final volume of 100 uL and the proteolytic activity was measured by spectrofluometry for 10 minutes. The substrate was preferably selected from each sample incubated only in PBS as 100% activity. **A)** Fraction 2 (F2) + (S1); **B)** F3 + S1.



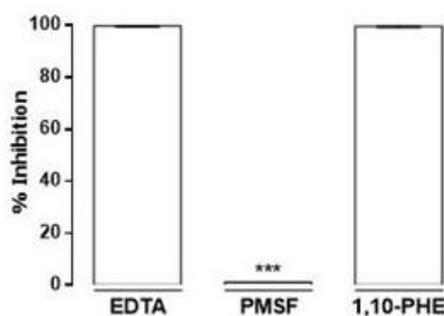
**Figure 7C.** BaV fractions (0.2 - 5ug/well), were incubated with EDTA (100 mM / well), PMSF (2 mM / well) and 1.10-PHE (2 mM/well). After thirty minutes of incubation at room temperature, 10 mM of FRET substrate (Abz-FRSSR-EDDnp (substrate 1) or Abz-RPPGFSPFR-EDDnp (substrate 2)) was added, in a final volume of 100 uL and the proteolytic activity was measured by spectrofluometry for 10 minutes. The substrate was preferably selected from each sample incubated only in PBS as 100% activity. **A)** F4 + S2 (S1); **B)** F5 + S2.

### 2.3. SVMP purification and characterization

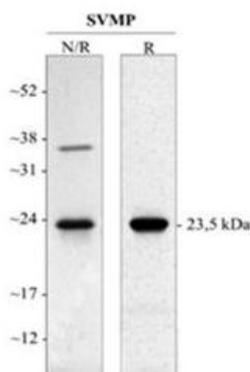
The second chromatographic step of fraction 5, selected to obtain SVMP, originated 7 subfractions. Among them, F5-4 was the one with the highest proteolytic activity (Figure 8A). This fraction was completely inhibited by selective SVMP inhibitors and the identification by mass spectrometry revealed that the peptides originated from the hydrolysis of this subfraction have similarity with two class I SVMP of botropic venoms (Figure 8B). The SVMP of *Bitis arietans* presented itself as a single band of 23.5 kDa in the reduced gel (Figure 8C). Like the SVSP, this SVMP was able to hydrolyze human fibrinogen, indicating its participation in hemostatic disorders, as well as fibronectin, indicating its participation in tissue damage and hemorrhage caused by the venom (manuscript in preparation.)



**Figure 8A.** The fifth chromatographic peak (F5), obtained from the first purification step, was subjected to a second cycle of molecular exclusion chromatography on a Superdex 75 column, equilibrated and eluted with ammonium acetate (50 mM) in an air-conditioned environment (22 ± 2°C). The samples were collected in a flow of 0.4 mL/min. and the protein content was monitored under 280 nm absorbance in a UPC-900 reader. **A)** Fraction 5.4 (F5-4) was shown to contain the highest proteolytic activity. **B)** The proteolytic activity of the fractions obtained from the F5 fractionation by molecular exclusion chromatography was evaluated by the cleavage of FRETs substrates. In 96-well white plates, the fractions (0.5 - 2 μg/well) were incubated with 10 μM of FRET substrate (Abz-RPPGFSPFR-EDDnp) in PBS (100 μL final volume). Proteolytic activity was measured by spectrofluorimetry for 10 minutes. Representative result of two independent tests expressed as mean ± SD and analyzed by One-Way Anova followed by the Tukey post-test (\* p < 0.05). UF: Arbitrary fluorescence unit; N.D.: not detectable.



**Figure 8B.** The fraction 5-4 (F5-4; 0.5 µg/well), was incubated with EDTA (100 mM/well), PMSF (2 mM/well) and 1.10-PHE (2mM/well) for thirty minutes at room temperature. Then, 10 µM of FRET substrate (Abz-RPPGFSPFR-EDDnp) was added in a final volume of 100 µL and the proteolytic activity was measured by spectrofluorimetry for 10 minutes. The calculation of the percentage of inhibition was performed considering the specific activity of F5-4 incubated only in PBS as 100% activity. Representative result of two independent tests expressed as the mean percentage of inhibition ± SEM and analyzed by One-Way ANOVA followed by Tukey's post-test. p <0.001 (\*\*\*)



**Figure 8C.** The fraction 5-4 (4 µg/well), corresponding to the metalloprotease isolated from fraction 5, was submitted to 12% polyacrylamide gel under reducing (R) and non-reducing (N/R) conditions. The band revealed by impregnation with silver nitrate.

#### 2.4. SVMP Activity Screening

The substrate Abz-RPPGFSPFR-QEDDnp, already defined as selective for the SVMPs present in the venom of *B. arietans* [16], was used for the selection of the chromatographic peaks of interest. EDTA, a site inhibitor targeting SVMP, was also used in the selection step. 5 µM of FRET substrate and 0.5 µg of the fractions in PBS (100 µL final volume) were used. The tests were performed in a fluorimeter (Hidex Sense, Hidex, Finland) in 96-well plates in a thermostabilized compartment (37 ° C) and under agitation and adjusted for excitation and emission reading 320 and 420 nm, respectively. All experiments were performed in duplicates (Table 1) [15].

**Table 1.** Specific activity (UF/min /µg) on FRET substrates. Proteolytic activity on the substrate Abz-FRSSR-EDDnp (venom<sup>1</sup>, fraction 3 and Kn-Ba) and Abz-RPPGFSPFR-EDDnp (venom<sup>2</sup>, fraction 5 and F5-4).

Sample	Purification step	Final protein (µg)	Specific activity	Final yield (%)
Venom <sup>1</sup>	-	20.000	259,1 ± 14,8	100
Fraction 3	1st filtration gel (Superose 12 HR)	3.053	27,8 ± 0,9	15,3
SVSP - Kn-Ba	2nd filtration gel (Superdex 75)	26	665,6 ± 4,5	0,1
Venom <sup>2</sup>	-	20.000	431,5 ± 29,9	100
Fraction 5	1st filtration gel (Superose 12 HR)	1.795	918 ± 50,8	0,9
SVMP - F5-4	2nd filtration gel (Superdex 75)	97	562,4 ± 25,3	0,5

### 3. Discussion

The global mortality rate caused by snake poisonings is extremely high, especially in underdeveloped countries located in Africa, Asia and Latin and South America [1]. The snake *Bitis arietans* is one of the main species of medical interest and also the most responsible for an accident. It is endemic to Sub-Saharan Africa, but is also found in Morocco and Saudi Arabia. Its venom can cause local and systemic effects, such as pain, fever, leukocytosis and cardiovascular and hypothermic disorders, which can culminate in the individual's death [4, 19]

So far, according to the literature, toxins from the *Bitis arietans* venom have been isolated and characterized, such as hemorrhage [20, 21] and non-hemorrhagic [22] metalloproteases, fibrinogenolytic serinoproteases, kinin-releasing and acting on insulin [23], serinoproteases with the ability to release kallidin [24], phospholipases A2 as bitanarin [23, 25], Ba25, as primary structure similar to venom type C lectins [26] and PAL (Puff adder lectin) that induces the release of  $Ca^{+++}$  in the sarcoplasmic reticulum [27] and bitistatin (P17497) also known as arietin [28].

In the present study, two specimens of the main classes of toxins present in the *Bitis arietans* venom were isolated and characterized: an SVSP of 33 kDa, and an SVMP of 23.5 kDa. The molecular mass of the two toxins is close to those found in the proteome and transcriptome experiments of the venom [29, 30].

Tests of biological activity of this venom were performed and demonstrated that the proteolytic activity of SVSP was completely inhibited by PSMF, while the proteolytic activity of SVMP was completely inhibited by EDTA and PHE (1,10-phenanthroline).

The SVMP purified from the venom in the due study, had a molecular weight of 23.5 kDa, similar to other class P-I SVMPs, such as insularinase A [31] and BaP1 [32].

### 4. Conclusion

To date, two toxins from the venom of the snake *Bitis arietans* have been purified, a SVSP of 33 kDa that makes up about 19.5% of the venom and an SVMP of 23.5 kDa that represents 38.5%. Tests of the biological activity of the two toxins using the EDTA, PMSF and PHE inhibitors confirmed the results of the proteome and the transcriptome performed, demonstrating the presence of both toxins in the venom. The first stage of molecular exclusion chromatography resulted in 9 subfractions that were directed to analysis by electrophoresis, demonstrating the presence of proteins from 180 to 6 kDa. The purification and characterization of the SVSP F3 fraction found gave rise to the F3-1 fraction, selected from its inhibition by PSMF, which has fibrinogenolytic action and is capable of promoting the release of vasoactive kinins. The second stage of the SVMP F5 fraction, selected from the inhibition by EDTA and PHE, gave rise to the F5-4 fraction, similar to other class PI SVMPs of botropic venoms. This SVMP is capable of hydrolyzing human fibrinogen and participating in hemostatic disorders. The selection of the chromatographic peaks was made using specific substrates for SVSP (Abz-FRSSR-QEDDnp) and SVMP (Abz-RPPGFSPFR-QEDDnp).

### 5. Materials and Methods

#### 5.1. Venom

The venom of *Bitis arietans* (Venom Supplies, SA, Australia) will be provided by the Soros section hyperimmune the Butantan Institute, developed as of adult animals, males and developments, each measuring approximately 60 cm and from the south of the African continent. Lyophilized poisons are stored in a  $-20^{\circ}\text{C}$  freezer. For the tests, the venom filter will be diluted in pyrogenic saline (0.15 M NaCl) and filtered through sterilizing filters, with  $0.22\ \mu\text{m}$  pores, and stored in a freezer.  $-80^{\circ}\text{C}$  until use.

#### 5.2. SVMP purification

Initially, 20mg of the lyophilized venom will be fractionated by molecular exclusion chromatography in a Superose 12 HR column, balanced and eluted with ammonium acetate (50 mM) in a climatized environment ( $22 \pm 2$  °C) and the proteolytic activity of the peaks was determined on FRET substrates. After selecting the peak containing the SVMP of interest, it will be submitted to a new chromatographic step, using Superdex 75. Then, from the inhibition of proteolytic activity with EDTA, PMSF and phenatroline, selective SVMP and SVSP inhibitors, the enzymatic class of the fractions will be determined. The identification of the selected toxins will be performed by mass spectrometry and the activity of the proteases on the physiological substrates related to the poisoning will be evaluated.

### 5.3. Immunization of mice

Female BALB / c mice, aged 4 to 6 weeks and weighing 18 to 20 g, will be supplied by the Central Animal Farm, Instituto Butantan. Subcutaneous injections of SVMP in the presence or absence of SBA-15 and the collection of blood samples for testing for anti-SVMP Abs will be done under anesthesia. Samples of the maxillary sinus will be performed on days 0, 30 and 60, and the injection of purified SVMP will be performed every two weeks. Adverse reactions will be under constant observation. To collect lymphocytes, the animals will be anesthetized and euthanized by lethal combination of the anesthetics Xylazine (30 mg / kg) and Ketamine (300 mg / kg) via intraperitoneal.

### 5.4. Preparation of peritoneal cells

Peritoneal cells will be obtained from the peritoneal cavity of BALB / c mice. 5 mL of the medium "Dulbecco's Modified Eagles Medium F-12 (DMEM-F12, GIBCO, BRL)" will be injected intraperitoneally, and the peritoneal fluid collected after a delicate massage for 1.5 min. The animal will be euthanized as described in the topic "Immunization of mice". The collected peritoneal liquid will be centrifuged at 1200 rpm for 5 min, the supernatants will be discarded and the cells suspended in 10mL of "DMEM-F12 medium containing 10% fetal bovine serum. Samples of these cells will be distributed in the 96-well plate wells and incubated for 24 h, at 37°C under a 5% CO<sub>2</sub> atmosphere.

### 5.5. Identification of anti-SVMP mAbs isotypes

Wells of 96-well plates (Costar) will be coated with 5 µg of the purified fraction of SVMP for 1 hour at room temperature, and the wells will be washed 3 times with PBS buffer, pH 7.4. Then, the plates will be blocked with PBS/BSA 5%, 200 µL/well, for 2h at 37°C. After 1 wash with PBS, pH 7.4, 100 µL of purified SVMP anti-fraction mAbs diluted in a 1: 2 ratio from the plateau dilution obtained in the ELISA assays will be added and the plates incubated 1h at 37 ° C. After incubation, the wells will be washed 3 times with 0.05% PBS / Tween, 200 µL / well. Next, 100 µL of anti-horse or anti-mouse IgG produced in rabbit and labeled with peroxidase (secondary [Ab] antibody) will be added to each well and the plates incubated for 1h at 37°C. Then the wells will be washed 3 times again with PBS / Tween 0.05%, 200 µL / well, and 50 µL / well of the substrate buffer for peroxidase, OPD (orthohyldiamine) (Sigma Chemical Co) and color development will be applied. observed after 15 min of reaction at room temperature under light. The reaction will be terminated with the application of 4 M sulfuric acid, 50 µL/well, and the absorbances (ABS) read at 490 nm. The values obtained will be projected on a graph with the X axis representing the corresponding dilutions versus the Y axis representing the absorbance. Dilutions giving maximum values of ABS 490 nm in the obtained curve will be considered titers of the tested Abs.

### 5.6. Obtaining purified anti-fraction hybridomas of the SVMP of the venom of *B. arietans*

#### 5.6.1. Hybridomas

Activated B cells will be fused with myeloma cells in the presence of HAT medium containing hypoxanthine, aminopterin, and thymidine and the positive hybridomas, identified based on viability and the production and secretion of mAbs, will be propagated. Representative hybridomas will be submitted to three sequential rounds of cloning. Clonal propagation will be performed by cultivation in 25 cm<sup>3</sup> plastic bottle bioreactors containing DMEM-F12 medium supplemented with 10% FCS, 10% gentamicin and 5 mM of 2-β-mercaptoethanol at 35°C under a 5% CO<sub>2</sub> atmosphere. The secreted mAbs will be collected, and the titers and affinities of these mAbs were measured using ELISA. Clonal propagation will be performed by cultivation in 25 cm<sup>3</sup> plastic bottle bioreactors containing DMEM-F12 medium supplemented with 10% FCS, 10% gentamicin and 5 mM of 2-β-mercaptoethanol at 35°C under a 5% CO<sub>2</sub> atmosphere. The secreted mAbs will be collected, and the titers and affinities of these mAbs were measured using ELISA. After the fusion of activated B cells with myeloma cells in the presence of HAT medium containing hypoxanthine, aminopterin and thymidine, positive hybridomas will be cloned by three successive limiting dilution steps. Clones resulting from the 3rd step will be expanded. The secreted mAbs will be harvested and the antibody titers, affinity, specificity, immunoglobulin isotypes and antigen recognition will be assayed [33,34].

#### 5.6.2. Cell fusion

The spleen of mice producing purified SVMP anti-fraction Abs with desirable titers and affinities will be collected after euthanasia. Splenic extracts will be prepared, lymphocytes will be organized and prepared for fusion [34].

#### 5.6.3. Identification of anti-SVMP myelomas

Myelomas will be multiplied in a culture medium containing hypoxanthine, aminopterin and thymine, conditions that select anti-SVMP hybridomas [33].

#### 5.6.4. Expansion of hybridomas

Selected hybridomas will be grown in 26 ml plastic bottles containing DMEM-F12 medium supplemented with 10% fetal bovine serum, 10% gentamicin and 5 mM 2-β-mercaptoethanol, at 35°C under 5% CO<sub>2</sub> for 3 days. Viable hybridomas that are secreting mAbs will be collected, kept in liquid nitrogen and will be sources of anti-SVMP mAbs [33].

#### 5.6.5. Characterization of anti-SVMP mAbs in the hybridoma culture medium

The culture medium will be concentrated, the mAbs purified by immunochemical methodology, their titers quantified by ELISA and isotypes of Igs identified [34].

#### 5.6.6. Evaluation of the antitoxic activity of anti-SVMP mAbs

Samples of anti-SVMP will be incubated with standardized samples of *B. arietans* venom or purified SVMP under test conditions. 100 μL of PBS containing 10 μL of the Abz-RPPGFSPFR-QEDDnp substrates will be pre-incubated at 37°C with buffer (control) or with 0.5 μg samples of the anti-SVMP mAbs and the reactions continuously monitored by fluorescence emission at 420 nm after excitation at 320 nm in a fluorimeter (Victor3™ Perkin-Elmer, Boston, MA, USA or FLUO star® Omega, BMG Labtech, HE, Germany). The Specific Activity Unit (UAE) will be expressed in fluorescence units of cleaved substrate per minute [14]. The antitoxic activities of the anti-SVMP mAbs will be reported in% inhibition using control results without anti-SVMP mAbs as 100%.

#### 5.7. Protein quantification

The protein content of the purified venom and mAbs will be quantified by the bicinchoninic acid method [35].

### 5.8. Titration of anti-SVMP mAbs by the ELISA method

96-well high-binding plates (Costar) will be incubated at room temperature for  $\pm$  12h with 100  $\mu$ L of PBS buffer, pH 7.4, containing 1  $\mu$ g of crude venom or purified fractions. Then, the plates will be blocked with PBS/BSA 5%, 200  $\mu$ L/well, for 2h at 37°C. After 1 wash with PBS, pH 7.4, equal volumes of dilutions of equine anti-*B arietans* serum or mAbs will be added in PBS/BSA 0.1% (primary Ab) and the plates incubated for 1h at 37°C. After that, the wells will be washed 3 times with PBS/Tween buffer 0.05%, 200  $\mu$ L/well. Next, 100  $\mu$ L of anti-horse or anti-mouse IgG produced in rabbit and labeled with peroxidase (secondary Ab) will be added to each well and the plates incubated for 1h at 37°C. Then the wells will be washed 3 times again with 0.05% PBS/Tween 200  $\mu$ L/well and 50  $\mu$ L/well of the substrate buffer for peroxidase, OPD (Sigma Chemical Co) and the color development will be applied after 15 min of reaction at room temperature under light. The reaction will be terminated with the application of 4 M sulfuric acid, 50  $\mu$ L/well and the absorbances read at 490 nm. The values obtained will be projected on a graph with the X axis representing the corresponding dilutions versus the Y axis representing the absorbance. Dilutions giving maximum values of ABS 490 nm in the obtained curve will be considered titers of the tested Abs.

### 5.9. Assessment of avidity of anti-SVMP mAbs obtained by the ELISA method

96-well high-binding plates (Costar) will be incubated at room temperature for  $\pm$  12h with 100  $\mu$ L of PBS buffer, pH 7.4, containing 1  $\mu$ g of crude venom or purified fractions. Then, the plates will be blocked with PBS/BSA 5%, 200  $\mu$ L/well, for 2h at 37°C. After 1 wash with PBS, pH 7.4, samples of anti-SVMP mAbs will be added in PBS/BSA 0.1% (primary Ab) and the plates incubated for 0 min, 20 min, 40 min or 60 min of incubation at 37°C. After incubation, the wells will be washed 3 times with 0.05% PBS/Tween buffer, 200  $\mu$ L/well. Next, 100  $\mu$ L of anti-horse or anti-mouse IgG produced in rabbit and labeled with peroxidase (secondary Ab) will be added to each well and the plates incubated for 1h at 37°C. Then the wells will be washed 3 times again with 0.05% PBS/Tween, 200  $\mu$ L/well, and 50  $\mu$ L/well of the substrate buffer for peroxidase, OPD (Sigma Chemical Co) and the color development observed after 15 minutes will be applied. min reaction at room temperature under light. The reaction will be terminated with the application of 4 M sulfuric acid 50  $\mu$ L/well, and the ABS read at 490 nm. The values obtained will be projected on a graph with X representing time and the Y axis representing absorbance. The avidity index indicates the shortest time for the toxin-antitoxin complexes to be formed.

### 5.10. Evaluation of the affinity of anti-SVMP mAbs obtained by the ELISA method

96-well high-binding plates (Costar) will be incubated at room temperature for  $\pm$  12h with 100  $\mu$ L of PBS buffer, pH 7.4, containing 1  $\mu$ g of venom or purified fractions. Then, the plates will be blocked with PBS/BSA 5%, 200  $\mu$ L/well, for 2h at 37°C. After 1 wash with PBS, pH 7.4, the mAbs that gave maximum values of ABS 490 nm in the curve obtained in the "Identification of the anti-SVMP immunoglobulin isotypes" assay will be added to the wells as primary Ab and the plates will be incubated for 1h at 37°C. After that, the wells will be washed 3 times with PBS/Tween buffer 0.05%, 200  $\mu$ L/well. Next, 100  $\mu$ L PBS/BSA 5% containing concentrations of 0.5 to 4.5 M of the potassium thiocyanate chaotropic agent (KSCN, Sigma Aldrich, St. Louis, MO, USA) will be added to each well, and the plates will be incubated for 10 minutes at room temperature, and washed 3 times with PBS pH 7.4. The ELISA assay continues as previously described. The affinity index will be defined by the highest mM concentration of the KSCN that breaks up the immune complexes.

### 5.11. Identification of SVMP protein bands recognized by mAbs

This "Western blotting" protocol will be conducted as originally described [20]. The SDS-PAGE analysis will be done according to the described methodology [35]. Samples

of purified SVMP or *B. arietans* venom (5µg) will be applied to SDS-PAGE gels (5% Upper, 10 to 15% Lower) in reducing and non-reducing conditions. The gels will be electrophoresed for 100V in a Power Pac Basic (Bio-Rad). The separated protein bands will be revealed by staining with Coomassie blue, and their molecular masses evaluated using the parallel band pattern profile in parallel. The identified bands will be electrotransferred “overnight” at 4°C, in a transfer tank TE 22 (Hoefer Pharmacia Biotech, San Francisco, CA), under a current of 150 mA for nitrocellulose membranes. Then, the membranes will be washed quickly with PBS, and treated with anti-SVMP mAbs. The membranes will be incubated for 1h at room temperature, and then washed 3 times with PBS/0.05% Tween. After that, the membranes will be incubated with anti-mouse or anti-horse IgG produced in rabbit and marked with alkaline phosphatase (secondary Ab) diluted in PBS/BSA 0.1% for 1h at room temperature, and then washed 3 times with 0.05% PBS/Tween and developed by adding NBT/BCIP solution (Sigma Aldrich, St. Louis, MO). The development of the bands will be monitored visually, and the reaction will end with the addition of distilled water.

#### 5.12. Statistical analysis

The data obtained will be analyzed by “one-way ANOVA”, complemented by “Dunnett's Multiple Comparison Test (standard antivenom as comparison)”, or by “two-way ANOVA”, complemented by “Bonferroni Post Test (standard antivenom as comparison)”. Differences will be considered significant if  $p < 0.05$ . The data will be analyzed using the “Graphic PAD Prism 5” model (Graph Pad Software).

**Author Contributions:** K.S.G. – development of the research project, experiments, article and thesis; F.R.G. – development of monoclonal antibodies; A.A.A.M. – purifications and characterization of toxins; F.V.C.P. – CDRs sequencing-modeling; W.D.S. – article review and project development; All authors have read and agreed to the published version of the manuscript.

**Funding:** This research is being funded by the Foundation for Research State São Paulo (FAPESP), Toxins Center, immune response and Cell Signaling (CETICS) and Higher Education Personnel Improvement Coordination (CAPES).

**Acknowledgments:** I would like to thank all the collaborators who worked directly and indirectly in the execution of the project so far, especially my advisor Dr. Prof. Wilmar Dias da Silva, who has been with me since the beginning and who is always willing to help, and to all the employees of the Butantan Institute's Immunochemistry Laboratory, technicians, secretaries, researchers and students.

**Conflicts of Interest:** The authors declare no competing interest.

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