DENIS ALEXIS MOLINA MOLINA

Produção de anticorpos contra Metaloproteases e Crotoxina usando antígenos sintéticos e caracterização proteômica e toxicológica do veneno de *Bothrops brazili.*

Belo Horizonte

Minas Gerais – Brasil

Setembro – 2020

DENIS ALEXIS MOLINA MOLINA

Produção de anticorpos contra Metaloproteases e Crotoxina usando antígenos sintéticos e caracterização proteômica e toxicológica do veneno de *Bothrops brazili.*

ORIENTADOR Dr. CARLOS D. CHÁVEZ OLÓRTEGUI

Tese submetida ao Departamento Bioquímica e Imunologia do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, como requisito parcial para obtenção do grau de Doutor em Bioquímica e Imunologia.

Universidade Federal de Minas Gerais

Belo Horizonte

Setembro - 2020

043 Molina, Denis Alexis Molina.
Produção de anticorpos contra metaloproteases e crotoxina usando antígenos sintéticos e caracterização proteômica e toxicológica do veneno de *Bothrops brazili* [manuscrito] / Denis Alexis Molina Molina. – 2020.

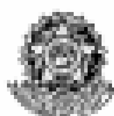
95 f. : il. ; 29,5 cm.

Orientador: Dr. Carlos Delfin Chávez Olórtegui.
Tese (doutorado) – Universidade Federal de Minas Gerais, Instituto de Ciências Biológicas. Programa de Pós-Graduação em Bioquímica e Imunologia.

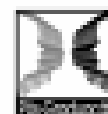
1. Serpentes. 2. Hemorragia. 3. Crotoxina. 4. Metaloproteases. 5. Formação de Anticorpos. I. Chávez–Olórtegui, Carlos Delfin. II. Universidade Federal de Minas Gerais. Instituto de Ciências Biológicas. III. Título.

CDU: 577.1

Ficha catalográfica elaborada pela bibliotecária Rosilene Moreira Coelho de Sá – CRB 6 – 2726



Universidade Federal de Minas Gerais
Curso de Pós-Graduação em Bioquímica e Imunologia ICB/UFMG
Av. Antônio Carlos, 6627 – Pampulha
31270-901 – Belo Horizonte – MG
e-mail: pg-biq@icb.ufmg.br (31)3409-2615



ATA DA DEFESA DA TESE DE DOUTORADO DE DENIS ALEXIS MOLINA MOLINA. Ao um dia do mês de setembro de 2020 às 13:00 horas, reuniu-se no Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, a Comissão Examinadora da tese de Doutorado, indicada *ad referendum* do Colegiado do Curso, para julgar, em exame final, o trabalho intitulado "' Produção de anticorpos contra Metaloproteases e Crotoxina usando antigenos sintéticos e caracterização proteômica e toxicológica do veneno de Bothrops brazili.", requisito final para a obtenção do grau de Doutor em Ciências: Bioquimica. Abrindo a sessão, o Presidente da Comissão, Prof. Carlos Delfin Chavez Olortegui, da Universidade Federal de Minas Gerais, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra ao candidato para apresentação de seu trabalho. Seguiu-se a arguição pelos examinadores, com a respectiva defesa do candidato. Logo após a Comissão se reuniu, sem a presença do candidato e do público, para julgamento e expedição do resultado final. Foram atribuídas as seguintes indicações: Dr. Stephen Hyslop (UNICAMP), aprovado; Dr. Adolfo Borges Strauss (CEDIC – Asunción, Paraguai), aprovado; Dr. Luis Henrique Franco (Universidade Federal de Minas Gerais), aprovado; Dr. Jader dos Santos Cruz (Universidade Federal de Minas Gerais), aprovado; Dr. Carlos Delfin Chavez Olortegui - Orientador (Universidade Federal de Minas Gerais), aprovado. Pelas indicações o candidato foi considerado:

(X) APROVADO
( ) REPROVADO

O resultado final foi comunicado publicamente ao candidato pelo Presidente da Comissão. Nada mais havendo a tratar, o Presidente da Comissão encerrou a reunião e lavrou a presente Ata que será assinada por todos os membros participantes da Comissão Examinadora. Belo Horizonte, 01 de setembro de 2020.

Dr. Stephen Hyslop (UNICAMP)

Dr. Adolfo Borges Strauss (CEDIC –Asunción, Paraguai)

Dr. Luis Henrique Franco (UFMG)

Dr. Jader dos Santos Cruz (UFMG)

Dr. Carlos Delfin Chavez Olortegui - Orientador (UFMG)

DEDICATÓRIA

A mis padres por siempre me motivar a estudiar y apoyarme en mis metas,

formando parte de cada uno de mis logros.

AGRADECIMENTO

Ao meu orientador Dr. Carlos Chávez Olortégui, pela orientação e ensinamentos que tornaram possível a realização deste trabalho.

Aos colegas do laboratório do LABIMQ, pelo companheirismo ajuda e incentivo, além dos bons momentos compartilhados.

Ao Jamil e Adriana por toda sua ajuda e sábios conselhos em cromatografia e espectrometria de massa, fazendo o trabalho bem mais agradável.

Aos Doutores Stephanie, Luís Felipe, Fernanda, Dayane, Elizangela, Eliseu, Clara, Francisco e Ricardo, por toda sua ajuda durante todo este período, na pós-graduação, colaborando muito na elaboração deste trabalho.

A doutoranda Carolina Rego, companheira e amiga de laboratório, com quem dividi muitas horas de trabalho e discussões que levaram a publicação de nossa pesquisa.

Aos meus amigos que são minha família aqui no Brasil, Daniela, Hector, Fatima, Nubia, Giovana, Rodrigo, Rafael, Adriano e Fernanda londrina, por todos os bons momentos fazendo me sentir em casa, fazendo que todo este tempo aqui no Brasil fora bem agradável.

Aos membros da banca pelas correções e contribuições com esta tese.

À todos aqueles que participaram, direta ou indiretamente, na realização deste trabalho.

Education is the passport to the future,

for tomorrow belongs to those who prepare for it today.

Malcolm X

RESUMO

As serpentes pertencentes aos gêneros *Bothrops*, *Crotalus, Lachesis e Micrurus* são responsáveis pela maioria dos casos de ofídismos no Brasil. Serpentes do gênero *Crotalus spp e Micrurus spp* possuem veneno neurotóxico com alta letalidade se não neutalizado a tempo. As serpentes do gênero *Bothrops* e *Lachesis* apresentam veneno com ação proteolítica, ocasionando danos permanentes mesmo após o tratamento com soros antiofídicos.

O uso tradicional de venenos na imunização de animais para a produção de soros antiofídicos apresenta alguns problemas, uma vez que os animais imunizados podem sofrer com toxicidade dos componentes do veneno, o que diminui sua expectativa de vida. Além disso, a extensa variabilidade inter e intraespecífica na composição bioquímica dos venenos de serpentes podem afetar a eficácia neutralizante dos soros antiofídicos. Dessa forma, é de suma relevância a utilização de novas tecnologias para buscar e produzir imunógenos atóxicos alternativos para serem utilizados na produção de soros antiofídicos. O uso de peptídeos sintéticos e proteínas multiepitópicas que mimetizam epítopos das principais toxinas de cada veneno constituiria uma alternativa para produção de anticorpos neutralizantes com menor impacto sobre os animais experimentais.

Esta tese apresenta-se na forma de três artigos, cada um está contido em um capítulo da parte dos resultados. Cada capítulo apresenta seu resumo individualizado. Segue a descrição do conteúdo de cada capítulo:

Capítulo 1, (Artigo 1 - Identification of a linear B-cell epitope in the catalytic domain of bothropasin, a metalloproteinase from *Bothrops jararaca* snake venom),

neste artigo descreve-se o mapeamento e síntese de um epítopo linear para células B da bothropasina capaz de induzir anticorpos neutralizantes.

Capítulo 2, (Artigo 2 - Engineered protein containing Crotoxin epitopes induces neutralizing antibodies in immunized rabbits), neste artigo descreve-se a produção de uma proteína recombinante multiepitopo da crotoxina capaz de induzir anticorpos neutralizantes do efeito letal da crotoxina.

Capítulo 3, (Artigo 3 - Proteomic and toxinological characterization of Peruvian pitviper *Bothrops brazili* ("jergón shushupe"), venom.), neste artigo, os componentes do veneno de *Bothrops brazili* (BbV) foram analisados por RP-HPLC, SDS-PAGE e MALDI-TOF/TOF, mostrando que as metaloproteases do veneno de serpentes (SVMPs) foram as proteínas mais abundantes no veneno de *B. brazili*.

Cada artigo apresenta sua discussão em separado. Ao final do terceiro artigo uma discussão geral dos três artigos é apresentada.

ABSTRACT

Snakes belonging to the genera *Bothrops, Crotalus, Lachesis* and *Micrurus* are responsible for most cases of snakebites in Brazil. Snakes of the genus *Crotalus spp* and *Micrurus spp* have neurotoxic venom with high lethality if not neutralized in time. *Bothrops* and *Lachesis* venom display proteolytic action, causing permanent damage even after treatment with antiofidic sera.

The traditional use of venoms in the immunization of animals for the production of antiofidic sera have drawbacks, since the immunized animals may suffer because of toxic components of the venom, which reduces their life expectancy. Beside, the extensive inter and intraspecific variability in the biochemical composition of snake venoms can affect the neutralizing efficacy of antiofidic sera. Thus, it is crucial to use new technologies to search for and produce alternative non-toxic immunogens that can be used in the production of antiofidic serums. The use of synthetic peptides and multiepitopic proteins that mimic epitopes of the main toxins of each venom would be an alternative for the production of neutralizing antibodies with less impact on experimental animals.

This work is presented in the format of three articles, each one containing in a separated chapter of results. Each chapter presents its individualized abstract. The content description of each chapter is shown below:

Chapter 1, (Article 1 - Identification of a linear B-cell epitope in the catalytic domain of bothropasin, metalloproteinase from *Bothrops jararaca* snake venom), this article describes the mapping and synthesis of a linear B cell epitope for bothropasin, capable of to induce neutralizing antibodies.

Chapter 2, (Article 2 - Engineered protein containing Crotoxin epitopes induces neutralizing antibodies in immunized rabbits), this article descridbes the production of a multiepitopic recombinant crotoxin protein, capable of inducing neutralizing antibodies.

Chapter 3, (Article 3 - Proteomic and toxinological characterization of Peruvian pitviper *Bothrops brazili* ("jergón shushupe"), venom.), In this article, the components of *Bothrops brazili* (BbV) venom were analyzed by RP-HPLC, SDS-PAGE and MALDI-TOF/TOF, showing that snake venom metalloproteinases (SVMPs) were the most abundant proteins in the venom of *B. brazili.*

Each article presents its discussion separately. At the end of the third article a general discussion of the three articles is presented.

Lista de figuras

Figura 1. Distribuição mundial das serpentes. ... 16

Figura 2. Tipo de dentição das serpentes. ... 18

Figura 3. Mapa de distribuição das principais serpentes do gênero *Bothrops*. ... 22

Figura 4. *Bothrops brazili*. ... 24

Figura 5. *Bothrops jararaca*. ... 25

Figura 6. Diagrama esquemático das estruturas de domínio de SVMP P-I, P-II e P-III. 27

Figura 7. Modelo estrutural da bothropasina. ... 28

Figura 8. *Crotalus durrisus terrificus*. ... 32

Figura 9. Composição proteica do veneno da *Crotalus durisus terrificus*. ... 33

Figura 10. Modelo estrutural da crotoxina.. ... 34

Lista de siglas e abreviaturas

- Ag-Ac: antígeno anticorpo
- BCIP: 5-*Bromo-4-chloro-3-indolyl phosphate*
- BSA: Do inglês *Bovine Serum Albumin*
- CA: subunidade ácida da crotoxina
- CB: subunidade básica da crotoxina
- *C.d.t*: *Crotalus durissus terrificus*
- Ctx: crotoxina
- CTLs: Lectinas tipo C
- DL_{50}: Dose letal 50
- DMF: Do ingles *dimetilformamide*
- ELISA: Do inglês *Enzyme linked immunosorbent assay*
- F(ab´)2: Do inglês *Fragment of antigen binding* (bivalente)
- Fmoc: Grupo Fluorenilmetoxicarbonil
- Funed: Fundação Ezequiel Dias, de Belo Horizonte MG.
- His: Histidina
- HPLC: Do inglês *High performance liquid chromatography*
- IB: Instituto Butantan, São Paulo SP.
- ICV: intra cérebro ventricular
- IgG: imunoglobulina G
- IRA: insuficiência renal aguda
- kDa: Kilodalton
- MALDI-TOF: Do inglês *Matrix Assisted Laser Desorption ionized Time of Flight- Time of Flight*
- MHD: Dose minima hemorrágica
- MTT: Do inglês *metil thiazolyldiphenil tetrazolium*
- OMS: Organização Mundial de Saúde
- OPD: Do inglês *o-Phenylenediamine dihydrochloride*
- PBS: Tampão fosfato salino
- PDB: Do inglês *Protein Data Bank*
- PI: Ponto isoelétrico
- PLA_2: Fosfolipase A_2

- S.C.: subcutâneo
- SDS: dodecil sulfato de sódio
- SDS-PAGE: Eletroforese em gel de poliacrilamida, com dodecil sulfato sódio
- SVMPs: Do inglês *Snake venom Serine Metaloroteinases*
- SVSPs: Do inglês *Snake venom Serine Proteinases*
- TFA: Ácido trifluoroacético

Sumario

1. Introdução 16
1.1 Ofidismo 16
1.2 A soroterapia 20
1.3 Gênero *Bothrops* 22
1.3.1 *Bothrops brazili* 23
1.3.2 *Bothrops jararaca* 25
1.3.3 Metaloproteases 26
1.3.4 Serino proteases 28
1.3.5 fosfolipases (PLA_{2s}) 29
1.3.6 L-aminoácido oxidase(LAAO) 30
1.4 Gênero *Crotalus* 31
1.4.1 Crotoxina 33
1.4.2 crotamina 34
1.5 Identificação proteômica dos componentes dos venenos 35
1.6 Predição de epítopos 37
1.7 Epítopos de proteínas para linfócitos B 37
2. Objetivos 39
2.1 Objetivos específicos 39
3. Resultados 40
3.1 Artigo 1. Identification of a linear B-cell epitope in the catalytic domain of bothropasin, metalloproteinase from *Bothrops jararaca* snake venom 41
3.2 Artigo 2. Engineered protein containing Crotoxin epitopes induces neutralizing antibodies in immunized rabbits 49
3.3 Artigo 3. Proteomic and toxinological characterization of Peruvian pitviper *Bothrops brazili* ("jergón shushupe"), venom 60
4. Discussão 70
5. Conclusões 81
6. Perspectivas 82
7. Referencias 83

1. INTRODUÇÃO

1.1 Ofidismo

As serpentes são animais pertencentes à Subordem Ophidia classe Reptilia, caracterizados pela falta de membros locomotores e corpo alongado (VALENTA, 2010). As serpentes podem ser classificadas em dois grupos básicos: as peçonhentas, que são aquelas que conseguem inocular seu veneno no corpo de uma presa ou vítima e as não peçonhentas, que são aquelas que podem até produzir veneno, mas não possuem um aparato físico que o inoculam. Ainda assim, as não-peçonhentas podem morder e causar sintomas de febre, inchaço, ou mesmo contaminar com doenças como o tétano. Ambos tipos de serpentes são encontradas em todas as regiões brasileiras, nos mais diferentes tipos de habitat, inclusive em ambientes urbanos (FUNASA, 2001).

A Organização Mundial de Saúde (OMS) estima que no mundo existam aproximadamente 3000 espécies de serpentes, sendo que 10 a 14% são consideradas peçonhentas. As serpentes são encontradas em todos os continentes, exceto na antártica (Figura 1) (WHO, 2016a).

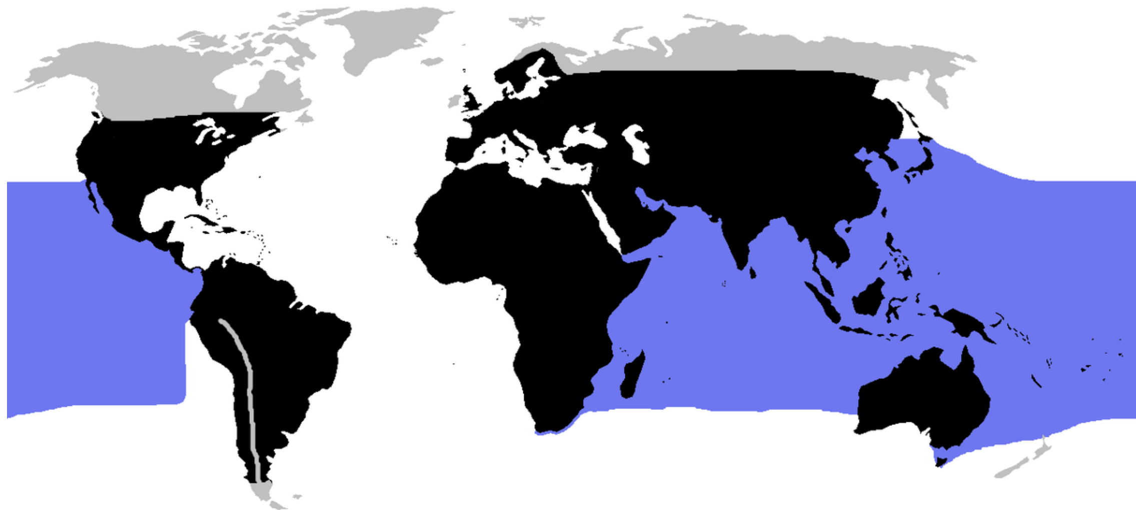


Figura 1. Distribuição mundial das serpentes. Em azul, o habitat das serpentes marinhas e em preto as serpentes terrestres. Disponível em: https://apps.who.int/bloodproducts/snakeantivenoms/database/

Venenos de serpentes é uma mistura rica e complexa de peptídeos e proteínas com diferentes propriedades biológicas, os quais correspondem aproximadamente 90% do peso seco (MARKLAND, 1998; NÚÑEZ et al., 2009; QUEIROZ et al., 2008). A extensa diversidade de proteínas e peptídeos encontrados nos venenos de serpentes está classificada em algumas poucas famílias proteicas que variam de espécie em espécie (CALVETE, 2013; CALVETE et al., 2009; CALVETE AND JU; SANZ, 2007; FRY; WÜSTER, 2004).

O veneno das serpentes é uma adaptação evolutiva que tem um papel chave na evolução e diversificação das serpentes, o qual é uma secreção produzida em glândulas especializadas de um animal e injetada em outro, que causa distúrbios nos processos fisiológicos e/ou bioquímicos de modo que é utilizado na captura das presas pela ação das toxinas que além de imobilização também ajuda na digestão, o veneno também é usado como defensa do animal produtor. (FRY et al., 2006, 2009; FRY; WÜSTER, 2004).

Associados à glândulas produtora de veneno podem existir dentes modificados (presas) especializados na inoculação do veneno, quando existentes, o par de presas inoculadoras de veneno fixam-se na maxila e podem ser rostral (anterior) ou caudalmente (posteriormente), nas serpentes que possuem o sistema de injeção do veneno rostral, as presas inoculadoras são ocas, possuindo um canal central por onde o veneno passa andes de ser injetado no alvo da serpente. As presas inoculadoras rostrais podem ser fixas e de pequeno tamanho (dentição proteróglifa, típica da família Elipadae) ou (dentição solenóglifa, típica da família Viperidae) (Figura 2).

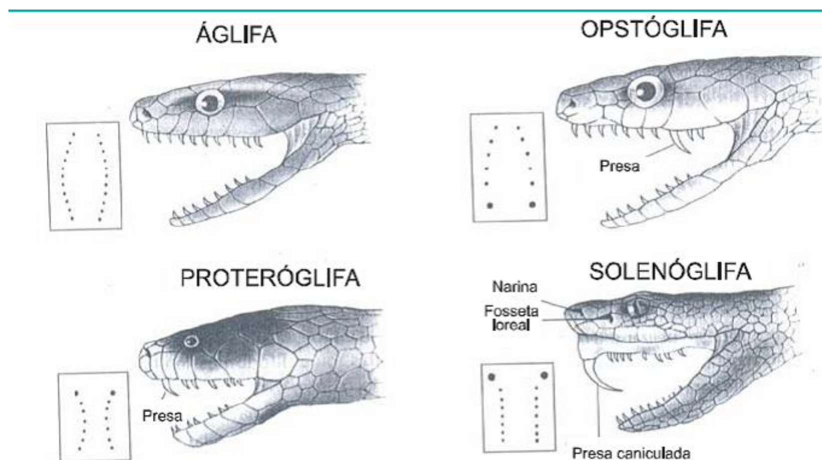


Figura 2. Tipo de dentição das serpentes. Áglifa: dentição mais ou menos uniforme, sem especialização de dentes para a inoculação de peçonha. Solenóglifa: dentição na qual há dentes inoculadores (canaliculados) retratáveis e compridos localizados na parte anterior da boca; são responsáveis pela inoculação de peçonha. Este tipo de dentição é característico da família Crotalidae. Exemplo: gêneros *'Bothrops' e 'Crotalus'*. Opistóglifa: dentição na qual há uma especialização de alguns dentes localizadas na parte posterior da boca, e que são capazes de inocular peçonha. Proteróglifa: dentição na qual há dentes inoculadores (canaliculados) imóveis curtos localizados na parte anterior da boca; são responsáveis pela inoculação de peçonha. Este tipo de dentição é característico da família Elapidae: gênero *'Micrurus'* (corais verdadeiras). Disponível em: https://biologiaentenderrespeitar.wordpress.com/2018/03/29/diferencas-de-denticao-de-serpentes/

No mundo, anualmente se estima que possam ocorrer ao redor de 1,8 milhões de casos de envenenamento por acidentes ofídicos, com mais de 80.000 óbitos (WHO, 2019). No Brasil as serpentes do gênero *Bothrops* são as principais responsáveis pelos acidentes ofídicos (MINISTÉRIO DA SAÚDE., 2009). Na região amazônica, a serpente *Bothrops atrox* é a serpente mais comumente envolvida nos envenenamentos, respondendo por até 80% do total de casos registrados (GUTIÉRREZ; THEAKSTON; WARRELL, 2006; WALDEZ, FABIANO AND VOGT, 2009). Estes acidentes representam um sério problema de saúde pública nos países tropicais, pela frequência com que ocorrem e pela morbidade e mortalidade que causam.

No Brasil, cerca do 16% das espécies de serpentes locais podem ser consideradas potencialmente capazes de produzir envenenamentos que necessitem de

uma intervenção médica (LIRA-DA-SILVA et al., 2009). Dentre as serpentes de importância médica estão aquelas que apresentam glândulas que produzem toxinas, que estão agrupadas nas Famílias Viperidae (*Bothrops, Bothriopsis, Bothrocophias, Lachesis* e *Crotalus*), Elapidae (*Micrurus* e *Leptomicrurus*) e Dipsadidae (*Boiruna* e *Philodryas*). Existem atualmente 23 espécies de *Bothrops*, 2 de *Bothriopsis* e 1 de *Bothrocophias* (jararacas), 24 de *Micrurus* (corais) e 3 de *Leptomicrurus* (corais). As serpentes *Lachesis muta* (surucucus) e *Crotalus durissus* (cascavéis) são uniespecíficas (LIRA-DA-SILVA et al., 2009).

Os venenos das cascavéis sul-americanas são notórios por sua capacidade de causar miotoxicidade sistêmica e desabilitar a transmissão neuromuscular levando à paralisia progressiva. A neurotoxicidade nas picadas é atribuída principalmente à presença de altas concentrações de crotoxina, o sintoma de hemorragia, contudo os sintomas neurotóxicos prevalecem e são os mais graves (CALVETE et al., 2010a).

As sintomatologias dos acidentes botrópicos e laquéticos diferem do crotálicos, já que os dois primeiros apresentam um quadro clínico caracterizado por dor local, edema, hemorragia e necrose no local da picada, e nos casos mais graves podem apresentar choque cardiovascular, disfunção na coagulação, hemólise e falência renal (DAMICO et al., 2007), o envenenamento botrópico induz reações locais severas nas vitimas humanas, incluindo formação de edema e inchaço (~95% dos casos), equimose, inflação e dor. Mas os sintomas locais mais graves são a hemorragia (~34%) e dano tecidual local, podendo acarretar em necrose (dermo e mionecrose ~10%). (OTERO-PATIÑO, 2009; WHITE, 2005). Em relação aos sinais locais, a progressão e a extensão do edema, bem como a presença de tecido necrosado, definem a gravidade do envenenamento (OTERO-PATIÑO, 2009).

O acidente crotálico apresenta o maior índice de letalidade devido à frequência com que evolui para insuficiência renal aguda (IRA), dada à maior nefrotoxicidade do envenenamento. O veneno crotálico apresenta três ações principais, sendo elas neurotóxica, miotóxica e coagulante, mas talvez seja a rabdomiólise o principal mecanismo de lesão renal neste tipo de acidente (SANTOS.; FARANI.; ROCHA., 2009).

1.2 A soroterapia

Desenvolvida no final do século XIX é trazida ao Brasil por Vital Brazil no ano de 1905. Atualmente, apesar dos avanços tecnológicos na área da saúde, o único tratamento disponível e recomendado pela OMS para os casos de acidentes ofídicos é a administração intravenosa de soro antiofídico, de acordo com o gênero da serpente.

Atualmente, muito laboratórios processam os anticorpos a serem utilizados na soroterapia e, assim, três tipos de antivenenos são produzidos em todo o mundo: (1) antivenenos composto por moléculas inteiras de IgG de cavalo obtidos por meio de fracionamento de plasma; (2) fragmentos $F(ab')_2$ de IgG de cavalos derivados de sua digestão por pepsina; e (3) fragmento Fab de origem ovina, obtido pela digestão de molécula de IgG com papaína (ESPINO-SOLIS et al., 2009; OTERO-PATIÑO, 2009). A OMS indica que o soro antiofídico utilizado em cada pais seja avaliado em estudos pré-clínicos e que sua capacidade neutralizante seja eficiente contra os principais efeitos tóxicos das serpentes de maior revelância epidemiológica nas regiões onde ele é utilizado (THEAKSTON; WARRELL; GRIFFITHS, 2003; WHO, 2016b).

No Brasil são produzidos os soros antibotrópico, antibotrópico/crotálico, antibotrópico/laquético anticrotálico e antielapídico. Os soros antivenenos contendo fragmentos $F(ab')_2$ são produzidos pela hiperimunizacão de cavalos e reações alérgicas

podem ocorrer em até 36% dos casos (OTERO-PATIÑO, 2009; THEAKSTON; WARRELL; GRIFFITHS, 2003). Se administrados nas primeiras horas após o acidente os antivenenos são eficientes em diminuir a taxa de mortalidade, mas o retardo no tratamento é relacionado com ao desenvolvimento de quadros de falência renal aguda e hemorragia no sistema nervoso central o que pode levar a morte do paciente (OTERO-PATIÑO, 2009). Por outro, lado os sintomas locais não são completamente neutralizados pelo soro antibotrópico, fazendo que estes sejam clinicamente e socioeconomicamente de grande importância devido a que no acidente botrópico se estima que até um 40% dos casos pode apresentar dano tecidual permanente, com consequente amputação de membros (BATTELLINO et al., 2003; HARRISON et al., 2009; MUNIZ et al., 2000; ZAMUNÉR et al., 2004).

Entretanto, a soroterapia pode desencadear alguns efeitos adversos, tanto precoces (tem caráter anafilático ou analifilactoide, que podem ser graves), quanto tardios, como “Doença do Soro” que pode aparecer entre 5 a 24 dias após administração do soro e caracterizam-se por: febre, urticária, dores articulares, aumento dos gânglios e, raramente, comprometimento neurológico ou renal. Esta reação é tratada de acordo com a sua intensidade, através da administração de corticosteroides, analgésicos e anti-histamínicos. (MINISTÉRIO DA SAÚDE., 2009).

Além dos efeitos adversos observados, por vezes os soros antiofídicos disponíveis não protegem satisfatoriamente os pacientes. Sendo assim, se faz relevante o estudo das proteínas/peptídeos presentes em cada um dos venenos para os quais deve-se obter um anticorpo neutralizante (ESPINO-SOLIS et al., 2009). Diante disso, o desenvolvimento de novas abordagens é importante para a produção de antivenenos, com o intuito de melhorar a eficância, segurança/confiabilidade e a acessibilidade dos

antivenenos, visando à melhoria da terapia nos casos de envenenamento por animais peçonhentos.

O presente trabalho se desenvolveu com foco nas serpentes responsáveis pela maioria dos envenenamentos no Brasil e Peru, pertencentes aos gêneros *Bothrops* e *Crotalus* que serão discutidas mais aprofundadamente a seguir.

1.3 Gênero *Bothrops*

As serpentes do gênero *Bothrops* são peçonhentas, extremamente diversas e amplamente distribuídas nas regiões centrais e do sul do continente americano (Figura 3), sendo o principal grupo de serpentes de importância médica da região. Compreendem pelo menos 50 espécies, algumas das quais descritas recentemente, e apresentam grande variação de tamanho, podendo atingir até 2 metros de comprimento (CARRASCO et al., 2016).

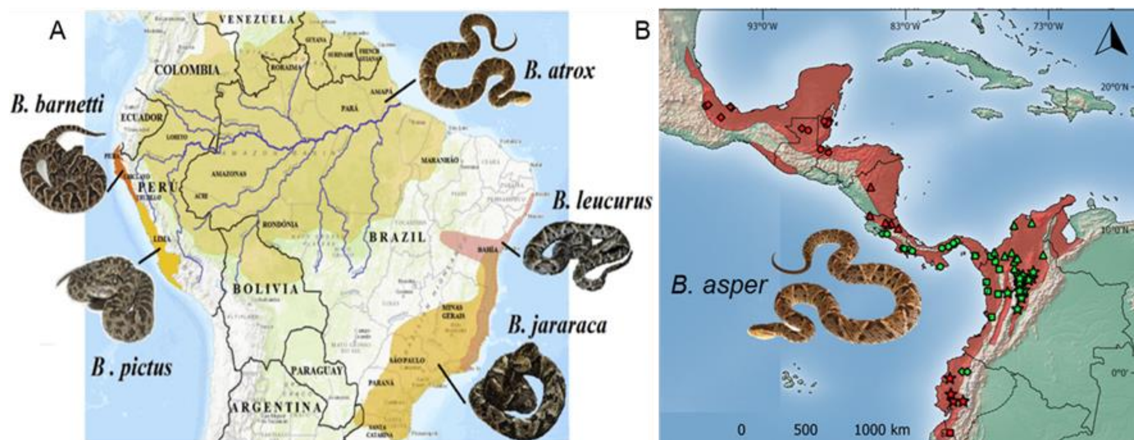


Figura 3. Mapa de distribuição das principais serpentes do gênero *Bothrops*. (A) Principais espécies do gênero na América do Sul são: *Bothrops atrox* e *Bothrops jararaca*, podendo também ser encontrada *Bothrops barnetti, Bothrops pictus* e *Bothrops leucurus* (B) Na América Central é encontrada a espécie *Bothrops asper*. Fonte: (ESTEVAO-COSTA et al., 2016; SALDARRIAGA-CÓRDOBA et al., 2017).

A variabilidade do veneno botrópico é muita complexa, dada a sua composição, uma vez que pode haver consideráveis diferenças entre as espécies (interespecífica e intraespecífica). Esta variação faz com que a eficácia dos antivenenos seja também

afetada, devido à utilização de diferentes venenos para a sua produção (SOUSA et al., 2018).

Os principais efeitos clínicos do envenenamento por serpentes do gênero *Bothrops* incluem danos teciduais locais (edema, hemorragia e mionecrose) e efeitos sistêmicos que resultam principalmente em distúrbios da coagulação sanguínea. As principais famílias de proteínas envolvidas nestes distúrbios são as metaloproteases (SVMPs – *Snake Venom Metalloproteinases*), serino proteases (SVSPs – *Snake Venom Serine Proteases*), fosfolipases (PLA_{2s}) e as lectinas do tipo C (CTLs).

Essas toxinas são capazes de atuar nos componentes da cascata de coagulação, promover hemorragia, interferir na formação do tampão hemostático e na agregação plaquetária (SOUSA et al., 2018).

1.3.1 *Bothrops brazili*

A serpente *B. brazili* nomeada assim em homenagem a Vital Brazil Mineiro de Campanha, um grande cientista brasileiro e fundador do Instituto Butantan (São Paulo), foi descrita por Alphonse Hoge em 1954. Os espécimes adultos desta serpente tem um comprimento entre 70 a 90 cm (Figura 4). Esta espécie venenosa amplamente distribuída nas florestas equatoriais do sul da Colômbia, leste do Equador, leste do Peru, norte da Bolívia, sul e leste da Venezuela, Guiana, Suriname, Guiana Francesa e Brasil, esta serpente comparte uma distribuição geográfica semelhante a *B. atrox* (BERNARDE; NEGREIROS DE ALMEIDA, 2020).

Figura 4. ***Bothrops brazili*.** O nome específico, brazili, é uma homenagem ao Dr. Vital Brazil, médico brasileiro e fundador do Instituto Butantan de São Paulo. Fonte: http://reptile-database.reptarium.cz/species?genus=Bothrops&species=brazili

A coloração da camuflagem do *B brazili* torna difícil para suas presas ou predadores detectá-lo quando está enrolado na serapilheira. Isso também contribui para picadas de cobra em pessoas incautas que andam nesses ambientes (SILVA et al., 2019). Dados de espécimes dos estados brasileiros do Maranhão, Pará e Rondônia (MARTINS; MARQUES; SAZIMA, 2002), e da bacia do alto Amazonas, Região de Iquitos, Peru, indicaram que as jararacas brasileiras exibem mudança ontogenética na dieta de tipo de presa de ectotérmicos invertebrados para vertebrados ecto e endotérmicos. Centopéias são presas comuns de juvenis, enquanto os adultos são generalistas, alimentando-se principalmente de roedores, anuros e lagartos (SANZ et al., 2020).

B. brazili peruano produz grandes quantidades de veneno (3-4 mL) com uma potente dose letal (LD50) em camundongos de 15,27 µg/18-20 g de camundongo em comparação com 49,90 µg/camundongo de *B. atrox*, (LAING et al., 2004). No modelo murino, a *B. brazili* peruano exibiu dose hemorrágica mínima (MHD) de 7,40 µg/camundongo), dose dermonecrótica mínima (MND) de 152,15 µg/camundongo,

dose mínima de coagulante contra plasma (MCD-P) e fibrinogênio (MCD- F) de 19,20 e 1020,0 μg/mL, respectivamente, e dose mínima de desfibrinogenação (MDD) de 7,0 μg/camundongo (LAING et al., 2004). Poucos estudos foram relatados sobre o arsenal de toxinas do veneno d e *B. brazili*, e estes foram focados principalmente nos efeitos farmacológicos e possíveis aplicações biotecnológicas de toxinas isoladas. (CALDERON et al., 2014; SANZ et al., 2020).

1.3.2 *Bothrops jararaca*

A serpente *B. jaracaca* conhecida popularmente como jararaca da mata, é uma serpente de grande porte pode chagar a medir até 1,6 m. Possui corpo marrom com manchas triangulares escuras e região ao redor da boca com escamas de cor ocre uniforme, peculiaridades que propicia uma excelente camuflagem (Figura 5), esta serpente é altamente agressiva, sendo uma das principais causadoras de acidentes ofídicos no Brasil, esta serpente produz entre 25 a 300 mg de veneno altamente tóxico com ação proteolítica determinado pela alta concentração de metaloproteases no veneno, é estimada a dose letal média (DL50) para camundongos é de 1,4 mg/kg intraperitoneal (1,2 mg/kg intravenoso, 3 mg/kg subcutâneo) (GONÇALVES-MACHADO et al., 2016)

Figura 5. *Bothrops jararaca*. Espécimen camuflado em folhas secas. Fonte http://reptile-database.reptarium.cz/species?genus=Bothrops&species=jararaca

B. jararaca se encontra amplamente distribuída no Brasil, principalmente nas regiões sul e sudeste (Bahia, Espirito Santo, Rio de Janeiro, Minas Gerais, São Paulo, Parana, Santa Catarina, Rio Grande do Sul, E Mato Grosso), chegando a ser encontrada também em Paraguay e Argentina (Misiones) (GONÇALVES-MACHADO et al., 2016)

1.3.3 Metaloproteases

As SVMPs da Família Viperidae são enzimas proteolíticas dependentes de metais, como o zinco. Causam ruptura da membrana basal vascular, através da degradação das proteínas da matriz extracelular resultando em hemorragia típica (ASSAKURA et al., 2003).

As SVMPs variam em tamanho de 20 a 100 kDa e são classificadas em três grupos, P-I, P-II e P-III, de acordo com seus domínios (Figura 6). As SVMPs de classe P-I é a menor estruturalmente, com apenas o domínio M (catalítico); as SVMPs da classe P-II contém um domínio M e um domínio D (desintegrina), as SVMPs classe P-III além dos domínios M e D, contém um domínio C (rico em cisteína) que está localizado no lado carboxílico do domínio D. Além disso, as SVMPs classe P-III foram divididas em subclasses com base em suas modificações pós-traducionais, se estão sujeitas a dimerização (P-IIIc) ou processamento proteolítico (P-IIIb) (OYAMA; TAKAHASHI, 2017).

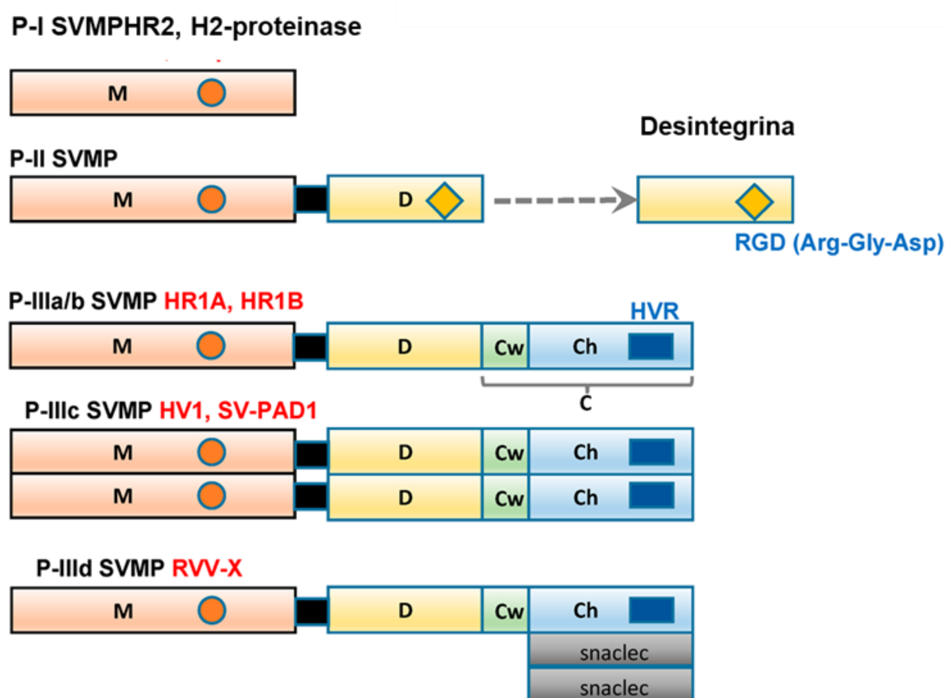


Figura 6. Diagrama esquemático das estruturas de domínio de SVMP P-I, P-II e P-III. Cada domínio ou subdomínio é representado por uma cor diferente. Domínio M, de metaloprotease (laranja), domínio D, da desintegrina (amarelo), domínio C rico em cisteína, o subdomínio Cw *"wrist"* rico em cisteína (verde claro), subdomínio Ch *"hand"* rico em cisteína (azul claro), região hipervariável HVR (azul escuro), domínio semelhante à lectinas de tipo C "snaclec" (cinza). Fonte: (OYAMA; TAKAHASHI, 2017).

Entre as SVMPs descritas no veneno de *B. jararaca*, se encontra descrita a bothropasina (EC 3.4.24.49) é uma SVMP classe III (PIII) dependente de zinco, de 48 kDa, (Figura 7) (Mandelbaum et al., 1982). Esta proteína pertence às adamalisinas ou à subfamília M12B (banco de dados MEROPS) das metaloproteases (Rawlings et al., 2016; Silva et al., 2017). A estrutura cristalográfica da bothropasina foi determinada (PDB 3DLS) e foi possível a identificação dos três domínios: (i) domínio metaloprotease, (ii) domínio semelhante à desintegrina e (iii) domínio rico em cisteína (MUNIZ et al., 2008). Essa enzima é responsável por causar hemorragia na pele de coelho com uma dose mínima hemorrágica (MHD) de 1 μg, (ASSAKURA et al., 2003).

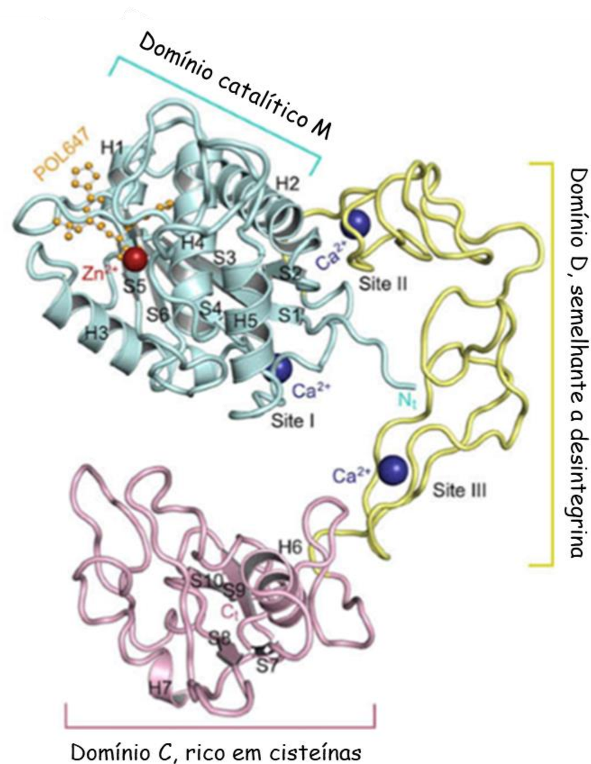


Figura 7. Modelo estrutural da bothropasina. O domínio catalítico (domínio M) em azul claro; o domínio semelhante à desintegrina (domínio D) em amarelo e o domínio rico em cisteína (domínio C) em rosa claro. Fonte: (MUNIZ et al., 2008).

1.3.4 Serino proteases

As Serino proteases presentes nos venenos de serpentes são enzimas glicoproteicas, que possuem uma região de sítio catalítico bem conservada (Ser195, His57 e Asp102) (SERRANO; MAROUN, 2005), estão agrupadas em seis clãs principais e subdivididos em famílias com base na sequência e semelhanças funcionais (classificação MEROPS, http://merops.sanger.ac.uk) (RAWLINGS; TOLLE; BARRETT, 2004).

Estas enzimas catalisam a clivagem de ligações peptídicas covalentes em proteínas e desempenham papéis importantes em diversos processos biológicos que vão desde a digestão até o controle e regulação da coagulação do sangue, o sistema imunológico e a inflamação(KANG et al., 2011). As Serino proteases são encontradas

em diversas isoformas e alterações estruturais podem influenciar sua atividade, com massa molecular entre 26 e 67 kDa, dependendo da quantidade de glicosilações presentes (SERRANO, 2013).

Essas enzimas interferem na regulação e no controle das principais reações biológicas da cascata de coagulação do sangue, do sistema fibrinolítico e da ativação das plaquetas sanguíneas (LU; CLEMETSON; CLEMETSON, 2005), sendo frequentemente associadas a distúrbios hemostáticos. Com base em seus papéis biológicos, eles foram classificados como ativadores do sistema fibrinolítico, pró-coagulante, anticoagulante e enzimas agregadoras de plaquetas (MARSH; WILLIAMS, 2005)

As Serino proteases possuem diferentes formas de ação. As do tipo thrombin-like por exemplo clivam o fibrinogênio no mesmo local que a trombina, levando a uma diminuição do fibrinogênio de forma rápida e a um efeito anticoagulante (KANG et al., 2011). Algumas Serino proteases thrombin-like podem clivar duas cadeias do fibrinogênio (Aα e Bβ), mas a maioria atua apenas sobre uma (Aα ou Bβ) (CHERNYSHENKO et al., 2014). Já as Serino proteases do tipo Kallikrein-like levam a uma liberação de bradicinina e a degradação de angiotensina, causando uma diminuição da pressão sanguínea (SANT'ANA et al., 2008).

1.3.5 fosfolipases (PLA_{2s})

As PLA_{2s} (fosfatídeo 2-acil-hidrolase, EC 3.1.14) representam uma superfamília de enzimas lipolíticas que catalisar especificamente a hidrólise da ligação éster na posição sn-2 dos glicerofosfolipídios resultantes na geração de ácido graxo (araquidonato) e lisofosfolipídios (KANG et al., 2011). As fosfolipases A_2 são enzimas de 14 a 18 kDa que hidrolisam os fosfolipídios de membrana, gerando mediadores

envolvidos nos processos inflamatórios locais e sistêmicos (CHISARI et al., 1998), e são encontradas principalmente em venenos de serpentes, abelha, escorpião ou vespa (KANG et al., 2011; KINI, 2006; SANTOS-FILHO et al., 2008). Normalmente contêm cinco a oito ligações dissulfeto e, para funcionar, essas proteínas precisam da disponibilidade do íon Ca^{2+} para a hidrólise dos fosfolipídios (KANG et al., 2011).

Existem diferentes grupos de PLA_2, de forma que essas enzimas são encontradas tanto em células e fluidos biológicos quanto em venenos. O veneno de Bothrops possui PLA_2 estruturalmente semelhantes àquelas presentes nos mamíferos (MOREIRA, 2007). Vários efeitos fisiológicos tem sido relacionados a ação dessas enzimas, como a hemorragia interna, miotoxicidade, neurotoxicidade, hipotensão, atividade anticoagulante, antiplaquetária, inflamatória e edematogênica (KINI, 2006; TAN; SAIFUDDIN; YONG, 1991; TEIXEIRA et al., 2003).

1.3.6 L-aminoácido oxidase(LAAO)

A L-aminoácido oxidase (LAAO, EC1.4.3.2) é uma flavoenzima que catalisa a desaminação oxidativa estereoespecífica de l-aminoácidos para dar o a-cetoácido correspondente, são encontradas em vários organismos e em venenos de serpentes (SOUSA et al., 2013; YANG et al., 2011), a enzima do veneno de serpente exibe uma preferência por aminoácidos aromáticos e hidrofóbicos, como fenilalanina e leucina (KANG et al., 2011).

Estudos mostraram que a enzima passa por um complexo ternário de enzima, substrato e oxigênio e que a redução da flavina envolve a formação de uma semiquinona (RIBEIRO et al., 2016). Como a proteína é uma flavoenzima oxidase, o cofator FAD reduzido reoxidado com dioxigênio durante a meia reação redutiva, resultando na formação de peróxido de hidrogênio.

Várias atividades biológicas da LAAO relacionadas com o H_2O_2 produzido durante a reação catalisada por esta enzima. Entre elas estão a indução de apoptose (SUHR; KIM, 1999), inibição e indução de agregação plaquetária (STÁBELI et al., 2004) atividade anticoagulante (SAKURAI et al., 2003) atividade anti-viral, anti-parasitária e hemolítica (TEMPONE et al., 2001; ZHANG et al., 2003).

Outras proteínas como as Snaclecs, que atuam sobre a função plaquetária (BRINKHOUS et al., 1983), as fosfodiesterases, capazes de atacar os ácidos nucleicos (MAMILLAPALLI et al., 1998), as fosfolipases B, conhecidas por atuar na lise de eritrócitos e causar citotoxicidade em células musculares (BERNHEIMER et al., 1987), e as CRISPs que podem se ligar a canais de cátion não específicos dependentes de nucleotídeos cíclicos (CNG) (BROWN et al., 2003), também são encontradas nos venenos de serpentes e contribuem com os sintomas do envenenamento

1.4 Gênero *Crotalus*

Este é o gênero das cascavéis, habitam os campos abertos, regiões secas e pedregosas e também os pastos, as serpentes deste gênero chegam a atingir, na fase adulta até 1,60m de comprimento. Sua característica mais importante é a presença de um guizo, ou chocalho, na ponta da cauda.

As serpentes do gênero *Crotalus* estão agrupadas dentro da Família Viperidae, e compreendem aproximadamente 70 espécies. Estas serpentes são facilmente reconhecidas pela presença de um "chocalho" na ponta da cauda, usado como um dispositivo de alerta quando ameaçada (Figura 8). São venenosas e amplamente distribuídas descontinuamente do Sul do Canadá ao Norte da Argentina (CALVETE et al., 2010a).

Figura 8. ***Crotalus durrisus terrificus.*** Comumente conhecida como cascavel. Fonte: https://www.researchgate.net/figure/Crotalus-durissus-terrificus-rattlesnake_fig2_250043694

Na América do Sul a subespécie *Crotalus durrisus terrificus* está amplamente distribuída desde o Nordeste do Brasil até o Norte da Argentina e Uruguai (PINHO; PEREIRA, 2001). Possui um veneno notório por sua capacidade de dissociar a transmissão neuromuscular da presa. A neurotoxicidade e miotoxicidade sistêmica é frequentemente acompanhada de rabdomiólise causando necrose tubular aguda e insuficiência renal que representa a principal causa de morte por serpentes *Crotalus durissus ssp*. Estes efeitos são atribuídas principalmente à alta concentração de crotoxina, uma molécula de PLA_2 heterodimérica exibindo β-neurotoxicidade pré-sináptica (SAMPAIO et al., 2010). A crotoxina normalmente é representa 70–90% das toxinas do veneno de *C. durissus ssp* (CALVETE et al., 2010b), os venenos também contêm quantidades variáveis de crotamina, uma toxina mionecrótica que atua nos canais de Na + sensíveis à voltagem (BOLDRINI-FRANÇA et al., 2010; OGUIURA, N. BONI-MITAKE; RADIS, 2005).

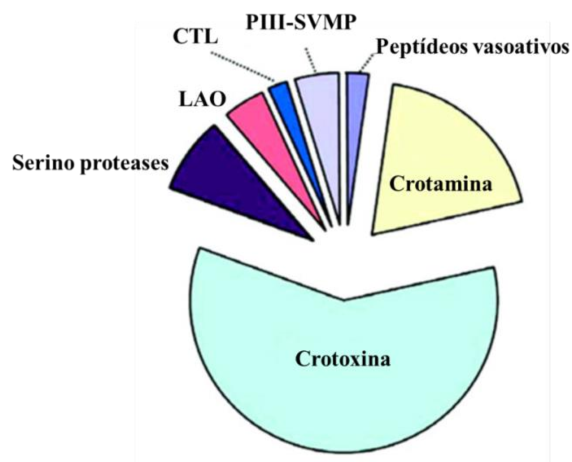


Figura 9. Composição proteica do veneno da *Crotalus durisus terrificus*. Crotoxina e crotamina são os principais componentes tóxicos do veneno, que juntos compõem aproximadamente 80% do veneno total. Fonte: (CALVETE et al., 2010a).

1.4.1 Crotoxina

A crotoxina (Ctx) é a principal toxina do veneno de *C. d. terrificus*. É uma proteína heterodimérica, não covalente, formada por duas subunidades: uma subunidade ácida (CA ou crotapotina) não tóxica, e uma subunidade básica (CB) tóxica com atividade de fosfolipase A_2 que depende da subunidade CA para ancorar-se na membrana celular (Figura 10). O efeito letal da Ctx é relacionado com sua ação nas junções neuromusculares por inibição da liberação de acetilcolina (LOMEO et al., 2014).

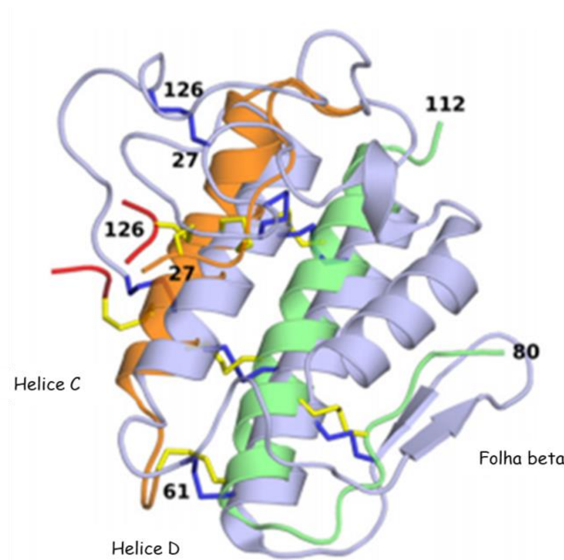


Figura 10. Modelo estrutural da crotoxina. Superposição da subunidade ácida de CA2 da crotoxina com a subunidade CBb mostrada em azul. Os três polipeptídeos são ligados por pontes dissulfeto. Cadeias (α, β e γ) de CA2 mostradas em laranja, verde e vermelho, respectivamente e ligações dissulfeto que ligam as três cadeias de CA2 são mostradas em amarelo. As ligações dissulfeto equivalentes a subunidade CBb são mostradas em azul. Fonte: (FAURE; XU; SAUL, 2011).

Em *C. d. terrificus,* 16 isoformas diferente de Ctx foram identificadas, resultantes da associação aleatória de várias isoformas das subunidades CA e CB. Quatro isoformas CA (CA1, CA2, CA3 e CA4) e quatro isoformas CB (CBa2, CBb, CBc e CBd). A combinação dessas isoformas leva a diferentes complexos de crotoxina que têm propriedades farmacológicas e biológicas significativamente diferentes. A crotoxina é grupada em duas classes de complexos de crotoxina: classe I (contendo as isoformas CBb, CBc, CBd e CA) que é altamente tóxica (DL_{50} de 93 ± 20 µg/kg injetável em camundongo) e enzimaticamente menos ativa, e classe II (contendo as isoformas CBa2 e CA), menos tóxica (DL_{50} de 435 ± 65 µg/kg) e enzimaticamente mais ativa (FAURE; XU; SAUL, 2011).

1.4.2 crotamina

Crotamina (Crt), um dos componentes do veneno de *Crotalus durisus ssp*. A estrutura desta proteína foi determinada por espectroscopia de RMN de prótons, é uma

proteína monomérica de 42 aminoácidos com três pontes dissulfeto (LAURE, 1975). A estrutura secundária compreende uma curta hélice alfa N-terminal e uma pequena folha beta antiparalela de cadeia tripla arranjada na topologia αβ1β2β3 (NICASTRO et al., 2003). Esta proteína está incluída em uma família de pequenas miotoxinas do veneno de cascavel com alta identidade em sua sequência de aminoácidos (MATAVEL et al., 1998; NICASTRO et al., 2003). É caracterizada por toxicidade leve, comparada a outros membros da mesma família, sendo capaz de modificar especificamente o canal de Na^+ voltagem-sensível, sugerindo sua atividade analgésica e mionecrótica (NICASTRO et al., 2003).

A crotamina é uma pequena proteína rica em aminoácidos básicos, uma toxina não enzimática que causa paralisia e espasmos musculares no músculo esquelético dos membros posteriores de camundongos, reduzindo o potencial de membrana em repouso, por interferência com o funcionamento dos canais de sódio sensíveis à voltagem (CORONADO et al., 2012; OGUIURA et al., 2005).

A crotamina apresenta vários níveis de polimorfismo no Crotalus do Brasil, relacionados à distribuição geográfica desse tipo de serpente, o que nos leva a encontrar várias crotaminas identificadas no banco de dados de proteínas (OGUIURA et al., 2005).

1.5 Identificação proteômica dos componentes dos venenos

Desde a antiguidade, substâncias naturais vêm sendo utilizadas como agentes terapêuticos e, apesar de possuírem efeitos tóxicos, os venenos tem muito a contribuir neste aspecto. Um exemplo disto é a utilização de componentes do veneno na pesquisa e tratamento da trombose (HUANG; HSU; KUO, 2016) e até mesmo câncer (BRIGATTE et al., 2016). Somente através do estudo dos venenos é possível conhecer a patologia

associada ao envenenamento e buscar a melhor linha terapêutica a ser empregada. Pesquisas sobre a composição dos venenos são, portanto, fundamentais, não somente pelo seu potencial na biotecnologia/farmacologia, como também para o desenvolvimento de soros mais efetivos e seguros na neutralização das toxinas da serpente.

O entendimento dos venenos nos permite conhecer o mecanismo de ação do mesmo associado à patologia do envenenamento e direcionar a linha terapêutica. Estudar a composição dos venenos é fundamental, não somente pelo seu potencial na biotecnologia/farmacologia, como também para o desenvolvimento de soros mais efetivos e seguros na neutralização das toxinas da serpente.

O modo tradicional de estudo de venenos consistia no isolamento e caracterização bioquímica das proteínas, principalmente as mais abundantes e que são maior relevância medica. Atualmente os avanços na tecnologia transcriptômica combinados com métodos proteômicos, como cromatografia líquida de alta eficiência em fase reversa (RP-HPLC) e espectrometria de massa (MS), permitem uma rápida identificação de diferentes toxinas em venenos de serpentes, bem como a capacidade de medir rapidamente sua abundância relativa, o que permite uma melhor compreensão das relações evolutivas das serpentes (filogenia) e da diversidade de componentes tóxicos presentes em venenos de serpentes medicamente importantes, comumente encontradas na América do Sul (TASOULIS AND ISBISTER, 2017). A composição do veneno varia entre as serpentes de todos os níveis taxonômicos e é influenciada pela idade, habitat, dieta e dimorfismo sexual (AMORIM et al., 2018). Desta forma, o estudo dos componentes dos venenos é um recurso importante para identificar alternativas que atendam a demanda de soros antiofídicos específica de cada região.

1.6 Predição de epítopos

Os epítopos são sítios específicos dos antígenos, que são reconhecidos pelo sistema imunológico, que se ligam à receptores na superfície de linfócitos, levando a produção de anticorpos capazes de reconhecer estes antígenos. Quando ocorre a imunização com uma proteína, a maioria dos linfócitos são específicos para algumas sequências de aminoácidos, denominadas epítopos imunodominantes (AKRAM; INMAN, 2012). Em alguns casos, a formação de epítopos em uma proteína depende somente da sequência de aminoácidos, sendo denominados epítopos lineares ou contínuos. Em outros casos, a formação do epítopo depende da estrutura tridimensional devido à justaposição de aminoácidos, e são denominados epítopos conformacionais ou descontínuos (GERSHONI et al., 2007).

1.7 Epítopos de proteínas para linfócitos B

Peptídeos realizam diversos papéis na imunologia, sendo o de direcionar a resposta imune adaptativa como o epítopos, o mais relevante. Os epítopos peptídicos executam suas funções primárias via interações com o complexo de histocompatibilidade, no caso de epítopos de células-T, ou via anticorpos, no caso de epítopos de células-B (ALVARENGA et al., 2010; HARRISON et al., 2011; MENDES et al., 2013), o epítopo para células-B de uma proteína é uma porção desta proteína antigênica reconhecida por uma molécula de anticorpo, o qual resulta na ativação da resposta imune (REGENMORTEL, 2001).

A maioria dos epítopos presente em uma proteína é descontínuo ou conformacionais e são identificados por técnicas laboriosas de cristalografia de raios X do complexo antígeno-anticorpo. Devido às limitações na identificação e síntese dos epítopos descontínuos, os epítopos sintéticos contínuos ou lineares são usados no estudo

das propriedades imunológicas das proteínas, diagnóstico de doenças e como candidatos para vacinas sintéticas (REGENMORTEL, 2001).

Os epítopos são interessantes ao ser utilizados como ferramenta, já que podem ser reproduzido através de peptídeos sintéticos usando tecnologias, como síntese de peptídeos e *Phage-Display*. Estas moléculas podem ser utilizadas para imunização de animais gerando anticorpos, úteis para o estudo de vacinas, biossensores, métodos imuno-terapêuticos e moléculas para diagnóstico.

Nos epítopos, alguns aminoácidos são considerados mais importantes, uma vez que suas cadeias laterais interagem com o anticorpo é por isso são denominados aminoácidos críticos ou de contato. Quando um deles é substituído, as características do epítopo são alteradas podendo impedir sua ligação ao anticorpo (ANDERSEN; NIELSEN; LUND, 2006). Identificar estes aminoácidos críticos no epítopo é importante ao desenhar os peptídeos e proteínas voltadas para a produção de anticorpos (TRIER; HANSEN; HOUEN, 2012).

Os epítopos contínuos ou lineares, em sua maioria, são descritos por conterem de 4 a 5 resíduos de aminoácidos que são importantes na ligação antígeno-anticorpo, o que define a especificidade da interação com o anticorpo e os outros aminoácidos que formam a região epitópica (GEYSEN; MASON; RODDA, 1988).

Assim, a identificação de epítopos é uma parte importante na busca de imunógenos alternativos para produção do antiveneno, visto que algumas dessas biomoléculas podem ser desprovidas de toxicidade. O presente trabalho propõe a identificação de epítopos lineares dos principais componentes tóxicos presentes no veneno de serpentes brasileiras, assim como a produção de anticorpos policlonais capazes de neutralizar as atividades tóxicas das toxinas estudadas.

2. OBJETIVOS

Identificar e caracterizar epítopos lineares das principais toxinas dos venenos das serpentes da família Viperidae (*Crotalus spp* e *Bothrops spp)* e caracterização proteômica e toxicológica do veneno de *Bothrops brazili*.

2.1 Objetivos específicos

- Mapear, usando o método de *Spot synthesis*, epítopos para células B das toxinas selecionadas dos venenos das serpentes *Crotalus spp.* e *Bothrops spp;*
- Sintetizar os peptídeos correspondentes aos epítopos detectados;
- Avaliar a capacidade antigênica/imunogênica dos peptídeos sintetizados;
- Produzir soros específicos correspondentes aos epítopos identificados;
- Avaliar o potencial de neutralização de anticorpos policlonais anti-peptídeo;
- Mapear, usando o método de implantes subcutâneos, epítopos para células B da crotoxina do veneno da serpente *Crotalus durissus terrificus;*
- Produzir uma proteína recombinante multiepitópica, a partir das sequências dos epítopos identificados;
- Testar o potencial de neutralização dos anticorpos gerados pela imunização de coelhos com a proteína multiepitópica.
- Avaliar o perfil proteômico do veneno da serpente *Bothrops brazili*

3. RESULTADOS

Neste capítulo são apresentados os três artigos, os quais abordam diferentes análises de mapeamento de epítopos lineares de toxinas de venenos de serpentes, entre as que se encontram metaloproteases de classe P-III e a crotoxina, (artigos 1 e 2), assim como a análise proteômica do veneno de *B. brazili* (artigo 3).

3.1 Artigo 1. Identification of a linear B-cell epitope in the catalytic domain of bothropasin, metalloproteinase from *Bothrops jararaca* snake venom

(2018) Denis A. Molina Molina, Clara Guerra-Duarte, Dayane L. Naves de Souza, Fernanda Costal-Oliveira, Giovana Reis de Ávila, Vanete T. Soccol, Ricardo A. Machado-de-Ávila, Carlos Chávez-Olórtegui.

A serpente *Bothrops jararaca* é de importância médica pelo grande número de acidentes que causa, mas o antiveneno disponível não apresenta uma proteção satisfatória dos efeitos locais, o que leva a necessidade de melhorar os antivenenos. Este primeiro artigo descreve a produção de anticorpos capazes de neutralizar a bothropasina uma metaloprotease hemorrágica de serpentes (SVMPs).

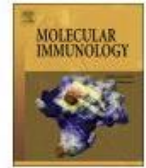
Bothropasina é uma PIII-SVMP de 48 kDa que contém 600 aminoácidos formando uma estrutura de multidomínios: peptídeo sinal seguido do pro peptídeo, metaloprotease, desintegrina e domínio rico em cisteína. As diferenças nos domínios do tipo desintegrina ou rico em cisteína podem estar relacionados com ligação a molécula alvo, que por sua vez pode gerar diversidade ou especificidade de substrato para o domínio catalítico. Esta toxina apresenta um alto grau de similaridade dentro do grupo das PIII-SVMP, além de possuir diversas atividades biológicas dentre as quais destaca-se a atividade hemorrágica e mionecrótica.

Molecular Immunology 104 (2018) 20–26

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm



Identification of a linear B-cell epitope in the catalytic domain of bothropasin, a metalloproteinase from *Bothrops jararaca* snake venom

Denis A. Molina Molina[a], Clara Guerra-Duarte[b], Dayane L. Naves de Souza[a], Fernanda Costal-Oliveira[a], Giovana Reis de Ávila[a], Vanete T. Soccol[c], Ricardo A. Machado-de-Ávila[d], Carlos Chávez-Olórtegui[a,*]

[a] Departamento de Bioquímica-Imunologia, ICB, Universidade Federal de Minas Gerais, Av. Antônio Carlos 6627, CEP: 31270-901 Belo Horizonte, Minas Gerais, Brazil
[b] Centro de Pesquisa e Desenvolvimento, Fundação Ezequiel Dias, 30510-010, Belo Horizonte, MG, Brazil
[c] Universidade Federal do Paraná (UFPR), Curitiba, PR, Brazil
[d] Laboratório de Fisiopatologia Experimental, Programa de Pós-Graduação em Ciências da Saúde, Universidade do Extremo Sul Catarinense (UNESC), Brazil

ARTICLE INFO

Keywords:
Linear B-cell epitope mapping
Snake venom metalloproteinases
Peptide synthesis
Hemorrhage

ABSTRACT

Bothropasin is a hemorrhagic snake venom metalloproteinase (SVMP) from *Bothrops jararaca* venom, the snake responsible for most bites in Southeastern Brazil. SVMPs, such as bothropasin, are involved in the main bothropic envenoming symptoms, which include hemorrhage, inflammation, necrosis and blood coagulation deficiency. B-cell epitope mapping of SVMPs can lead to the identification of peptides capable of inducing neutralizing antibodies without causing toxic effects, therefore improving anti-venom production. Here, using the SPOT synthesis technique, we have identified an epitope located in the catalytic domain of bothropasin (^{202}KARMYELANIVNEILRYLYMH^{222}) which was synthesized and named BotEp1. The peptide was used to immunize Swiss mice and Anti-BotEp1 serum cross-reacted with bothropasin and crude venoms from *B. jararaca* and *B. atrox* venoms. Furthermore, Anti-BotEp1 antibodies were able to completely neutralize the hemorrhagic activity of a chromatographic fraction from *B. jararaca* venom, which contains hemorrhagic SVMPs. In addition, the coagulation activity of the hemorrhagic fraction showed to be diminished when tested in serum from rabbit immunized with BotEp1 (compared to serum from non-immunized animal). Our results show the identification of neutralizing epitopes in bothropasin and provide basis for the use of synthetic peptides to improve the production of immunotherapeutics.

1. Introduction

Snakes from *Bothrops* genus are responsible for approximately 80% of snakebites yearly in Brazil (Ministério da Saúde, 2009). Accidents involving *B. jararaca* are the main cause of bothropic envenoming in the most populated regions of southeastern Brazil and is mainly found in Southern Bahia, Espírito Santo, Rio de Janeiro, Minas Gerais, São Paulo, Paraná, Santa Catarina and Rio Grande do Sul (Estevao-Costa et al., 2016). Envenoming by *Bothrops* snakes is characterized by severe inflammatory reactions with complex tissue damage, e.g. drastic hemostatic disturbances, hemorrhage, edema and myonecrosis, extending from the bite site (Warrell, 2004).

Snake Venom Metalloproteinases (SVMPs) toxins are abundantly found within bothropic venoms and directly related to the hydrolysis of basal membrane proteins from micro vessels, leading to hemorrhage, the most remarkable effect of bothropic envenoming (Freitas-de-Sousa et al., 2017). Furthermore, these toxins present other biological activities such disruption of hemostasis mediated by procoagulant or anticoagulant effects, platelet aggregation, apoptotic and pro-inflammatory activities (Kang et al., 2011).

Bothropasin (EC 3.4.24.49) is a zinc dependent class III (PIII) SVMP of 48 kDa isolated from *B. jararaca* venom (Mandelbaum et al., 1982). This protein belongs to the adamalysins or the M12B subfamily (MEROPS database) of metalloproteinases (Rawlings et al., 2016; Silva et al., 2017). The crystallographic structure of bothropasin (PDB 3DLS) revealed three domains: (i) metalloproteinase domain, (ii) disintegrin-like domain and (iii) cysteine-rich domain (Muniz et al., 2008). Bothropasin shares approximately 95% of identity with jararhagin (EC 3.4.24.73), a P-III SVMP from *B. jararaca* venom (Oliveira et al., 2010).

Monoclonal and polyclonal antibodies against several SVMPs have

* Corresponding author at: Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais (UFMG), Avenida Antônio Carlos, 6627, Belo Horizonte, CEP: 31270-901, Brazil.
E-mail address: olortegi@icb.ufmg.br (C. Chávez-Olórtegui).

https://doi.org/10.1016/j.molimm.2018.10.019
Received 31 August 2018; Received in revised form 16 October 2018; Accepted 23 October 2018
0161-5890/ © 2018 Published by Elsevier Ltd.

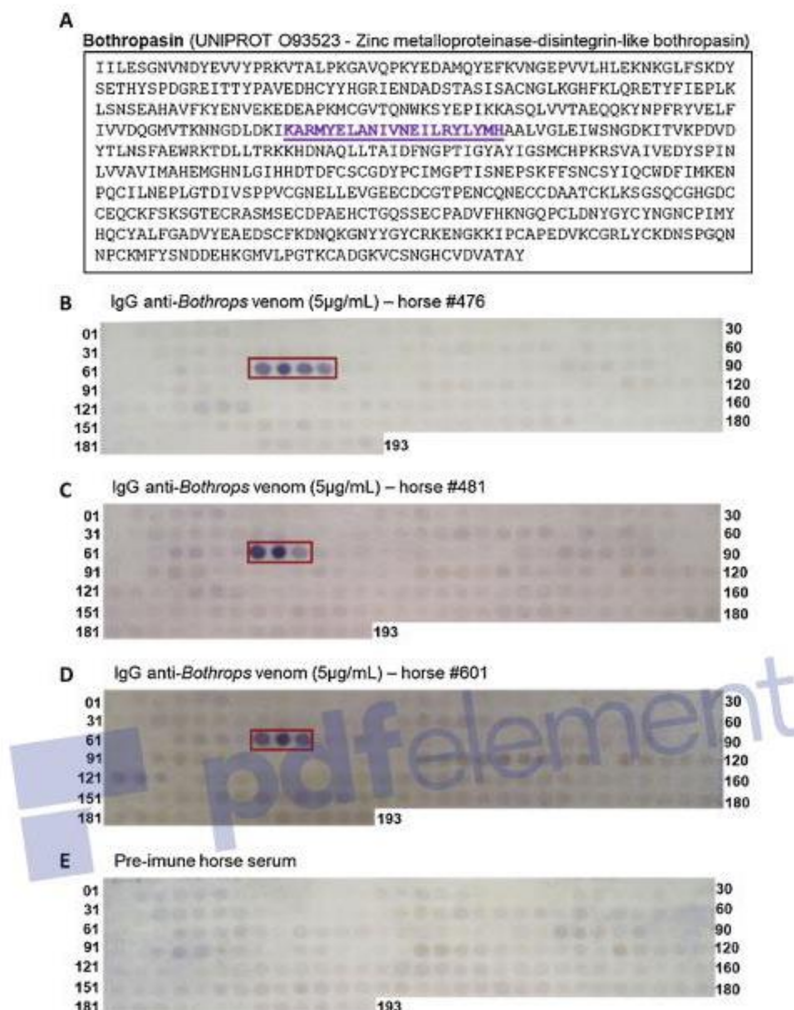


Fig. 1. Identification of linear B-cell epitopes of Bothropasin. (A) Primary sequence of Bothropasin. The identified epitope BotEp1 is highlighted in purple. Membrane containing 193 overlapping peptides frame-shifted by three residues covering the complete sequence of Bothropasin. The membrane was probed with different horse anti-Bothrops venom IgG (5 µg/mL). (B) 476, (C) 481, (D) 601 e (E) pre-immune horse serum (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

pdfelement

been produced (Fernandes et al., 2010; Ferreira et al., 2006; Machado De Avila et al., 2011; Morine et al., 2008; Schneider et al., 2016), presenting cross-immunorecognition and neutralizing the hemorrhagic effect of several Bothrops crude venoms.

These findings suggest that immunoanalytical studies of SVMPs may have enormous potential for the improvement of antivenom production for passive immunotherapy. Currently, antivenom immunotherapy is the only effective treatment for snakebites accidents recommended by World Health Organization (2016) and has been used since the earlier 20th century (Stephano et al., 2005). Antivenoms are obtained by hyper immunization of animals (horse, sheep, donkey, goat or rabbit) (WHO, 2016) with venom from a single or a pool of venoms from distinct species of snakes found in a specific country/region. Despite its widespread use, this therapy still needs to be improved, mainly in terms of its efficiency against the multiple local effects (Espino-Solis et al., 2009; Harrison et al., 2011).

The identification of neutralizing epitopes B-cell epitopes might help the design and production of non-toxic immunogens that can improve the production of antibothropic therapeutic sera (Felicori et al., 2009; Mendes et al., 2013). Thus, in this work, we used the SPOT-synthesis technique for a systematic mapping of continuous B-cell epitopes of bothropasin using antibothropic antivenoms.

2. Material and methods

2.1. *Animals, venoms, and antivenoms*

Swiss mice (18–22 g) were obtained and maintained at Centro de Bioterismo of Instituto de Ciências Biológicas of Universidade Federal de Minas Gerais (UFMG). All animals received food and water *ad libitum*, under controlled environmental conditions. Experimental protocols were performed after approval by the Ethics Committee in Animal Experimentation of UFMG (445/2015-CETEA/UFMG).

B. jararaca and *Bothrops atrox* venoms samples and horse anti-*B.*

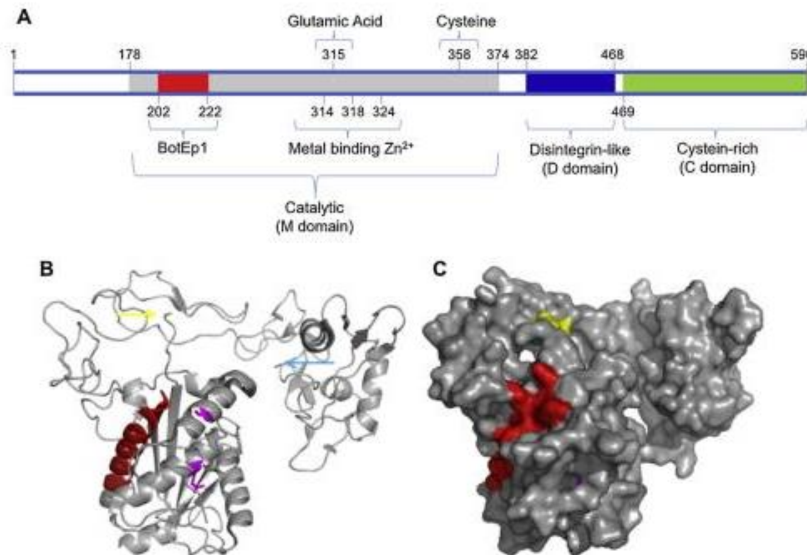


Fig. 2. Bothropasin structure showing the location of BotEp1. (A) Bothropasin primary sequence schema showing the position of the three domains and BotEp1 in red, M domain in gray, D domain in blue and C domain in green. (B) Visualization of BotEp1 on the tridimensional structure of Bothropasin using Pymol. In magenta, glutamic acid (315) and cysteine (358), forming the catalytic site, in red the sequence of amino acids corresponding to BotEp1, in yellow the N-terminal and in blue the C-terminal. (C) shows the surface configuration where the amino acids of BotEp1 are marked in red, indicating that they are exposed on the surface of the protein (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

SPECIE	UNIPROT	TOXIN	SEQUENCE	
B.jararaca	093523	Bothropasin	^{222}KARMYELANIVNEILRYLYMH^{242}	
B.jararaca	P30431	Jararhagin	^{183}KARMYELANIVNEIFRYLYMH^{203}	PIII
B.insularis	Q8QG88	BITM06A	^{222}KARMYELANIVNEILRYLYMH^{242}	PIII
B.atrox	A0A0K2JNB8	Batroxrhagin	^{222}KARMYELANIVNEIFRYLYMH^{242}	PIII
C.durissus	Q2QA02	ZMD-crotalus	^{222}KTRMYELANTVNEIYRYMYIH^{242}	PIII
B.jararaca	Q98UF9	HF3	^{223}RTKIYEIVNILNEIFRYLYIR^{243}	PIII
B.insularis	Q5XUW8	Insularinase-A	^{222}RTRVHEMVNTLNGFFRSVNVD^{242}	PII
B.asper	Q072L5	SVMP-asper	^{222}RTRVHEMLNTVNGFYRSVNVT^{242}	PII
B.lateralis	U5PZ28	BlatH1	^{223}RIKIYEIVNILNEMFRYLYIR^{243}	PII
B.asper	P83512	BaP1	^{222}RTRVHEMLNTVNGFYRSVDVH^{242}	PI
B.leucurus	P84907	Leucurolysin-a	^{30}RKWVHEMLNTVNGFFRSMNVD^{50}	PI
B.atrox	P85420	Atroxlysin-I	^{30}RRRIHQMVNIMKEAYSTMYID^{50}	PI
			: :::: * :: : :	

Fig. 3. Alignment of BotEp1 with several SVMPs. The region comprising BotEp1 of bothropasin was aligned with several PI, PII and PIII SVMPs. Positively charged amino acids are highlighted in blue and negatively charged amino acids are highlighted in red (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

jararaca antivenom were kindly provided by Dr. Eladio Flores from Fundação Ezequiel Dias-FUNED (Belo Horizonte, Brazil).

The chromatographic fraction from *B. jararaca* venom, which contained P-III Metalloproteinase (P2 fraction), was obtained by size exclusion chromatography using Superdex™ 75 HR10/30 column operated by a Fast Performance Liquid Chromatography (FPLC) system as described by Ciscotto et al. (2009). Out of the six fractions obtained, the P2 was the most hemorrhagic fraction.

2.2. Identification of B-cell epitopes in bothropasin

A set of 193 overlapping pentadecapeptides frameshifted by 3 residues covering the primary sequence of bothropasin (UNIPROT 093523) was synthesized on cellulose membrane, using the SPOT synthesis technique as previously described (Laune et al., 2002), by a ResPep (Intavis) robot. For the immunodetection assays, the membrane was blocked overnight [PBS-bovine serum albumin (3%), sucrose (5%)]. Then, the membrane was probed with anti-*B. jararaca* horse serum (5 μg/mL). Antibody binding was detected using goat anti-horse IgG conjugated to alkaline–phosphatase (Sigma, 1:5000) for 90 min, at 37 °C. After washing, 5-bromo-4-chloro-3-indolyl phosphate (Sigma) and 3-(4,5- dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (Sigma) were added as substrate and a blue precipitate was formed on reactive peptides. After photographing the probed membrane, it was regenerated using dimethylformamide, then 1% SDS, 0.1% β-mercaptoethanol in 8 M urea, followed by ethanol/water/acetic acid (50:40:10 vol/vol/vol) and ethanol. As a negative control, pre-immune horse serum was used.

The primary structure of the epitope mapped on bothropasin was compared with sequences from other SVMPs. Searching was performed using BLASTP 2.6.1 + (Altschul et al., 1997), against the UniProtKB/SwissProt database and Clustal omega (1.2.4) (Altschul et al., 2005) was used for sequence alignment. The location of the identified epitope was analyzed in the three-dimensional crystal structure of bothropasin, available in PDB (3DSL), and visualized with Pymol (Molecular Graphics System, Version 2.0, Schrödinger, LLC)

2.3. Soluble peptide synthesis

Based on the results analysis of cellulose-bound peptides assay, the following sequence was identified as a putative epitope and was chosen to be synthesized in the soluble form:

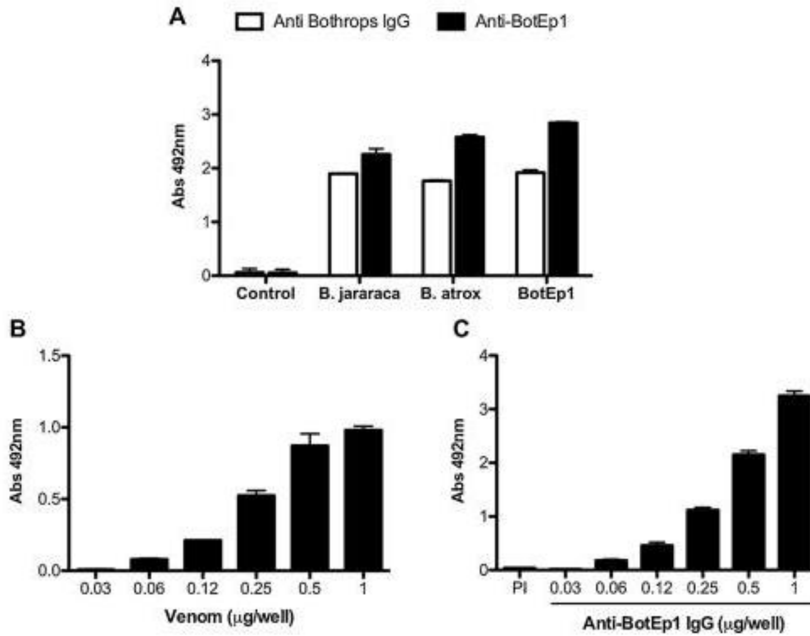


Fig. 4. Reactivity of anti-BotEp1 mouse antibody in ELISA. (A). It was sensitized with BotEp1 10 μg/well, venom of *B. jararaca* and *B. atrox*, each at a concentration of 0.5 μg/well. Anti-*Bothrops* 1:2000 and anti-BotEp1 IgG at a 1:500 dilution, (B) IgG anti-BotEp1 (1:1000) against variable concentrations of *B. jararaca* venom. (C) Reactivity of varying concentrations of IgG anti-BotEp1 against venom of *B. jararaca* (0.5 μg/well). Negative control mouse pre-immune serum.

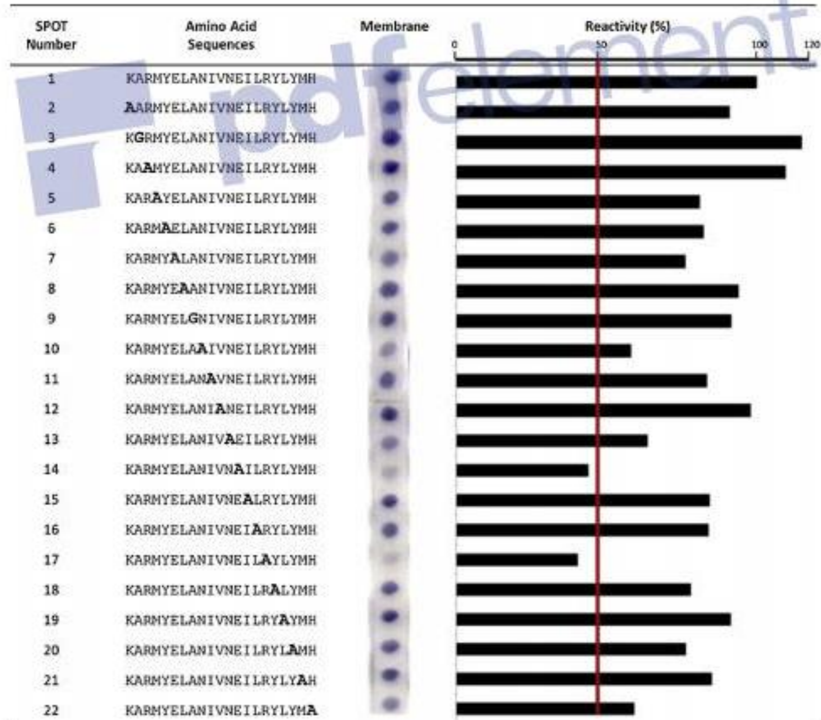


Fig. 5. Alanine scanning of BotEp1. Alanine analogs of BotEp1 were synthesized on SPOT membrane. The membrane was probed with mouse anti-BotEp1 IgG (0.3 μg/mL).

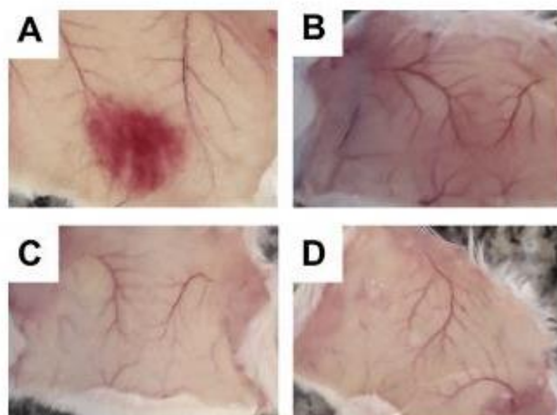


Fig. 6. Neutralization of Bothropasin hemorrhagic activity. (A) Positive control using 2MHD of P2 hemorrhagic fraction. Residual hemorrhage was evaluated after incubating anti-BotEp1 with 2 MHD of P2 fraction subcutaneously in three mice (B, C, D). After 3 h, mice were euthanized and skins were removed. Negative control for hemorrhage was performed injecting saline (not shown).

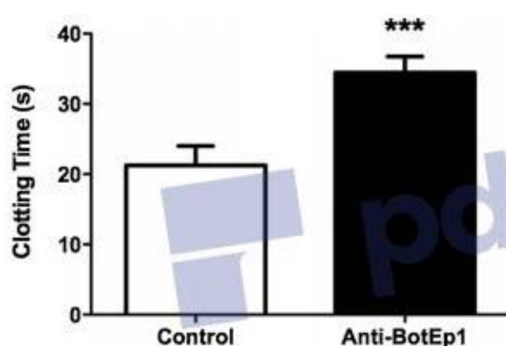


Fig. 7. Evaluation of coagulant activity of P2 fraction in serum from rabbit immunized with BotEp1. P2 hemorrhagic fraction (10 μg) was mixed with 200 μL of rabbit plasma immunized with BotEp1. The effect of the coagulation activity induced by the P2 hemorrhagic fraction was greatly reduced compared to the control group which was non-immunized rabbit plasma (***$p < 0.0001$).

BotEp1-^{202}KARMYELANIVNEILRYLYMH^{222}. The peptide was prepared by Fmoc solid-phase synthesis, immobilized to a Rink Amide resin (Novabiochem). Amino acids were purchased from Sigma Aldrich and coupling steps were performed using a ResPep robot (Intavis), as described elsewhere (Gausepohl et al., 1992). The Peptide was N-terminally acetylated and C-terminally amidated during synthesis. The peptide was released from the resin and amino acid side-chain deprotection was achieved using a 95% TFA, 2.5% triisopropylsilane and 2.5% water cocktail. BotEp1 molecular mass was confirmed by spectrometry (MALDI-TOF) linear mode.

2.4. Anti-BotEp1 sera production and purification

A group of 5 Swiss mice was immunized with BotEp1. The designed peptide was polymerized with glutaraldehyde, as described elsewhere (Machado De Avila et al., 2004). Animals received 10 subcutaneous doses (100 μg/dose/animal), with intervals of 14 days. The first dose was administered with Freund's Complete Adjuvant 1:1 (adjuvant: BotEp1), and nine subsequent doses with incomplete adjuvant; controls animals were immunized with adjuvant and PBS.

Mice polyclonal IgGs antibodies against BotEp1 were purified by immunoaffinity on protein G coupled to super-paramagnetic agarose beads (LOABeads™ Protein G UltraRapid), according to the manufacturer directions.

In addition, anti-BotEp1 serum was also produced in rabbit. After collection of pre-immune serum, ten doses of BotEp1 (100 μg/dose/animal) with Montanide adjuvant were administered with intervals of 14 days.

2.5. Immunocharacterization of anti-BotEp1

ELISA plates were coated with BotEp1 (100 μg/mL), *B. jararaca* (5 μg/mL) or *B. atrox* venoms (5 μg/mL), for 48 h at 4 °C in coating buffer (0.05 M Na_2CO_3, pH 9.6). Plates were later blocked with 1% powdered milk solution in PBS-tween 0.1%. Anti-BotEp1 serum (1:1000) was incubated for 1 h at 37 °C and goat IgG anti-mouse conjugated to peroxidase (Sigma-Aldrich, dilution 1:12,000) and OPD substrate (Sigma-Aldrich) were used to detect the reaction.

Alanine scanning was performed as described by Schneider et al. (2016). Alanine analogs of the identified epitope were synthesized in a membrane, as described in the item 2.2, replacing each amino acid by Alanine (or Glycine, in case of alanine in the original sequence). The synthesis and probing steps were performed as mentioned above.

2.6. Neutralizing assays

Hemorrhagic activity was performed as described by Nikai et al. (1985) with modifications. Firstly, different concentrations of the fraction containing SVMP (P2) were tested to determine its minimum hemorrhagic dose (MHD) (data not shown). For the neutralization assay, Swiss mice (n = 4) were injected intradermally in the dorsal region with two MHD (1 μg) of P2 hemorrhagic fraction of *B. jararaca* venom previously incubated with anti-BotEp1 antibodies at a ratio of 1:50 (venom: IgG, w/w) for 60 min at 37 °C. After 2 h, animals were anesthetized [ketamine 10% (0.05 mL/kg) + xylazine 2% (0.025 mL/kg)] and euthanized. Skins were removed, and the hemorrhagic halo was measured. Negative controls were performed with non-immune mice serum.

2.7. Clotting activity

To evaluate the clotting activity, the P2 hemorrhagic fraction of *B. jararaca* venom was mixed with normal citrated rabbit plasma immunized with BotEp1 or pre-immune rabbit plasma, as negative control. P2 fraction (10 μg) was mixed with 200 μL of rabbit plasma and the coagulation time was measured. Results were expressed as clotting time (s)/plasma.

3. Results

3.1. Epitope mapping and molecular analysis of BotEp1

A linear epitope was mapped in the primary sequence of bothropasin, a P-III metalloproteinase from *B. jararaca*, by interaction with anti-*B. jararaca* sera (FUNED) elicited in three different horses, using a series of peptides covering the complete amino acid sequence of bothropasin synthesized in a cellulose membrane by the SPOT technique. The primary structure of bothropasin is shown in Fig. 1A. A unique region with twenty-one amino acid residues was highly recognized by all the tested sera (Fig. 1B-D).

The epitope named BotEp1 (^{202}KARMYELANIVNEILRYLYMH^{222}) is located in the M catalytic domain of bothropasin (Fig. 2A). The resolved crystallographic structure of bothropasin (Protein Data Bank accession number 3DSL) showed BotEp1 is based partially in the first α-helix and partially in the loop connecting the end of the first α-helix and the beginning of the first parallel β-sheet, as shown in (Fig. 2B). The surface

representation of bothropasin structure showed that the epitope is exposed (Fig. 2C).

Bothropasin was submitted to BlastP, to search for other similar molecules. The results showed that BotEp1 sequence is conserved among the group of PIII SMVP, as can be observed in Fig. 3. The 21-amino acid sequence of BotEp1 was aligned with other SVMPs of the PI, PII and PIII family, with Clustal omega, finding that positively and negatively charged amino acids are conserved in the selected immunogenic region.

3.2. Production and characterization of anti-BotEp1 sera

The corresponding sequence of BotEp1 was synthesized as a soluble peptide. The peptide is composed of 21 amino acids and has a molecular mass of 2641.12 g/mol, a pI of 9.28 and a good solubility in water (parameters calculated on PepCalc, which is available at http//pepcalc.com). The synthetic peptide was used to immunize Swiss mice and the antibody production was monitored by ELISA. Anti-BotEp1 antibodies recognize BotEp1, *B. jararaca* and *B. atrox* crude venoms (Fig. 4A). To evaluate anti-BotEp1 antibodies titers, an ELISA was performed with different concentrations of *B. jararaca* venom, against a fixed concentration of anti-BotEp1 IgG (1:1000) (Fig. 4B). Subsequently, *B. jararaca* venom at 0.5 μg/well was tested against different concentrations of anti-BotEp1 (Fig. 4C).

A series of alanine analogs peptides of BotEp1 (1–21) was prepared by Spot synthesis and assayed for reactivity with anti-BotEp1 polyclonal antibodies. The results (Fig. 5) indicated that the alanine substitution of some residues in C-terminal caused more than 50% decrease in antibody binding. This is the case of residues Glu^{13} and Arg^{16} in the C-terminal sequence, suggesting that these residues are key contributors to the main antigenic regions of BotEp1. On the other hand, when Ala^{2} was substituted by Gly and Arg^{3} by Ala in the N-terminal sequence of BotEp1, the peptides reactivity with anti-BotEp1 was increased.

3.3. Neutralization assays

We have analyzed the ability of anti-BotEp1 to neutralize the hemorrhagic activity induced by P2 fraction from *B. jararaca* venom. Two minimum hemorrhagic doses (2MHD) were pre-incubated with anti-BotEp1 serum at a rate of 1:50 and injected subcutaneously in the dorsal region of mice. After removing the skin around the injected area, no residual bleeding was observed (Fig. 6B–D), showing that anti-BotEp1 antibodies were able to neutralize the hemorrhagic activity caused by P2.

3.4. Clotting activity

Clotting activity was verified by incubating 10 μg of the P2 fraction with 200 μL of plasma. The coagulation time of P2 fraction mixed with plasma from rabbit immunized with BotEp1 was statically higher than control (pre-immune plasma) ($p < 0.0001$), as shown in Fig. 7.

4. Discussion

Bothropasin and Jararhagin, from *B. jararaca* venom, share 95.5% of identity. These proteins are P-III SVMP, a toxins class responsible for hemorrhage, edema and necrosis (Muniz et al., 2008). Due to their role in envenomation, the efficient neutralization of these SVMPs should be critical for antivenom therapy success. As the identification of B-cell epitopes is a fundamental step for development of epitope-based therapeutic antibodies or diagnostic tools, in the present work we aimed to map linear B-cell epitopes from bothropasin and validate the epitope-based antibodies as a promising class of biopharmaceuticals.

Bothropasin B-cell epitope mapping was performed using IgGs anti-*B. jararaca* (FUNED) elicited in three different horses. For this end, the antibody ability to bind to sets of immobilized overlapping peptides of uniform size, covering the complete amino acid sequence of bothropasin, was evaluated.

This systematic mapping of continuous epitopes recognized by anti-*B. jararaca* antibodies indicated that a region of twenty-one residues (^{202}KARMYELANIVNEILRYLYMH^{222}) was recognized by antibodies of all tested animals. The amino acid sequence corresponding to linear BotEp1 seems to be highly conserved in other PIII-SVMPs (Fig. 3). It is located in the catalytic domain (M) of the toxin (Muniz et al., 2008) and have five charged residues. These residues seem to be critical for antigen-antibody binding (Machado De Avila et al., 2004). Based on the 3D structure of bothropasin, (Muniz et al., 2008) we were able to demonstrate the surface exposure of BotE1 showing its availability for binding to anti-bothropasin antibodies.

The exact amino acid sequence of BotEp1 was found within the sequence of the PIII-SVMP BITM06 A (UNIPROT: Q8QG88) of *Bothrops insularis* venom. Remarkably, among the twenty-one residues of the epitope, only Leu^{15} is substituted for Phe, in this immunodominant region in Jararhagin and Batroxrhagin, (from *B. atrox* venom) (Fig. 3). Data show that these proteins share a possible common and immunodominant epitope. Interestingly, this region is highly conserved among P-III SVMPs, when compared with P-I and P-II SVMPs.

The defined BotEp1 epitope was chemically synthesized in its soluble form and administered as immunogen (using Freund's adjuvants) in mice. The antibody response of immunized mice against the synthetic BotEp1 peptide and against crude *B. jararaca* venom was evaluated by ELISA. Through these assays, we could detect a strong antibody response of the immunized sera against *B. jararaca* venom and with the synthetic peptide. We observed that BotEp1, a single peptide, was able to elicit a strong antibody response. The antibody response was found to be cross-reactive, since it was possible to detect by ELISA circulating antibodies against *B. atrox* crude venom, that together with *B. jararaca* are the species responsible for the majority of the accidents in Brazil (Estevao-Costa et al., 2016; Ministério da Saúde, 2009).

We also examined the fine specificity of anti-peptide antibodies elicited in mice through their binding to a set of immobilized alanine analogs of the BotEp1. These results led us to pinpoint charged residues (Glu^{214} and Arg^{217}) as critical amino acids for the recognition of the continuous epitope BotEp1 by antibodies. These two amino acid residues, found important by alanine scanning, are conserved in P-III class of SVMPs as shown in Fig. 3. The four proteins compared with bothropasin are almost identical in this region, suggesting the importance of these charged amino acids. The molecular basis for the neutralizing capacity of anti-BotEp1 antibodies can be explained by considering that the antibody binds to the two amino acids present in the N-terminal part of the catalytic domain involved in the proteolytic activity of bothropasin, possibly causing a steric hindrance for the substrate (Laustsen et al., 2018).

In the last part of this study, the neutralization of hemorrhagic and coagulant activities by anti-BotEp1 antibodies was evaluated. These antibodies were able to neutralize the hemorrhagic activity caused by the hemorrhagic fraction of *B. jararaca* venom. Mice from control group, that received hemorrhagic fraction diluted in PBS, did not show any protection. Coagulant activity of P2 hemorrhagic fraction mixed with plasma from rabbit immunized with BotEp1 showed to be diminished when compared to control (non-immune plasma), suggesting that the immunization with BotEp1 induced antibodies capable of decrease the coagulant activity of P2 fraction.

The use of epitopes representing complex venoms for obtaining neutralizing sera has already been attempted. Wagstaff et al. (2006) engineered seven epitopes, predicted by bioinformatics tools, to represent the variety of metalloproteases present in the African snake *Echis occelatus* venom as a single synthetic DNA immunogen, which they called epitope string. The produced antivenom was capable of neutralizing hemorrhage caused by *E. occelatus* venom and *Cerastes cerastes* as well, indicating an important cross-reactivity of the antivenom (Wagstaff et al., 2006).

Our group has also recently engineered, by molecular biology techniques, a recombinant multiepitopic protein composed by validated epitopes from different toxins found in the *Loxosceles spp* spider venoms. Using a rabbit model, preincubation of anti-chimeric protein serum was capable of neutralizing 100% of the toxic activities of *L. intermedia* spider crude venom (Lima et al., 2018). Taken together, previous publications (Ferreira et al., 2006; Machado De Avila et al., 2011; Schneider et al., 2016) indicate that the use of molecules mimicking multiple epitopes is an efficient approach for antivenom production.

The present work identified an epitope (BotEp1) that elicited neutralizing antibodies against the hemorrhagic fraction of *B. jararaca* venom. Our results provide a rational basis to the identification of a neutralizing epitopes on SVMPs snake toxins and show that their corresponding synthetic peptides could improve the generation of immunotherapeutics against these snake venoms. In the future, this peptide can be combined with other epitopes from other toxin families, such as snake venom serine proteinases (SVSP) with neutralizing potential against other deleterious actions of *B. jararaca* venom. The development of a new generation of therapeutic anti-venoms by the precise identification of linear or conformational epitopes directed to the production of protective antibodies (protective B-cell epitopes) is an interesting perspective in the Toxinology field.

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgements

This research was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil (CNPq) (n° 17/2013, Process: 490269/2013-3), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil (CAPES) (TOXINOLOGIA n°23038000825/2011-63) and by Fundação de Amparo à Pesquisa do Estado de Minas Gerais, Brazil (FAPEMIG) (n° PPM: 00270-16).

References

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25 (17), 3389–3402. https://doi.org/10.1093/nar/25.17.3389.

Altschul, S.F., Wootton, J.C., Gertz, E.M., Agarwala, R., Morgulis, A., Schaffer, A.A., Yu, Y.-K., 2005. Protein database searches using compositionally adjusted substitution matrices. FEBS J. 272, 5101–5109. https://doi.org/10.1111/j.1742-4658.2005.04945.x.

Ciscotto, P., Machado de Avila, Ra., Coelho, E., a, F., Oliveira, J., Diniz, C.G., Farías, L.M., de Carvalho, M.A.R., Maria, W.S., Sanchez, E.F., Borges, a., Chávez-Olórtegui, C., 2009. Antigenic, microbicidal and antiparasitic properties of an l-amino acid oxidase isolated from Bothrops jararaca snake venom. Toxicon 53, 330–341. https://doi.org/10.1016/j.toxicon.2008.12.004.

Espino-Solis, G.P., Riaño-Umbarila, L., Becerril, B., Possani, L.D., 2009. Antidotes against venomous animals: state of the art and prospectives. J. Proteomics. https://doi.org/10.1016/j.jprot.2009.01.020.

Estevao-Costa, M.I., Gontijo, S.S., Correia, B.L., Yarleque, A., Vivas-Ruiz, D., Rodrigues, E., Chávez-Olortegui, C., Oliveira, L.S., Sanchez, E.F., 2016. Neutralization of toxicological activities of medically-relevant Bothrops snake venoms and relevant toxins by two polyvalent bothropic antivenoms produced in Peru and Brazil. Toxicon 122, 67–77. https://doi.org/10.1016/j.toxicon.2016.09.010.

Felicori, L., Fernandes, P.B., Giusta, M.S., Duarte, C.G., Kalapothakis, E., Nguyen, C., Molina, F., Granier, C., Chávez-Olórtegui, C., 2009. An in vivo protective response against toxic effects of the dermonecrotic protein from Loxosceles intermedia spider venom elicited by synthetic epitopes. Vaccine 27, 4201–4208. https://doi.org/10.1016/j.vaccine.2009.04.038.

Fernandes, I., Assumpção, G.G., Silveira, C.R.F., Faquim-Mauro, E.L., Tanjoni, I., Carmona, A.K., Alves, M.F.M., Takehara, H.A., Rucavado, A., Ramos, O.H.P., Moura-da-Silva, A.M., Gutiérrez, J.M., 2010. Immunochemical and biological characterization of monoclonal antibodies against BaP1, a metalloproteinase from Bothrops asper snake venom. Toxicon 56, 1059–1065. https://doi.org/10.1016/j.toxicon.2010.07.014.

Ferreira, R.N., Machado de Avila, R.A., Sanchez, E.F., Maria, W.S., Molina, F., Granier, C., Chávez-Olórtegui, C., 2006. Antibodies against synthetic epitopes inhibit the enzymatic activity of mutalysin II, a metalloproteinase from bushmaster snake venom. Toxicon 48, 1098–1103. https://doi.org/10.1016/j.toxicon.2006.07.040.

Freitas-de-Sousa, L.A., Colombini, M., Lopes-Ferreira, M., Serrano, S.M.T., Moura-da-Silva, A.M., 2017. Insights into the mechanisms involved in strong hemorrhage and dermonecrosis induced by atroxlysin-Ia, a PI-class snake venom metalloproteinase. Toxins (Basel) 9. https://doi.org/10.3390/toxins9080239.

Gausepohl, H., Boulin, C., Kraft, M., Frank, R.W., 1992. Automated multiple peptide synthesis. Pept. Res. 5, 315–320.

Harrison, R.A., Cook, D.A., Renjifo, C., Casewell, N.R., Currier, R.B., Wagstaff, S.C., 2011. Research strategies to improve snakebite treatment: challenges and progress. J. Proteomics 74, 1768–1780. https://doi.org/10.1016/j.jprot.2011.06.019.

Kang, T.S., Georgieva, D., Genov, N., Murakami, M.T., Sinha, M., Kumar, R.P., Kaur, P., Kumar, S., Dey, S., Sharma, S., Vrielink, A., Betzel, C., Takeda, S., Arni, R.K., Singh, T.P., Kini, R.M., 2011. Enzymatic toxins from snake venom: structural characterization and mechanism of catalysis. FEBS J. 278, 4544–4576. https://doi.org/10.1111/j.1742-4658.2011.08115.x.

Laune, D., Molina, F., Ferrières, G., Villard, S., Bès, C., Rieunier, F., Chardès, T., Granier, C., 2002. Application of the Spot method to the identification of peptides and amino acids from the antibody paratope that contribute to antigen binding. J. Immunol. Methods 267, 53–70. https://doi.org/10.1016/S0022-1759(02)00140-0.

Laustsen, A.H., María Gutiérrez, J., Knudsen, C., Johansen, K.H., Bermúdez-Méndez, E., Cerni, F.A., Jürgensen, J.A., Ledsgaard, L., Martos-Esteban, A., Øhlenschlæger, M., Pus, U., Andersen, M.R., Lomonte, B., Engmark, M., Pucca, M.B., 2018. Pros and cons of different therapeutic antibody formats for recombinant antivenom development. Toxicon 146, 151–175. https://doi.org/10.1016/j.toxicon.2018.03.004.

Lima, S., de, A., Guerra-Duarte, C., Costal-Oliveira, F., Mendes, T.M., Luís, L.F., Oliveira, D., de Avila, R.A.M., Ferrer, V.P., Trevisan-Silva, D., Veiga, S.S., Minozzo, J.C., Kalapothakis, E., Chávez-Olórtegui, C., 2018. Recombinant protein containing B-cell epitopes of different Loxosceles spider toxins generates neutralizing antibodies in immunized rabbits. Front. Immunol. 9. https://doi.org/10.3389/fimmu.2018.00653.

Machado De Avila, R.A., Alvarenga, L.M., Tavares, C.A.P., Molina, F., Granier, C., Chávez-Olórtegui, C., 2004. Molecular characterization of protective antibodies raised in mice by Tityus serrulatus scorpion venom toxins conjugated to bovine serum albumin. Toxicon 44, 233–241. https://doi.org/10.1016/j.toxicon.2004.04.015.

Machado De Avila, R.A., Stransky, S., Velloso, M., Castanheira, P., Schneider, F.S., Kalapothakis, E., Sanchez, E.F., Nguyen, C., Molina, F., Granier, C., Chávez-Olórtegui, C., 2011. Mimotopes of mutalysin-II from Lachesis muta snake venom induce hemorrhage inhibitory antibodies upon vaccination of rabbits. Peptides 32, 1640–1646. https://doi.org/10.1016/j.peptides.2011.06.028.

Mandelbaum, F.R., Reichel, A.P., Assakura, M.T., 1982. Isolation and characterization of a proteolytic enzyme from the venom of the snake Bothrops jararaca (Jararaca). Toxicon 20, 955–972. https://doi.org/10.1016/0041-0101(82)90098-8.

Mendes, T.M., Oliveira, D., Figueiredo, L.F.M., Machado-de-Avila, R.A., Duarte, C.G., Dias-Lopes, C., Guimarães, G., Felicori, L., Minozzo, J.C., Chávez-Olórtegui, C., 2013. Generation and characterization of a recombinant chimeric protein (rCpLi) consisting of B-cell epitopes of a dermonecrotic protein from Loxosceles intermedia spider venom. Vaccine 31, 2749–2755. https://doi.org/10.1016/j.vaccine.2013.03.048.

Ministério da Saúde, 2009. . Guia de Vigilância Epidemiológica, Série A. Normas e Manuais Técnicos. https://doi.org/10.1590/S1806-37132004000700003.

Morine, N., Matsuda, S., Terada, K., Eto, A., Ishida, I., Oku, H., 2008. Neutralization of hemorrhagic snake venom metalloproteinase HR1a from protobothrops flavoviridis by human monoclonal antibody. Toxicon 51, 345–352. https://doi.org/10.1016/j.toxicon.2007.10.009.

Muniz, R.C., Ambrosio, A.L.B., Selistre-de-araujo, H.S., Moura-da-silva, A.M., Oliva, G., Garratt, R.C., Souza, D.H.F., 2008. The three-dimensional structure of bothropasin, the main hemorrhagic factor from Bothrops jararaca venom: insights for a new classification of snake venom metalloprotease subgroups. Toxicon 52, 807–816. https://doi.org/10.1016/j.toxicon.2008.08.021.

Nikai, T., Mori, N., Kishida, M., Tsuboi, M., Sugihara, H., 1985. Isolation and characterization of hemorrhagic toxin g from the venom of Crotalus atrox (western diamondback rattlesnake). Am. J. Trop. Med. Hyg. 34, 1167–1172. https://doi.org/10.4269/ajtmh.1985.34.1167.

Oliveira, A., Paes Leme, A., Asega, A., Camargo, A., Fox, J., Serrano, S., 2010. New insights into the structural elements involved in the skin haemorrhage induced by snake venom metalloproteinases. Thromb. Haemost. 104, 485–497. https://doi.org/10.1160/TH09-12-0855.

Rawlings, N.D., Barrett, A.J., Finn, R., 2016. Twenty years of the MEROPS database of proteolytic enzymes, their substrates and inhibitors. Nucleic Acids Res. 44, D343–D350. https://doi.org/10.1093/nar/gkv1118.

Schneider, F.S., de Almeida Lima, S., Reis de Ávila, G., Castro, K.L., Guerra-Duarte, C., Sanchez, E.F., Nguyen, C., Granier, C., Molina, F., Chávez-Olórtegui, C., 2016. Identification of protective B-cell epitopes of Atroxlysin-I: a metalloproteinase from Bothrops atrox snake venom. Vaccine 34, 1680–1687. https://doi.org/10.1016/j.vaccine.2016.02.035.

Silva, C.C.F., Menezes, M.C., Palomino, M., Oliveira, A.K., Iwai, L.K., Faria, M., Portaro, F.V., 2017. Peptides derived from plasma proteins released by bothropasin, a metalloprotease present in the Bothrops jararaca venom. Toxicon 137, 65–72. https://doi.org/10.1016/j.toxicon.2017.07.009.

Stephano, M.A., Guidolin, R., Higashi, H.G., Tambourgi, D.V., Sant'Anna, O.A., 2005. The improvement of the therapeutic anti-Lachesis muta serum production in horses. Toxicon 45, 467–473. https://doi.org/10.1016/j.toxicon.2004.12.006.

Wagstaff, S.C., Laing, G.D., Theakston, R.D.G., Papaspyridis, C., Harrison, R.A., 2006. Bioinformatics and multiepitope DNA immunization to design rational snake antivenom. PLoS Med. 3, 0832–0844. https://doi.org/10.1371/journal.pmed.0030184.

Warrell, D.A., 2004. Snakebites in Central and South America: epidemiology, clinical features, and clinical management. The Venomous Reptiles of the Western Hemisfephere. Comstok, London p. 425.

WHO, 2016. Post ECBS Version Expert Committee on Biological Standardization Geneva, 17 to 21 October 2016 WHO Guidelines for the Production, Control and Regulation of Snake Antivenom Immunoglobulins. World Heal. Organ., pp. 1–138.

3.2 Artigo 2. Engineered protein containing Crotoxin epitopes induces neutralizing antibodies in immunized rabbits

(2020) Denis A. Molina Molina, Clara Guerra-Duarte, Fernanda Costal-Oliveira, Elizângela Almeida Rocha, Carolina Rego Rodrigues, Ricardo A Machado-de-Ávila, Vanete T Soccol, Carlos Chávez-Olórtegui.

A crotoxina é uma potente neurotoxina com atividade fosfolipase A_2, formada por duas subunidades que exerce uma ação letal ao bloquear a transmissão neuromuscular a nível pré-sináptico. É o principal componente do veneno da serpente de cascavel sul-americana *Crotalus durissus terrificus.* Além da neurotoxicidade, verificou-se que a crotoxina exerce nefrotoxicidade e toxicidade cardiovascular. Adicionalmente, foram relatados efeitos anti-inflamatórios, imunomoduladores, antitumorais e analgésicos da crotoxina.

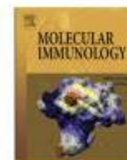
Após o uso da técnica de *Spot synthesis* no mapeamento de epítopos lineares e o uso de peptídeos sintéticos para induzir a produção anticorpos neutralizantes anti-bothropasina, propomos neste trabalho o mapeamento de epítopos utilizando implantes subcutâneos para então produzir uma proteína multiepitópica recombinate contendo os principais epítopos da crotoxina. Esta proteína recombinante, nomeada rMEPCtx, foi utilizada para imunizar coelhos e induziu a produção de anticorpos neutralizantes contra a ação letal da crotoxina. Deve-se ressaltar que esta metodologia pode ser aplicada a outros tipos de toxinas de venenos, sendo uma alternativa para a produção de soro antiofídico baseados no potencial imunogênico de proteínas recombinantes formadas por epítopos de diferentes famílias de toxinas.

Molecular Immunology 119 (2020) 144–153

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm



Engineered protein containing crotoxin epitopes induces neutralizing antibodies in immunized rabbits

Denis A. Molina Molina[a], Clara Guerra-Duarte[b], Fernanda Costal-Oliveira[a], Elizângela Almeida Rocha[a], Carolina Rego Rodrigues[a], Ricardo A. Machado-de-Ávila[c], Vanete T. Soccol[d], Carlos Chávez-Olórtegui[a,*]

[a] Departamento de Bioquímica e Imunologia, ICB, Universidade Federal de Minas Gerais, Av. Antônio Carlos 6627, CEP: 31270-901, Belo Horizonte, MG, Brazil
[b] Centro de Pesquisa e Desenvolvimento, Fundação Ezequiel Dias, 30510-010, Belo Horizonte, MG, Brazil
[c] Universidade Federal do Paraná (UFPR), Curitiba, PR, Brazil
[d] Universidade do Extremo Sul Catarinense (UNESC), Criciúma, SC, Brazil

ARTICLE INFO

Keywords:
Crotalus snake venom
Crotoxin
Immunome
B-cell epitopes
Multiepitopic recombinant protein
Neutralizing antibodies

ABSTRACT

Crotoxin (Ctx) is the main lethal component of *Crotalus durissus terrificus* venom. It is a neurotoxin, composed of two subunits associated by noncovalent interactions, the non-toxic acid subunit (CA), named Crotapotin, and the basic subunit (CB), with phospholipase A_2 (PLA_2) activity. Employing the SPOT synthesis technique, we determined two epitopes located in the C-terminal of each Ctx subunit. In addition, 3 other epitopes were mapped in different regions of Ctx using subcutaneous spot implants surgically inserted in mice. All epitopes mapped here were expressed together as recombinant multi-epitopic protein (rMEPCtx), which was used to immunize New Zealand rabbits. Anti-rMEPCtx rabbit serum cross-reacted with Ctx and crude venoms from *C. d. terrificus*, *Crotalus durissus ruruima*, Peruvian *C. durissus* and *Bothrops jararaca* (with lower intensity). Furthermore, anti-rMEPCtx serum was able to neutralize Ctx lethal activity. As the recombinant multiepitopic protein is not toxic, it can be administered in larger doses without causing adverse effects on the immunized animals health. Therefore, our work evidences the identification of neutralizing epitopes of Ctx and support the use of recombinant multiepitopic proteins as an innovation to immunotherapeutics production.

1. Introduction

In Brazil, the rattlesnake, *Crotalus durissus terrificus* is responsible for almost 10 % of over 28,000 snakebites registered each year (DATASUS Brasil, 2019; Ministerio de Saude, 2019). Antivenom is the only specific treatment recommended by World Health Organization (WHO, 2016). Before its use, the lethality of snakebite accidents in Brazil was approximately 73 %. Currently, due to the implementation of systematic antivenom treatment protocols, lethal cases decreased to around 1.5 % (Amaral et al., 1997). In Minas Gerais state, *C. d. terrificus* is the most frequently encountered subspecies from *C. durissus*. Several clinical studies have reported acute renal failure as the leading cause of death in this type of accident, however, acute respiratory failure, due to respiratory muscle paralysis, can also account for lethality (Amaral et al., 1986; Amaral and Magalhães, 1991).

The symptomatology presented by patients after accidents caused by *Crotalus* genus is attributed to two main proteins present in its venom: crotoxin and crotamine. Variability related to the concentrations of these toxins in crotalic venom has been previously studied (Teixeira-Araújo et al., 2017). Crotoxin (Ctx), the main toxin of *C. d. terrificus* venom, is a neurotoxin composed of two non-covalently associated subunits, a non-toxic acid subunit (CA), known as crotapotin, and a basic subunit (CB) with phospholipase A_2 (PLA_2) activity, which accounts for 70–90 % of the venom proteome (Teixeira-Araújo et al., 2017). In *C. d. terrificus* venom, it was already identified 16 isoforms of Ctx, due to the random combination of 4 isoforms of CA (CA1, CA2, CA3 and CA4) and 4 isoforms of CB (CBa2, CBb, CBc and CBd) (Faure et al., 2011; Faure and Bon, 1987). In addition to Ctx, significant variation in the amount of PIII class snake venom metalloproteinase (SVMPs), LAAO, and thrombin-like serine proteinases toxins were observed in *C. d. terrificus* proteome (Calvete et al., 2010). Futthermore, a positive correlation between the concentration of Ctx in envenomed patients and severity of the clinical manifestations was acknowledged (Amaral et al., 1997).

In Brazil, treatment of envenoming by all *C. durissus* subspecies (*C. d. terrificus*, *C. d. cascavella* and *C. d. collilineatus*) is performed by the

* Corresponding author.
E-mail address: olortegi@icb.ufmg.br (C. Chávez-Olórtegui).

https://doi.org/10.1016/j.molimm.2020.01.012
Received 2 October 2019; Received in revised form 27 December 2019; Accepted 20 January 2020
0161-5890/ © 2020 Elsevier Ltd. All rights reserved.

use of a monospecific crotalic antivenom produced by horse hyperimmunization with *C. d. terrificus* venom (Boldrini-França et al., 2010). Fundação Ezequiel Dias (FUNED) is one of the three Brazilian institutions that manufacture crotalic anti-venom, together with Butantan and Vital Brazil Institutes (Teixeira-Araújo et al., 2017). Although Brazilian antivenom has shown to be effective in preventing signs and symptoms of envenoming and lethality, its production is limited and faces some issues, including the toxicity to the producer animal caused by the venom used as immunogen (Gazarian et al., 2005; Chippaux, 2010), the occupational risk of maintaining venomous animals for venom extraction and the differential immunogenicity of venom toxins, which may lead to the lack of neutralizing antibodies directed to some important toxins. In addition, crotalic venom presents lower immunogenicity in horses, when compared to bothropic venom (Rangel-santos et al., 2004; Rangel-Santos and Mota, 2000).

These raised concerns lead to the need of new immunization strategies, such as synthetic antigens that mimic the venoms native proteins, but with no toxicity to the producer animal. According to Ainsworth (2018), the rational choice of immunogens for anti-venom production, using key toxins or synthetic/recombinant fragments derived from their sequences instead of crude venom, can lead to antibodies (or its fragments) with improved neutralizing capacity and can also modulate cross-reactivity. Indeed, previous works have shown that synthetic epitopes derived from toxic antigens sequences have allowed the development of efficient anti-venoms (Lima et al., 2018; Molina et al., 2018).

With this in mind, we first report the localization of linear B-cell epitopes of Ctx using the SPOT method of multiple peptide synthesis (Frank, 1992; Molina et al., 1996) and subcutaneous spot implants surgically inserted in mice. After epitope identification, we show here the design and production of a recombinant multiepitopic, and non-toxic protein containing Ctx epitopes (rMEPCtx) to be used as immunogen for crotalic anti-venom production. Immunological studies using sera from rabbits immunized with rMEPCtx suggest that this multiepitopic protein can be used as immunogen to produce anti-venoms against potentially lethal *Crotalus* spp. snake venoms.

2. Material and methods

2.1. Animals, venoms, and antivenoms

Swiss mice (18 – 22 g) and New Zealand rabbits were used in the assays reported here. Mice were obtained from the Centro de Bioterismo of the Instituto de Ciências Biológicas of Universidade Federal de Minas Gerais (UFMG) and rabbits from Escola de Medicina Veterinária of UFMG. All animals received water and food in *ad libitum* conditions. Experimental protocols were approved by the Ethics Committee on Animal Experimentation of UFMG (445/2015-CETEA / UFMG).

The Fundação Ezequiel Dias (FUNED - Belo Horizonte, Brazil), kindly provided the venom samples of *C. d. terrificus* and anti-crotalic horse IgGs, precipitaded from horse sera. Ctx was previously purified by our laboratory group, following the method described in Quintana et al. (2012), with modifications.

2.2. Epitope mapping in PLA_2 and crotapotin subunits of Ctx

2.2.1. Peptide synthesis on cellulose membranes (SPOT)

Two SPOT membranes were synthesized, each membrane containing a set of peptides derived from Ctx. The first membrane consists of 30 pentadecapeptides overlapped by 3 residues, corresponding to the primary sequence of crotapotin acid subunit - CA, P08878 recovered from Uniprot and the second membrane is composed of 111 superimposed pentadecapeptides displaced by 3 residues, corresponding to the primary sequence of the 3 PLA_2 subunit isoforms (basic PLA_2 subunit - CB, P0CAS6, P24027 and P62022 recovered from Uniprot). These membranes were used for the epitope mapping assay (Tables 1–4).

Table 1
Thirty overlapping decapentapeptides synthesized corresponding to the amino acid sequence of the acid subunit of crotoxin (UniProt P08878).

Spot number	Peptide sequence in spot
1	SSYGCYCGAGGQGWP
2	GCYCGAGGQGWPQDA
3	CGAGGQGWPQDASDR
4	GGQGWPQDASDRCCF
5	GWPQDASDRCCFEHD
6	QDASDRCCFEHDCCY
7	SDRCCFEHDCCYAKL
8	CCFEHDCCYAKLTGC
9	EHDCCYAKLTGCDPT
10	CCYAKLTGCDPTTDV
11	AKLTGCDPTTDVYTY
12	TGCDPTTDVYTYRQE
13	DPTTDVYTYRQEDGE
14	TDVYTYRQEDGEIVC
15	YTYRQEDGEIVCGED
16	RQEDGEIVCGEDDPC
17	DGEIVCGEDDPCGTQ
18	IVCGEDDPCGTQICE
19	GEDDPCGTQICECDK
20	DPCGTQICECDKAAA
21	GTQICECDKAAAICF
22	ICECDKAAAICFRNS
23	CDKAAAICFRNSMDT
24	AAAICFRNSMDTYDY
25	ICFRNSMDTYDYKYL
26	RNSMDTYDYKYLQFS
27	MDTYDYKYLQFSPEN
28	YDYKYLQFSPENCQG
29	KYLQFSPENCQGESQ
30	LQFSPENCQGESQPC

In addition, other two membranes, one containing 30 pentadecapeptides of crotapotin CA, P08878 sequence and one containing 37 pentadecapeptides of PLA_2 (CB, P0CAS6) sequence, were synthesized and its spots were used for mice immunization. Each spot containing a different peptide was cut and implanted subcutaneously in animals, as described below (Section 2.2.3).

The general protocol to produce the membranes has been previously described in (Laune et al., 2002), using ResPep (Intavis) robot. Membranes were purchased from Abimed (Langenfeld, Germany). The synthesized peptides were acetylated at their N-terminus. Subsequently peptides were treated with trifluoroacetic acid, in order to eliminate the amino acids side chain protecting groups (Frank, 1992).

2.2.2. SPOT membrane immunoassay for epitopes identification in crotapotin (CA) and PLA_2 (CB)

Assays were performed as described by Molina et al. (2018). Membranes were tested by incubation with anti-Crotalus horse serum (1 μg/mL), diluted in 1:1000 in blocking buffer [bovine serum albumin (3 %) and sucrose (5 %) in PBS]. Anti-crotalic serum bound to the membrane were identified using secondary antibody anti-horse IgG conjugated to alkaline – phosphatase 1:10,000 antibody developed in rabbit code A-6063 - Sigma), for 90 min 37 °C. After washing, 5-bromo-4-chloro-3-indolyl phosphate (Sigma) and 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (Sigma) were added as chromogenic substrates. Peptide spots recognized by antibodies were detected by their blue color. After registration of reactive peptides, the membrane was regenerated using dimethylformamide, then 1 % SDS, 0.1 % β-mercaptoethanol in 8 M urea, followed by ethanol/water/acetic acid (50:40:10 vol/vol/vol) and ethanol.

pdfelement

Table 2
Thirty-seven overlapping decapentapeptides synthesized corresponding to the amino acid sequence of the basic subunit of crotoxin (UniProt P0CAS6).

Spot number	Peptide sequence in spot
1	SLLQFNKMIKFETRK
2	QFNKMIKFETRKNAV
3	KMIKFETRKNAVPFY
4	KFETRKNAVPFYAFY
5	TRKNAVPFYAFYGCY
6	NAVPFYAFYGCYCGW
7	PFYAFYGCYCGWGGR
8	AFYGCYCGWGGRRRP
9	GCYCGWGGRRRPKDA
10	CGWGGRRRPKDATDR
11	GGRRRPKDATDRCCF
12	RRPKDATDRCCFVHD
13	KDATDRCCFVHDCCY
14	TDRCCFVHDCCYEKV
15	CCFVHDCCYEKVTKC
16	VHDCCYEKVTKCNTK
17	CCYEKVTKCNTKWDI
18	EKVTKCNTKWDIYRY
19	TKCNTKWDIYRYSLK
20	NTKWDIYRYSLKSGY
21	WDIYRYSLKSGYITC
22	YRYSLKSGYITCGKG
23	SLKSGYITCGKGTWC
24	SGYITCGKGTWCKEQ
25	ITCGKGTWCKEQICE
26	GKGTWCKEQICECDR
27	TWCKEQICECDRVAA
28	KEQICECDRVAAECL
29	ICECDRVAAECLRRS
30	CDRVAAECLRRSLST
31	VAAECLRRSLSTYKN
32	ECLRRSLSTYKNGYM
33	RRSLSTYKNGYMFYP
34	LSTYKNGYMFYPDSR
35	YKNGYMFYPDSRCRG
36	GYMFYPDSRCRGPSE
37	MFYPDSRCRGPSETC

Table 3
Thirty-seven overlapping decapentapeptides synthesized corresponding to the amino acid sequence of the basic subunit of crotoxin (UniProt P24027).

Spot number	Peptide sequence in spot
1	GSLLQFNKMIKFETR
2	LQFNKMIKFETRKNA
3	NKMIKFETRKNAVPF
4	IKFETRKNAVPFYAF
5	ETRKNAVPFYAFYGC
6	KNAVPFYAFYGCYCG
7	VPFYAFYGCYCGWGG
8	YAFYGCYCGWGGQGR
9	YGCYCGWGGQGRPKD
10	YCGWGGQGRPKDATD
11	WGGQGRPKDATDRCC
12	QGRPKDATDRCCFVH
13	PKDATDRCCFVHDCC
14	ATDRCCFVHDCCYGK
15	RCCFVHDCCYGKLAK
16	FVHDCCYGKLAKCNT
17	DCCYGKLAKCNTKWD
18	YGKLAKCNTKWDIYR
19	LAKCNTKWDIYRYSL
20	CNTKWDIYRYSLKSG
21	KWDIYRYSLKSGYIT
22	IYRYSLKSGYITCGK
23	YSLKSGYITCGKGTW
24	KSGYITCGKGTWCKE
25	YITCGKGTWCKEQIC
26	CGKGTWCKEQICECD
27	GTWCKEQICECDRVA
28	CKEQICECDRVAAEC
29	QICECDRVAAECLRR
30	ECDRVAAECLRRSLS
31	RVAAECLRRSLSTYK
32	AECLRRSLSTYKNEY
33	LRRSLSTYKNEYMFY
34	SLSTYKNEYMFYPDS
35	TYKNEYMFYPDSRCR
36	NEYMFYPDSRCREPS
37	MFYPDSRCREPSETC

2.2.3. Subcutaneous immunization of mice with cellulose membranes containing synthetic peptides

Swiss mice, aged 6–8 weeks, were individually immunized subcutaneously with a single synthetic peptide coupled to cellulose membrane derived either from crotapotin (CA) or PLA_2 (CB) from Ctx. The sequence of each Spot is described in Tables 1 and 2. For mice immunization, cellulose membrane disks from each spot (5 mm of diameter) were sterilized by a treatment with 70 % ethanol for 1 h and 10 min of UV light. The membrane disks were washed 3 times in sterile PBS, before being implanted. After being anesthetized (xylazine 90 mg/kg and ketamine 90 mg/kg) animals received a subcutaneous implant surgically inserted in the dorsal region, following the method of Lima et al. (1999). It is estimated that approximately 50 nmol of each peptide is bound to the cellulose membrane disk (Frank, 1992). A total of 67 spots were individually inserted into 67 mice. As negative control, 5 mice received a cellulose membrane fragment without any peptide bound. Pre-immune serum from each animal was collected previously to the immunization. Another 3 blood collections were done 30, 60 and 90 days after receiving the implant.

2.2.4. Evaluation of spot-implant immunization efficiency

ELISA method was used to evaluate the production of antibodies induced by the cellulose-bound synthetic peptides implants. Plates were coated with 100 µL *C. d. terrificus* venom (5 µg/mL), overnight at 4 °C in coating buffer (0.05 M Na_2CO_3, pH 9.6), and then blocked with (1 % powdered milk solution in PBS-tween 0.1 %) at 37 °C. Anti-peptides sera diluted 1:100 were added to the wells and incubated for 1 h at 37 °C in 0.1 % blocking buffer. Plates were then incubated with secondary antibody anti-mouse IgG (whole molecule) peroxidase (1:5000), (produced in goat – code A4416 – 1 mL - Sigma), for 1 h at 37 °C in 0.1 % blocking buffer and OPD peroxidase substrate (SIGMAFAST from Sigma-Aldrich) was used to detect the reaction. The reaction was stopped with 25 µL of 2 M H_2SO_4 and intensity was estimated by absorbance reading at 490 nm in an iMark (Biorad) microplate reader.

2.3. Recombinant multiepitopic Ctx (rMEPCtx) design and construction

Based on the combined results of peptide array immunoassay and subcutaneous immunization, five linear epitopes of Ctx were used for the recombinant multiepitopic Ctx production. A DNA fragment was synthesized with the nucleotide sequence corresponding to the five epitopes, using two glycine codons as spacers between each epitope sequence (IDT – Integrated DNA Technologies), constituting a recombinant multiepitopic protein from Ctx (rMEPCtx). Sites for restriction enzymes, NdeI and XhoI were added at the 5′end and 3′end of the synthetic DNA, respectively. The DNA was cloned into the expression vector pET 28a(+)-TEV from GenScript™ (http://www.genscript.com/). The pET 28a-TEV vector contains 6 histidine residues that are bound in the N-terminal of the expressed protein.

2.4. Expression and purification of rMEPCtx

The vector pET 28a(+)-TEV containing the DNA fragment of rMEPCtx was chemically transformed into *E. coli* BL21 bacteria for expression, according Mendes et al. (2013).

The presence of rMEPCtx expression in the soluble or insoluble

pdfelement

Table 4
Thirty-seven overlapping decapentapeptides synthesized corresponding to the amino acid sequence of the basic subunit of crotoxin (UniProt P62022).

Spot number	Peptide sequence in spot
1	GHLLQFNKMIKFETR
2	LQFNKMIKFETRKNA
3	NKMIKFETRKNAIPF
4	IKFETRKNAIPFYAF
5	ETRKNAIPFYAFYGC
6	KNAIPFYAFYGCYCG
7	IPFYAFYGCYCGWGG
8	YAFYGCYCGWGGRGR
9	YGCYCGWGGRGRPKD
10	YCGWGGRGRPKDATD
11	WGGRGRPKDATDRCC
12	RGRPKDATDRCCFVH
13	PKDATDRCCFVHDCC
14	ATDRCCFVHDCCYGK
15	RCCFVHDCCYGKLAK
16	FVHDCCYGKLAKCNT
17	DCCYGKLAKCNTKWD
18	YGKLAKCNTKWDIYP
19	LAKCNTKWDIYPYSL
20	CNTKWDIYPYSLKSG
21	KWDIYPYSLKSGYIT
22	IYPYSLKSGYITCGK
23	YSLKSGYITCGKGTW
24	KSGYITCGKGTWCEE
25	YITCGKGTWCEEQIC
26	CGKGTWCEEQICECD
27	GTWCEEQICECDRVA
28	CEEQICECDRVAAEC
29	QICECDRVAAECLRR
30	ECDRVAAECLRRSLS
31	RVAAECLRRSLSTYK
32	AECLRRSLSTYKYGY
33	LRRSLSTYKYGYMFY
34	SLSTYKYGYMFYPDS
35	TYKYGYMFYPDSRCR
36	YGYMFYPDSRCRGPS
37	MFYPDSRCRGPSETC

fraction was evaluated by Western Blot (SDS-PAGE 15 % in reducing conditions), proteins were transferred to a nitrocellulose membrane and then incubated with anti-*C. d. terrificus* (1:1000) for 1 h at room temperature. Peroxidase-coupled anti-horse IgG secondary antibody from Millipore (1:6000) detected the reacting proteins. Immunoreaction was detected by DAB/chloronaphthol, according to the manufacturer's instructions.

Expressed rMEPCtx was purified according to Moura et al. (2014). A 5 mL HIS-Trap column (GE Healthcare Life Science) coupled to an ÄKTA Prime chromatography system (GE Healthcare Life Science). Column was equilibrated with 0.03 M imidazole [20 mM sodium phosphate; 0.5 M NaCl; 0.03 M imidazole] and the protein was eluted with 0.5 M imidazole [20 mM sodium phosphate; 0.5 M NaCl; 0.5 M imidazole] and stored in this buffer.

2.5. Anti-rMEPCtx sera production and immunocharacterization

Two groups of two rabbit each were immunized subcutaneously. Group 1, received seven doses of *C. d. terrificus* venom (25 μg/animal/dose); group 2, received seven doses of rMEPCtx (100 μg/animal/dose). Each immunization cycle was performed at 14 day interval. Montanide was used as adjuvant, at a ratio 1:1 (adjuvant: rMEPCtx).

Sera produced in rabbits (anti-rMEPCtx serum, anti-*Crotalus* serum) were evaluated by ELISA, as described above. Plates were coated with either rMEPCtx (10 μg/mL), *C. d. terrificus* (5 μg/mL), Peruvian *C. durissus* (5 μg/mL), *C. d. ruruima* (5 μg/mL), Ctx (5 μg/mL) and *B. jararaca* venoms (5 μg/mL). Different concentrations of anti-rMEPCtx serum were incubated with the antigens. Anti-Rabbit IgG (whole molecule) – Peroxidase secondary antibody (produced in goat - code A0545 – 1 mL - Sigma, 1:10,000 and OPD peroxidase substrate SIGMAFAST from Sigma-Aldrich were added in order to reveal the immunoreaction.

2.6. Median lethal dose (LD_{50}) and neutralizing assays

Median lethal dose (LD_{50}) of Ctx was determined using 5 groups of Swiss female mice 18–22 g (n = 4 for each dose). Animals were injected intraperitoneally (I.P.) with five different concentrations of Ctx (1, 1.4, 2, 2.8 and 3.9 μg/animal), dissolved in 0.15 M PBS. The experimental groups were kept under observation for 48 h, and the number of deaths per group was recorded. The LD_{50}, dose that killed 50 % of the animals was calculated by Probit analysis (Finney, 1971).

A concentration equivalent to $2LD_{50}$ of Ctx was incubated for 60 min at 37 °C with different rabbit sera (pre-immune, anti-*Crotalus*, anti-rMEPCtx). After this period, groups of Swiss mice (n = 4 per serum) were inoculated intraperitoneally with the samples (final volume of 500 μL). Lethality percentage occurring within 48 h after the injection of the toxin/serum mixture was recorded.

2.7. Statistical analysis

Data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using two-way ANOVA and Bonferroni post-test in GraphPad Prism software.

3. Results

3.1. Epitope mapping and molecular analysis of rMEPCtx

Two linear epitopes were mapped in the primary sequence of Ctx, a neurotoxin from *C. d. terrificus*, by measuring the interaction of anti-*Crotalus* horse serum (FUNED) in SPOT immunoassay. The amino acid sequence of Ctx is shown in Fig. 1A and B. A single region with eighteen amino acid residues was highly recognized in the crotapotin sequence (^{84}YKYLQFSPENCQGESQPC^{101}), and a single region of twenty amino acids was recognized in all PLA_2 isoforms. The sequence (^{103}YKNGYMFYPDSRCRGPSETC^{122}) of P 0CAS6 was selected as shown in Fig. 1B. The selected sequence and P24027 differ in only two amino acids, while P0CAS6 and P62022 isoforms differ in only one amino acid. The epitopes identified in the two Ctx subunits are located in the C-terminal region, as shown in Fig. 2A–D.

A second strategy to map Ctx immunogenic epitopes was also attempted. Fragments of cellulose membranes containing peptides derived from Ctx amino acid sequence were inserted subcutaneously into the upper dorsal region of mice. Each animal received a spot with a specific amino acid sequence as shown in Tables 1 and 2. Animals immune response was monitored by ELISA for 3 months, in order to evaluate the production of antibodies against the venom of *C. d. terrificus*. Due to their high reactivity by ELISA, 4 sequences of crotapotina (CA) and 5 of PLA_2 (CB) were considered positive, among them, the sequence 23 of crotapotin Fig. 3A and 16 and 26 of PLA_2 (Fig. 3B), were selected to compose rMEPCtx

Table 5 shows the epitopes selected by each mapping method applied, which were identified as CtxEp. The selected epitopes were located in the schematic model of Ctx primary sequence (Fig. 4A and B) and in its structure (Fig. 4C and D). Ctx crystallographic structure (Protein Data Bank accession number 3R0L) shows that the sequences of the mapped epitopes are located mainly on α-helices, including the region forming the Ctx catalytic site (Fig. 4C). The selected sequences are present on the surface of the Ctx structure (Fig. 4D). It was not possible to identify the complete CtxEp1 in the Ctx structure. Crotapotin CA subunit is a heterotrimer of three disulfide-linked chains (α, β and γ) generated by post-translational maturation of the precursor. CtxEp1 is located mainly in the γ-chain, which is the shorter one and

pdfelement

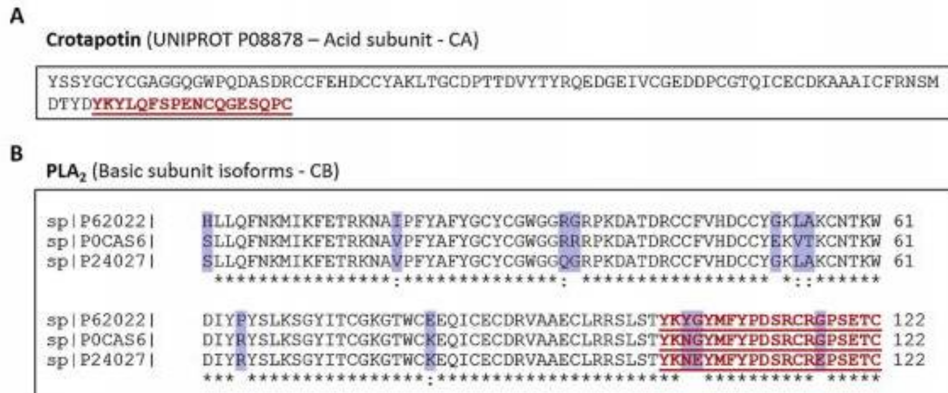


Fig. 1. Primary sequence of Ctx. A sequence of the acid subunit and three proteoforms sequences of the basic PLA_2 subunit, the identified epitopes are highlighted in red. (A) Acid subunit or crotapotin P08878. (B) Aligned sequences of the basic PLA_2 subunits (P0CAS6, P24027, P62022), the different amino acids between the sequences are marked in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2. Identification of linear B-cell epitopes of Ctx. Membrane containing 30 (15mer) overlapping peptides frameshifted by three residues covering the complete sequence of acid subunit or crotapotin and 37 (15mer) overlapping peptides frameshifted by three residues covering the complete sequence of basic subunit PLA_2. Each of the membranes was tested with horse 636 anti-*Crotalus* venom IgG (5 μg/mL) and pre-immune horse serum was used as negative control. (A) P08878, (B) P0CAS6, (C) P24027, (D) P62022.

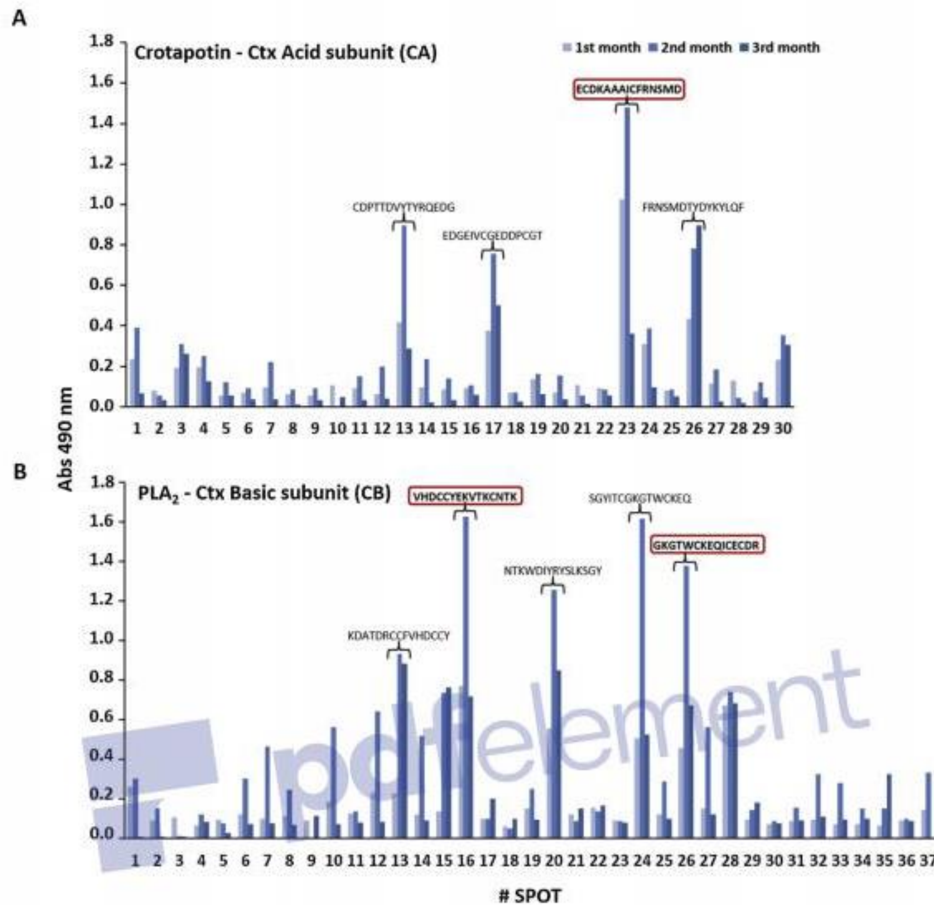


Fig. 3. Reactivity of sera from immunized mice. (A) Spots corresponding to the acid subunit or crotapotin. (B) Spots corresponding to the basic subunit PLA_2. Reactivity was assessed over time by ELISA. Sera were tested against the total venom of *C. d. terrificus* selecting as positive those sera with an absorbance greater than 0.6. The sequences of each subunit of the Ctx that were selected to be part of the multiepitopic protein rMEPCtx and highlight in red boxes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

contains flexible regions that were not represented in the PDB available structure (Faure et al., 2011).

The selected sequences shown in Table 5 were used to produce the rMEPCtx using the vector pET 28a (+)-TEV, Fig. 5A. Epitopes sequences were put *in tandem* and spaced by 2 glycine residues in order to compose rMEPCtx. The amino acid sequence and its codons are shown in Fig. 5B.

The rMEPCtx corresponding DNA was synthesized and the DNA fragment of interest was chemically transformed into *E. coli* BL21 bacteria for expression. Ten bacterial colonies were randomly selected and used as a template in PCR reactions. The forward (5′-CATATGTACAAGTATCTGCAGTTCAGC-3′) and reverse (5′-CTCGAGTTAACGGTCGCATTCGC-3′) primers were designed using Oligo Analyzer 3.1 (www.idtdna.com/calc/analyzer). The expression results were observed in GelRedTM (Uniscience) 2 % agarose gel electrophoresis (Fig. 5C) and the presence of rMEPCtx in the soluble or insoluble fraction was detected by Western Blotting using horse anti-*C. d. terrificus* serum 1:1000, (Fig. 5D).

3.2. Anti-rMEPCtx sera production and characterization

The corresponding rMEPCtx was expressed as a recombinant

Table 5
Crotoxin mapped epitopes. Sequences of amino acids identified as crotoxin epitopes, by the different methods of epitope mapping used.

Subunit to which it belongs	Sort by:	Epitopes	Sequence
Crotapotin	Spot Membrane	CtxEp1	YKYLQFSPENCQGESQPC
Crotapotin	Subcutaneous Implant 23	CtxEp2	CDKAAAICFRNSMDT
Phospholipase A_2	Spot Membrane	CtxEp3	YKNGYMFYPDSRCRGPSETC
Phospholipase A_2	Subcutaneous Implant 16	CtxEp4	VHDCCYEKVTKCNTK
Phospholipase A_2	Subcutaneous Implant 26	CtxEp5	GKGTWCKEQICECDR

pdfelement

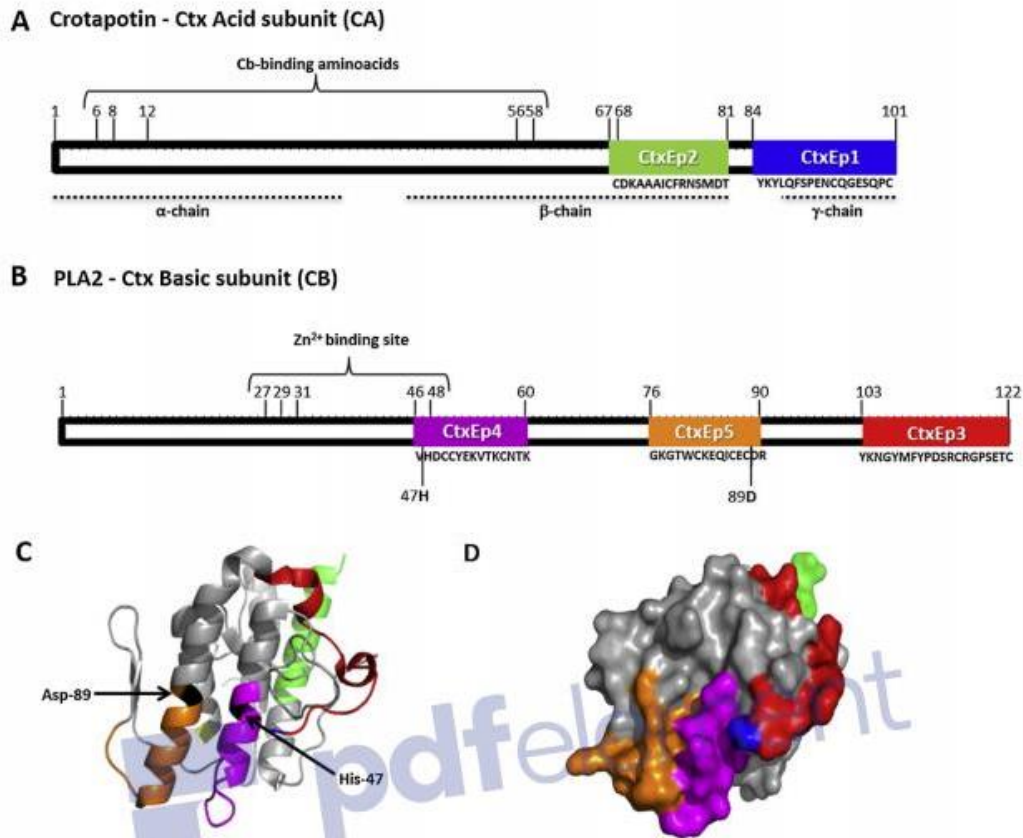


Fig. 4. Ctx structure showing the location of epitopes. (A) Scheme of the primary sequence of the acid subunit, which shows the position of the CtxEp1 and CtxEp2 epitopes in blue and green respectively. (B) Primary sequence of the basic subunit PLA_2, which shows the position of the epitopes CtxEp3, CtxEp4 and CtxEp5 in red, magenta and orange respectively, together with the position of the amino acids of the catalytic site. (C) Visualization of the sequences of the selected spots in the three-dimensional structure of Ctx using Pymol. In black histidine (47) and aspartic acid (89) that make up the catalytic site, the epitopes in the color described in part A and B, in yellow the N-terminal and in blue the C-terminal. (D) Surface configuration, where it can be observed that the sequences of the selected epitopes are exposed on the protein surface. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

protein, composed of 91 amino acids, presenting a molecular weight of 10053.24 g/mol, a pI of 7.13 and a good solubility in water (parameters calculated in PepCalc, available in https://pepcalc.com/). The recombinant protein was used to immunize New Zealand rabbit and the production of anti-rMEPCtx antibodies was monitored by ELISA.

Anti-rMEPCtx sera recognized the purified Ctx, *C. d. terrificus*, *C. d. ruruima*, Peruvian *C. durissus*, *B. jararaca* venoms and rMEPCtx by ELISA, as shown Fig. 6A. Anti-rMEPCtx showed high reactivity with Ctx, *C. d. terrificus* venom and rMEPCtx, although with lower intensity, anti-rMEPCtx was also able to recognize other crotalic venoms and *B. jararaca* venom in a statistically significant fashion. The analysis of anti-rMEPCtx sera titer by ELISA, using a fixed concentration of *C. d. terrificus* venom (0.5 μg/well) against different serum concentrations, showed a concentration dependent reactivity. Compared with the reactivity of the serum obtained by immunization with crude *C. d. terrificus* venom, anti-rMEPCtx serum presented no significant differences, indicating comparable antivenom titers (Fig. 6B).

3.3. Neutralization assays

Ctx lethality was investigated in mice and its LD_{50} was determined as 2.43 (± 0.15) μg/20 g (Table 6). We next analyzed the capacity of rabbit sera from the 2 groups of immunized animals to neutralize the lethal activity induced by Ctx. Pre-immune rabbit serum was used as a control. The number of surviving animals was quantified 48 h after the challenge.

Ctx ($2LD_{50}$) was 100 % lethality in the control groups, in which Ctx was pre-incubated either with PBS or pre-immune rabbit sera. Sera from animals immunized with *C. d. terrificus* venom, neutralized the lethal effect of Ctx in a ratio of $2LD_{50}$/400 μL of serum. Using a similar volume of serum, anti-rMEPCtx sera protected 75 % of challenged animals and, when used in a ratio of $2LD_{50}$/500 μL of serum, rabbit sera from animals immunized with rMEPCtx,completely neutralized Ctx lethal activity, as shown in Table 7.

4. Discussion

Lethality of *C. d. terrificus* venom is mainly due to Ctx neurotoxic activity, the major component of this venom (Calvete et al., 2010; Sampaio et al., 2010). Because of this, Ctx efficient neutralization is critical to the good performance of antivenom therapy. B-cell epitopes identification can help in the development of antibodies directed to specific venom proteins, for therapeutic purposes or as diagnostic tools. In this work, we sought to map B-cell linear epitopes from Ctx using two

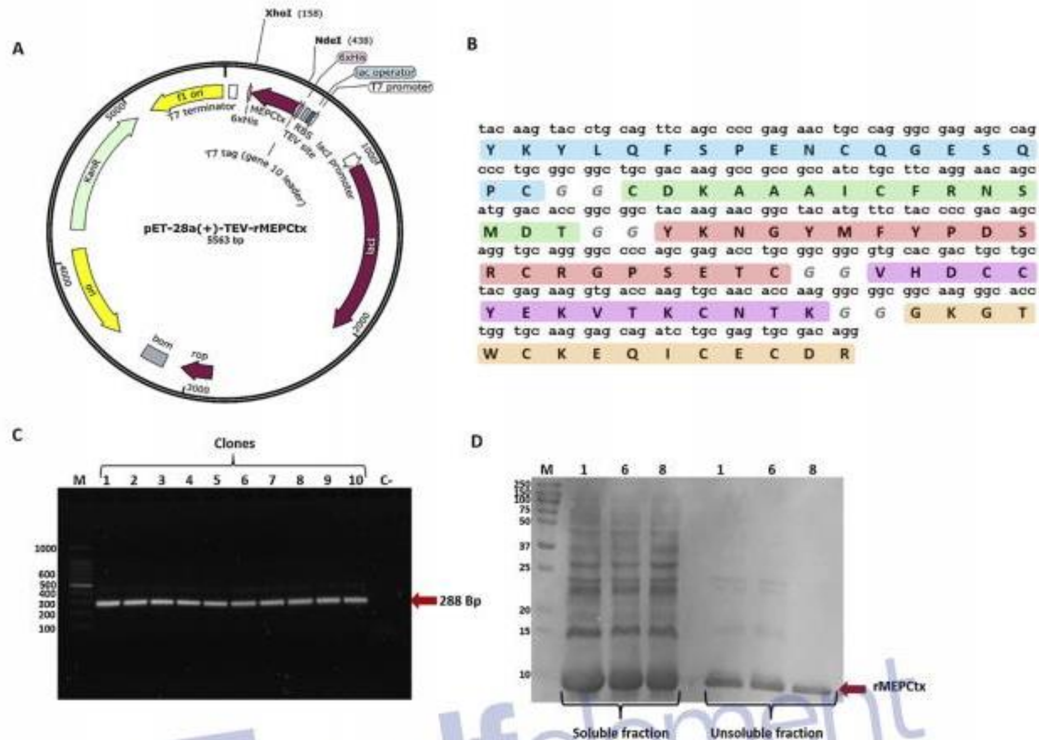


Fig. 5. rMEPCtx amino acid sequence. (A) Vector used for the expression of rMEPCtx. (B) Sequencing of codons and amino acids that make up rMEPCtx. (C) Insert of rMEPCtx in 2 % agarose gel. Starting with the molecular weight marker, column 1 −10 clones expressing rMEPCtx, negative control column 11, a red arrow pointing to the position of the 288 bp insert. (D) Western Blot analysis of the insoluble and soluble fraction of rMEPCtx clones 1, 6 and 8 against horse anti-*C. d. terrificus* serum (1:1000). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

pdfelement

different methods and validate epitope-based antibodies as an alternative or complementary therapy to conventional antivenoms.

The amino acid sequence corresponding to the C-terminal of the basic PLA_2 subunit of Ctx is highly conserved and it was recognized by the anti-crotalic serum in the SPOT assay used to map Ctx epitopes (Figs. 1 and 2). Data show that these proteins regions share a possible common and immunodominant epitope. Interestingly, the region identified is conserved among Ctx isoforms (Faure et al., 2011).

A second B-cell epitope mapping assay was performed, assessing the ability of subcutaneous implants, made of cellulose-bound peptides, to generate antibodies capable of recognizing Ctx (Fig. 3), (Lima et al.,

Table 6

Crotoxin lethal activity. Groups of 4 mice were injected intraperitoneally with different doses of crotoxin. The number of surviving mice was counted 48 h after the injection.

Crotoxin (μg/20 g mouse)	Dead/challenged	Death (%)
1	0/4	0
1.4	0/4	0
2	1/4	25
2.8	3/4	75
3.9	4/4	100

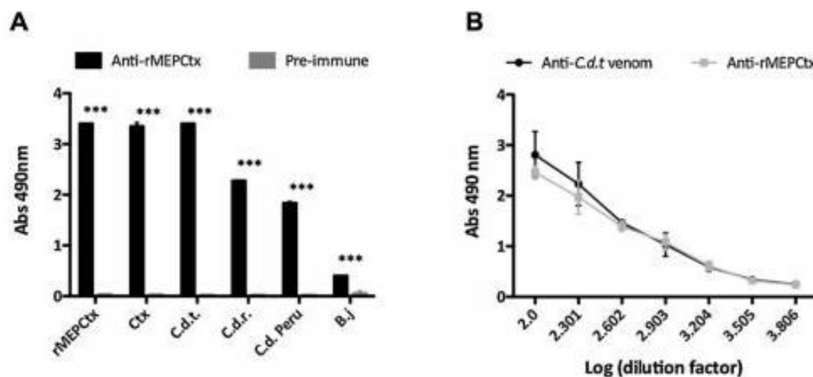


Fig. 6. Reactivity of anti-C.d.t venom and anti-rMEPCtx rabbit sera in ELISA. (A) Immune recognition of rMEPCtx (10 μg/well), Ctx, *C. d. terrificus*, *C. d. ruruima*, Peruvian *C. durissus and B. jararaca* venom, each at a concentration of 0.5 μg/well tested against anti-rMEPCtx serum and control rabbit preimmune serum 1:100. *** correspond to $P < 0.001$. (B) Reactivity of different dilutions of anti- *C. d. terrificus* and anti-rMEPCtx sera against *C. d. terrificus* venom (0.5 μg/well). Rabbit pre-immune serum was used as negative control.

Table 7
Neutralization of the neurotoxic activity of Ctx *in vivo*. Mice were injected intraperitoneally with $2LD_{50}$ of Ctx (4.8 µg), pre-incubated for 1 h at 37 °C with serum from different groups of immunized rabbits. The number of surviving mice was counted 48 h after the injection.

Experimental group	Challenge dose (Ctx)	Serum volume (µl)	Dead/challenged	Mice mortality after 48 h (%)
PBS	2 LD_{50}	500 µL	4/4	100
Pre-immune sera		500 µL	4/4	100
Anti-*C. d. terrificus* venom		400 µL	0/4	0
Anti-rMEPCtx		400 µL	1/4	25
		500 µL	0/4	0

1999). The reactivity of mice antibodies elicited by the implants against crotalic venom was evaluated by ELISA, identifying immunoreactive regions that differs from those found in the first method applied (SPOT assay).

Animals subjected to adjuvant immunization for a long period of time often show skin lesions followed by a reduction in their ability to produce antibodies (Bolanos and Cerdas, 1978). Iodination of proteins and encapsulation of the antigen in liposomes are examples of methods that have been proposed to avoid these problems but these strategies did not completely solve the issue (Lima et al., 1999). In response to this, we proposed the use of subcutaneous implants to promote immunization, which showed to be a viable alternative. We hypothesize that the release of antigens from the subcutaneous implants may be slower and continuous therefore promoting a longer and persistent stimulus to the immune system. This was corroborated by our observation that antibody production remained high at least until 3 months after animals have received the implant (Fig. 3). Antigen slow release represents an advantage as it maximizes antibodies production with a single antigen dose and allows that immunogenic substances remain in the body for a long period, giving the organism time to develop an adequate immune response (Lima et al., 1999).

Using our epitope mapping strategy, five B-cells epitopes were selected (Table 5): 2 epitopes based on the spot membrane result and 3 selected by the subcutaneous implants test. The latter were selected based on their reactivity intensity in ELISA and exposure in the 3D structure Ctx (Faure et al., 2011), which demonstrates their availability for binding. CtxEp4 epitope contains histidine 47 residue and CtxEp5 contains aspartic acid 89 residue (Fig. 4B and C), which are the amino acids that compose Ctx catalytic dyad (Faure et al., 2011). This makes these two epitopes very important targets, as their recognition by antibodies may lead to the neutralization of Ctx catalytic activity, which responsible for many of Ctx deleterious effects.

Sequences of the selected epitopes were expressed simultaneously (each sequence was spaced by two glycine residues), resulting in a new recombinant multiepitopic protein (rMEPCtx). This engineered protein has a total of 91 amino acids, 20 of which are charged residues. These charged residues might and play a fundamental role in the molecules antigenicity, as they seem to be critical for antigen-antibody binding (Machado De Avila et al., 2004; Molina et al., 2018). We were able to demonstrate the surface exposure of the selected epitopes, showing their availability antibody binding (Fig. 4D). We chose to include only 5 epitopes to form the rMEPCtx in order to avoid an excessively large and complex protein that could present unusual folds, masking possible relevant epitopes (Lima et al., 2018).

Since circulating antibodies able to bind Ctx could be detected, cross reactivity of anti-rMEPCtx sera against *C. d. terrificus* and other snake venoms from the Viperidae family was also evaluated. Anti-rMEPCtx sera were tested against venoms of *C. d. ruruima*, Peruvian *C. durissus*, which are other species of the *Crotalus* genus present in South America, and against *B. jararaca* venom, the species responsible for majority of snakebites in Brazil (Estevao-Costa et al., 2016; Fusco et al., 2015). We found that anti-rMEPCtx sera, recognized all tested venoms, although with different intensities. The reactivity against *C. d. ruruima* and Peruvian *C. durissus* was less intense when compared to the specific venom, indicating the possibility that *Crotalus* spp. crude venoms possess different proportion of Ctx, as suggested by (Boldrini-França et al., 2010), or subtle sequence variations that impaired reactivity, but supporting the notion that these toxic antigens share similar epitopes. A lower reactivity was also detected against *B. jararaca* venom compared to the reactivity found against crotalic venoms (Fig. 6A). This was expected since PLA_2 is not the main toxic component in this snake venom. It has been recorded that *B. jararaca* venom contains only 3.2 % of PLA_2 (Sousa et al., 2013), a remarkably smaller amount compared to the percentage of PLA_2 in crotalic venom. Nonetheless, a PLA_2 capable of inhibiting platelet-aggregation and inducing acute inflammation in mice was purified from *B. jararaca* venom (Cedro et al., 2018; Serrano et al., 1999), indicating that the neutralization of this enzyme may be beneficial to prevent deleterious *B. jararaca* venom effects.

In the final part of this investigation, neutralization of lethal activity of Ctx from *C. d. terrificus* venom by anti-rMEPCtx serum was evaluated. Control group mice, which received the $2LD_{50}$ of Ctx diluted in PBS or pre-immune serum, showed no protection. Pre-incubation of Ctx with 400 µL of anti-*C. d. terrificus* venom serum, used as positive control, completely neutralized crotoxin lethal effects, while the experimental group, containing Ctx pre-incubated with 400 µL of anti-rMEPCtx serum showed 75 % neutralization, being necessary to increase serum the volume. When challenging a new experimental group with Ctx pre-incubated with 500 µL of anti-rMEPCtx serum, a complete neutralization of the lethal activity was observed, as all animals survived. Although it was necessary to use a higher volume of anti-rMEPCtx serum, the use of the recombinant protein as antigen is advantageous.

This difference in efficiency between the anti-*C. d. terrificus* venom serum and the anti-rMEPCtx serum can be given by the fact that the anti-venom serum is produced from the immunization with the toxins in their native state. In this sense an immunological response where antibodies are directed against both linear or conformational Ctx epitopes take place, in comparison with the anti-rMEPCtx serum that is produced from a recombinant protein that contains exclusively previously identified linear epitopes. It is known that most B cell epitopes are conformational (~90 %) (Ansari and Raghava, 2010), but these are difficult to simulate as synthetic antigens.

In this work, the continuous epitopes of Ctx have shown to be efficient in inducing a neutralizing humoral response. The results encourage the use of such synthetic immunogens for the improvement of therapeutic sera production and/or for vaccination approaches, since rMEPCtx is not toxic and can be produced on a large scale.

However, it is important to remember that *C. d. terrificus* venom contains other components relevant to lethality, such as crotamine. Ctx has an LD_{50} of 2.4 µg/20 g and crotamine an LD_{50} of 820 µg/25 g, i.p. injection (Nicastro et al., 2003). This clear difference in toxicity directed our approach of prioritizing the neutralization of the toxic effects of Ctx in this first work. Notwithstanding addressing the neutralization of other relevant toxic components of crotalic venom is in our group perspectives.

Aiming the improvement of immunotherapy for snakebites, we envision an approach that can produce more potent antivenoms from synthetic molecules, reducing the use of venoms as immunogens. Although venoms have proven to be efficient immunogens, since they can elicit neutralizing antibodies, they require a complex infrastructure, suffer variability and are toxic to producer animals. Therefore, the

current immunization protocol for anti-crotalic antivenom production could be optimized by developing other synthetic immunogens from other toxins, such as crotamine, used together with the here-presented rMEPCtx.

Together with previous works (Lima et al., 2018; Molina et al., 2018), our results provide a conceivable basis for the design and production of multiepitopic proteins and can improve the conception of new immunotherapeutic elements against venoms.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgements

This research was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil (CNPq) (Process: 406163/2018-9), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil - CAPES (Program COFECUB Process: 88881.191812/2018-01) and by Fundação de Amparo à Pesquisa do Estado de Minas Gerais, Brazil (FAPEMIG).

References

Ainsworth, C., 2018. Building a better lymphoma vaccine. Nature 563, 552–554.

Amaral, F.S.C., Magalhães, R.A., 1991. Comprometimento respiratório secundário a acidentes ofídico crotálico (Crotalus durissus). J. Mater. Sci. Lett. 33, 251–255. https://doi.org/10.1023/A:1026313314513.

Amaral, C.F., de Rezende, N.A., da Silva, O.A., Ribeiro, M.M., Magalhães, R.A., dos Reis, R.J., Carneiro, J.G., Castro, J.R., 1986. Insuficiência renal aguda secundária a acidentes ofídicos botrópico e crotálico. Análise de 63 casos. Rev. Inst. Med. Trop. Sao Paulo 28, 220–227. https://doi.org/10.1590/S0036-46651986000400003.

Amaral, C.F.S., Campalina, D., Dias, M.B., Bueno, C.M., Chávez-Olortegui, C., Penaforte, C.L., Diniz, C.R., Rezende, N.A., 1997. Time factor in the detection of circulanting whole venom and crotoxin and efficacy of antivenom therapy in patients envenomed by crotalus durissus. Toxicon 35, 699–704.

Ansari, H.R., Raghava, G.P.S., 2010. Identification of conformational B-cell epitopes in an antigen from its primary sequence. Immunome Res. 6, 6. https://doi.org/10.1186/1745-7580-6-6.

Bolanos, R., Cerdas, L., 1978. The production and control of anti-venous sera. Dev. Biol. Stand. 41, 109–116.

Boldrini-França, J., Corrêa-Netto, C., Silva, M.M.S., Rodrigues, R.S., De La Torre, P., Pérez, A., Soares, A.M., Zingali, R.B., Nogueira, R.A., Rodrigues, V.M., Sanz, L., Calvete, J.J., 2010. Snake venomics and antivenomics of Crotalus durissus subspecies from Brazil: assessment of geographic variation and its implication on snakebite management. J. Proteom. 73, 1758–1776. https://doi.org/10.1016/j.jprot.2010.06.001.

Brasil, A.D.E.N., 2019. DATASUS Tecnologia da Informação a Serviço do SUS.

Calvete, J.J., Sanz, L., Cid, P., de la Torre, P., Flores-Díaz, M., Dos Santos, M.C., Borges, A., Bremo, A., Angulo, Y., Lomonte, B., Alape-Girón, A., Gutiérrez, J.M., 2010. Snake venomics of the Central American rattlesnake *Crotalus simus* and the South American *Crotalus durissus* complex points to neurotoxicity as an adaptive paedomorphic trend along *Crotalus* dispersal in South America. J. Proteome Res. 9, 528–544. https://doi.org/10.1021/pr9008749.

Cedro, R.C.A., Menaldo, D.L., Costa, T.R., Zoccal, K.F., Sartim, M.A., Santos-filho, N.A., Faccioli, L.H., Sampaio, S.V., 2018. Cytotoxic and inflammatory potential of a phospholipase A 2 from Bothrops jararaca snake venom. J. Venom. Anim. Toxins Incl. Trop. Dis. 24, 1–14. https://doi.org/10.1186/s40409-018-0170-y.

Chippaux, J.P., 2010. [Guidelines for the production, control and regulation of snake antivenom immunoglobulins]. Biol. Aujourdhui 204, 87–91.

Estevao-Costa, M.I., Gontijo, S.S., Correia, B.L., Yarleque, A., Vivas-Ruiz, D., Rodrigues, E., Chávez-Olortegui, C., Oliveira, L.S., Sanchez, E.F., 2016. Neutralization of toxicological activities of medically-relevant Bothrops snake venoms and relevant toxins by two polyvalent bothropic antivenoms produced in Peru and Brazil. Toxicon 122, 67–77. https://doi.org/10.1016/j.toxicon.2016.09.010.

Faure, G., Bon, C., 1987. Several isoforms of crotoxin are present in individual venoms from the South American rattlesnake Crotalus durissus terrificus. toxicon 25, 229–234.

Faure, G., Xu, H., Saul, F.A., 2011. Crystal structure of crotoxin reveals key residues involved in the stability and toxicity of this potent heterodimeric β-neurotoxin. J. Mol. Biol. 412, 176–191. https://doi.org/10.1016/j.jmb.2011.07.027.

Finney, D.J., 1971. Probit Analysis, 3rd ed. Cambridge University Presshttps://doi.org/10.1002/jps.2600600940. Journal of Pharmaceutical Sciences 60, 1432.

Frank, R., 1992. Spot-synthesis: an easy technique for the positionally addressable, parallel chemical synthesis on a membrane support. Tetrahedron 48, 9217–9232. https://doi.org/10.1016/S0040-4020(01)85612-X.

Fusco, L.S., Pablo, J., Torres-huaco, F., Huancahuire-vega, S., Teibler, P., Acosta, O., Marangoni, S., Ponce-soto, L.A., Leiva, L.C., 2015. P9a (Cdt -PLA 2) from Crotalus durissus terrificus as good immunogen to be employed in the production of crotalic anti-PLA2 IgG. Toxicol. Lett. 238, 7–16. https://doi.org/10.1016/j.toxlet.2015.06.528.

Gazarian, K.G., Gazarian, T., Hern, R., Possani, L.D., 2005. Immunology of scorpion toxins and perspectives for generation of anti-venom. vaccines 23, 3357–3368. https://doi.org/10.1016/j.vaccine.2004.12.027.

Laune, D., Molina, F., Ferrières, G., Villard, S., Bès, C., Rieunier, F., Chardès, T., Granier, C., 2002. Application of the Spot method to the identification of peptides and amino acids from the antibody paratope that contribute to antigen binding. J. Immunol. Methods 267, 53–70. https://doi.org/10.1016/S0022-1759(02)00140-0.

Lima, S.L., Machado, C.B., Pereira, M.A.C., Cara, D.C., Velarde, D.T., Andrade, S.P., Gontijo, C.M., 1999. Immunization by subcutaneous implants of polyester-polyurethane sponges coupled with antigen. Braz. J. Med. Biol. Res. 32, 443–447. https://doi.org/10.1590/S0100-879X1999000400011.

Lima, S., de, A., Guerra-Duarte, C., Costal-Oliveira, F., Mendes, T.M., Figueiredo, L.F.M., Oliveira, D., Machado de Avila, R.A., Ferrer, V.P., Trevisan-Silva, D., Veiga, S.S., Minozzo, J.C., Kalapothakis, E., Chávez-Olórtegui, C., 2018. Recombinant protein containing B-cell epitopes of different Loxosceles spider toxins generates neutralizing antibodies in immunized rabbits. Front. Immunol. 9. https://doi.org/10.3389/fimmu.2018.00653.

Machado De Avila, R.A., Alvarenga, L.M., Tavares, C.A.P., Molina, F., Granier, C., Chávez-Olórtegui, C., 2004. Molecular characterization of protective antibodies raised in mice by Tityus serrulatus scorpion venom toxins conjugated to bovine serum albumin. Toxicon 44, 233–241. https://doi.org/10.1016/j.toxicon.2004.04.015.

Mendes, T.M., Oliveira, D., Figueiredo, L.F.M., Machado-de-Avila, R.A., Duarte, C.G., Dias-Lopes, C., Guimarães, G., Felicori, L., Minozzo, J.C., Chávez-Olortegui, C., 2013. Generation and characterization of a recombinant chimeric protein (rCpLi) consisting of B-cell epitopes of a dermonecrotic protein from Loxosceles intermedia spider venom. Vaccine 31, 2749–2755. https://doi.org/10.1016/j.vaccine.2013.03.048.

Ministerio de Saude, 2019. Casos de ofidimos. Ministerio de saude do Brasil.

Molina, F., Laune, D., Gougat, C., Pau, B., Granier, C., 1996. Improved performances of spot multiple peptide synthesis. Pept. Res. 9, 151–155.

Molina, D.A., Guerra-duarte, C., Naves, D.L., Souza, D., Costal-oliveira, F., Reis, G., Ávila, D., Soccol, V.T., Machado-de-ávila, R.A., Chávez-olórtegui, C., 2018. Identification of a linear B-cell epitope in the catalytic domain of bothropasin, a metalloproteinase from Bothrops jararaca snake venom. Mol. Immunol. 104, 20–26. https://doi.org/10.1016/j.molimm.2018.10.019.

Moura, A., Rosa, A., Tenório, F., Rodrigues, G., Alves, R., Nagem, P., Daniel, R., Magalhães, M., Luis, J., Cunha, R., Castanheira, D., Monteiro, H., Andrade, D., 2014. Evaluation of three recombinant Leishmania infantum antigens in human and canine visceral leishmaniasis diagnosis. Acta Trop. 137, 25–30. https://doi.org/10.1016/j.actatropica.2014.04.028.

Nicastro, G., Franzoni, Lorella, de Chiara, Cesira, Mancin, Adriana C., Giglio, J.R., Spisni, A., 2003. Solution structure of crotamine, a Na+ channel affecting toxin from Crotalus durissus terrificus venom. Eur. J. Biochem. 270, 1969–1979. https://doi.org/10.1046/j.1432-1033.2003.03563.x.

Quintana, J.C., Chacón, A.M., Vargas, L., Segura, C., Gutiérrez, J.M., Alarcón, J.C., 2012. Antiplasmodial effect of the venom of Crotalus durissus cumanensis, crotoxin complex and Crotoxin B. Acta Trop. 124, 126–132. https://doi.org/10.1016/j.actatropica.2012.07.003.

Rangel-santos, A., Lima, C., Lopes-ferreira, M., Cardoso, D.F., 2004. Immunosuppresive role of principal toxin (crotoxin) of Crotalus durissus terrificus venom. Toxicon 44, 609–616. https://doi.org/10.1016/j.toxicon.2004.07.004.

Rangel-Santos, A., Mota, I., 2000. Effect of heating on the toxic, immunogenic and immunosuppressive activities of Crotalus durissus terrificus venom. Toxicon 38, 1451–1457.

Sampaio, S.C., Hyslop, S., Fontes, M.R.M., Prado-Franceschi, J., Zambelli, V.O., Magro, A.J., Brigatte, P., Gutierrez, V.P., Cury, Y., 2010. Crotoxin: novel activities for a classic β-neurotoxin. Toxicon 55, 1045–1060. https://doi.org/10.1016/j.toxicon.2010.01.011.

Serrano, S.M.T., Reichl, A.P., Mentele, R., Auerswald, E.A., Santoro, M.L., Sampaio, C.A.M., Camargo, A.C.M., Assakura, M.T., 1999. A novel phospholipase A2, BJ-PLA2, from the venom of the snake Bothrops jararaca: purification, primary structure analysis, and its characterization as a platelet-aggregation-Inhibiting factor 1. Arch. Biochem. Biophys. 367, 26–32. https://doi.org/10.1006/abbi.1999.1230.

Sousa, L.F., Nicolau, C.A., Peixoto, P.S., Bernardoni, J.L., Portes-junior, A., Moura, R.H.V., Oliveira, S., Chalkidis, M., Valente, R.H., Moura-da-silva, A.M., Sano-martins, I.S., 2013. Comparison of phylogeny, venom composition and neutralization by antivenom in diverse species of Bothrops complex. PLoS One 7, 1–16. https://doi.org/10.1371/journal.pntd.0002442.

Teixeira-Araújo, R., Castanheira, P., Brazil-Más, L., Pontes, F., Leitão de Araújo, M., Machado Alves, M.L., Zingali, R.B., Correa-Netto, C., 2017. Antivenomics as a tool to improve the neutralizing capacity of the crotalic antivenom: a study with crotamine. J. Venom. Anim. Toxins Incl. Trop. Dis. 23, 1–8. https://doi.org/10.1186/s40409-017-0118-7.

WHO, 2016. Post ECBS Version EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION Geneva, 17 to 21 October 2016 WHO Guidelines for the Production, Control and Regulation of Snake Antivenom Immunoglobulins. World Health Organization, pp. 1–138.

3.3 Artigo 3. Proteomic and toxinological characterization of Peruvian pitviper *Bothrops brazili* ("jergón shushupe"), venom

(2020) Carolina Rego Rodrigues, Denis Alexis Molina Molina, Thamyres C. Silva de Assis, Camila Liberato, Marcella N. Melo-Braga, César Bonilla Ferreyra, Javier Cárdenas, Fernanda Costal-Oliveira, Clara Guerra-Duarte, Carlos Chávez-Olórtegui

As serpentes do gênero *Bothrops* são as principais causadoras de acidentes ofídicos na América do Sul, com altas taxas de morbidade e mortalidade. Atualmente, 47 espécies são reconhecidas e a pesar de sua relevância, seus venenos não foram amplamente caracterizados. *Bothrops brazili* (Hoge, 1953), popularmente conhecida no Peru como "Jergon Shushupe", é uma espécie encontrada em toda a América do Sul.

Algumas espécies isoladas reprodutivamente por barreiras naturais apresentam variações na composição do veneno. Numerosos estudos demonstraram que há variação na composição dos venenos de diferentes espécies de serpentes de maneira intraespecífica, bem como as implicações dessas diferenças para o manejo clínico de acidentes do ponto de vista diagnóstico, terapêuticos e de produção de antivenenos.

Com base nessas implicações, foi analisada a composição do proteoma do veneno de *B. brazili* do Peru, utilizando as técnicas de HPLC, MALDI-TOF, SDS-PAGE, o que fornece uma visão geral do conteúdo de veneno da *B. brazili*.

Author's Personal Copy

Toxicon 184 (2020) 19–27

Contents lists available at ScienceDirect

Toxicon

journal homepage: http://www.elsevier.com/locate/toxicon

Proteomic and toxinological characterization of Peruvian pitviper *Bothrops brazili* ("jergón shushupe"), venom

Carolina Rego Rodrigues [a], Denis Alexis Molina Molina [a], Thamyres C. Silva de Assis [a], Camila Liberato [a], Marcella N. Melo-Braga [a], César Bonilla Ferreyra [b], Javier Cárdenas [c], Fernanda Costal-Oliveira [a], Clara Guerra-Duarte [d], Carlos Chávez-Olórtegui [a,*]

[a] *Departamento de Bioquímica e Imunologia, Instituto Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil*
[b] *Instituto Nacional de Salud, Lima, Peru*
[c] *Universidad Nacional del Callao, Callao, Peru*
[d] *Centro de Pesquisa e Desenvolvimento, Fundação Ezequiel Dias, 30510-010, Belo Horizonte, MG, Brazil*

ARTICLE INFO

Keywords:
Bothrops brazili
Amazonian region
Proteomic analyses
MALDI-TOF/TOF
Bothropic venoms

ABSTRACT

Bothrops brazili is a pitviper from Amazonian region, responsible for many accidents in Peru. Despite its relevance, its venom has not been extensively characterized. In the present work, *Bothrops brazili* venom (*BbV*) components were analyzed by RP-HPLC, SDS-PAGE and MALDI-TOF/TOF. Approximately 37 proteins were identified, belonging to 7 families. Snake venom metalloproteinases (SVMPs) were the most abundant proteins of the venom (33.05%), followed by snake venom serine proteinases (SVSPs, 26.11%), phospholipases A_2 (PLA_2, 25.57%), snake C-type lectins (CTLs, 9.61%), L-aminoacid oxidase (LAAO, 3.80%), cystein-rich secretory proteins (CRISP, 1.67%) and Bradykinin-potentiating peptide (BPP, 0.20%). *In vitro* enzymatic activities of *BbV* showed high levels of SVMP activity and reduced Hyal activity in comparison with other bothropic venoms. Furthermore, *BbV* reduced VERO cells viability. ELISA and Western Blotting showed that both Peruvian and Brazilian bothropic antivenoms were able to recognize *BbV* components. This work provides an overview of *BbV* venom content and indicates a potential efficiency of Peruvian and Brazilian antivenoms to treat accidents with this species.

1. Introduction

Bothrops brazili (Hoge, 1953), popularly known as "Jergón Shushupe", is a pitviper found throughout South America, especially in Colombia, Ecuador, Guyana, Peru, Suriname, French Guiana and Amazon region of Brazil (Campbell and Lamar, 2004). *B. brazili* can be found in Peru in the Departments of Amazonas, Madre de Dios, Loreto and Ucayali, at an altitude of 600 m above sea level (Ministerio de salud, 2005) (Fig. 1). It is a medium length pitviper (approximately 1.3 m) from the Viperidae family, with a thick brown body and dark triangular designs.

B. brazili is known for inoculating large amounts of venom (approximately 3–4 mL) in its victims when compared to other Viperids (Ministerio de salud, 2005; Zavaleta and Campos, 1992). Therefore, the cases of envenomation caused by this species could have serious consequences, both for the toxic components present in its venom and for the inoculated volume.

Specific treatment of bothropic envenomation in Peru is based on Peruvian bothropic antivenom (P-BAV). This antivenom is composed of an IgG solution obtained from horses immunized with a pool of venoms, consisted of 50% of *Bothrops atrox* venom and 12.5% of other species (*Bothrops pictus*, *Bothrops barnetti*, *B. brazili* and *Bothrocophias hyoprora*) venoms (Laing et al., 2004). Despite *B. brazili* presence in the antigenic pool used in P-BAV production, its venom composition and enzymatic activities, as well as its recognition by P-BAV, have been poorly studied.

Understanding the venom composition of medically relevant snakes from the South America equatorial zone, such as *B. brazili*, as well as the neutralization and cross-recognition of its toxins by antivenoms, could assist us in the manufacturing process of a shared antivenom for Latin America.

* Corresponding author. Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Avenida Antônio Carlos, 6627, Belo Horizonte, CEP: 31270-901, Brazil.
E-mail address: olortegi@icb.ufmg.br (C. Chávez-Olórtegui).

https://doi.org/10.1016/j.toxicon.2020.05.016
Received 6 February 2020; Received in revised form 16 May 2020; Accepted 21 May 2020
Available online 29 May 2020
0041-0101/© 2020 Elsevier Ltd. All rights reserved.

Author's Personal Copy

Based on the above considerations, this study shows the proteomic analyses and relative quantification of *B. brazili* venom (*Bb*V) proteins, some of its enzymatic activities and immunological characterization.

2. Material and methods

2.1. Animals, venom samples and antivenoms

Female Swiss mice (n = 4), weighing 18–22 g, were maintained in an animal facility within the Federal University of Minas Gerais (UFMG), Brazil. The animals were maintained under controlled environmental conditions and received water and food *ad libitum*. Experiments were performed in accordance with the Ethics Committee in Animal Experimentation (CEUA/UFMG 321/2018). 2.5 mg *B. brazili* venom, and *B. atrox* venom, used as control, were provided by the Instituto Nacional de Salud (INS) from Lima, Peru. These venoms were pooled from many *B. brazili* and *B. atrox* adults specimens respectively, and stored at −20 °C until use. The vial of Peruvian bothropic antivenom (P-BAV) was produced by INS (lot 10200221) and the vial of Brazilian bothropic antivenom (B-BAV) was produced by Fundação Ezequiel Dias (FUNED). B-BAV was obtained by immunization of horses with venoms from *B. jararaca* (50%), *B. alternatus, B. neuwiedi, B. moojeni* and *B. jararacussu* (12.5% each). Both antivenoms were stored at 4 °C until further use.

2.2. Proteomic characterization

2.2.1. RP-HPLC protein separation and SDS PAGE

Proteins from 2 mg of crude *Bb*V were dissolved in 0.1% trifluoroacetic acid (TFA) with 5% acetonitrile (ACN) and centrifuged at 1400 rpm for 4 min to remove insoluble materials. Venom proteins were separated using RP-HPLC C-8 (0.46 cm × 25 cm, 5 μm particle size, 300 Å pore size) column as previously described (Juárez et al., 2004; Rodrigues et al., 2018) with modifications at a flow rate of 1 mL/min. The sample was eluted using a linear gradient of 0.1% TFA in water (solution A) and ACN (solution B), following the protocol: 5% of B for 5 min, 5–30% for 5 min, 30–70% for 60 min and 70–100% for 5 min.

Protein detection was performed at 215 nm. Fractions were collected manually, vacuum-dried and re-dissolved in a solution containing 125 mM Tris–HCl, pH 6.8, 50% glycerol, 10% SDS, 0.1% bromophenol blue, 5% β-mercaptoethanol. The obtained fractions were further separated by SDS-PAGE (1-DE) under reducing conditions as described by Laemmli (1970). Gels were stained with Coomassie Brilliant Blue R-250 and densitometry was performed using the ImageLab (BioRad) program.

2.2.2. In-gel tryptic digestion

Selected bands were cut from SDS-PAGE gels and subjected to reduction with 10 mM DTT (Dithiothreitol) in 100 mM ammonium bicarbonate at 37 °C for 30 min and to alkylation with 100 mM iodoacetamide in 100 mM ammonium bicarbonate for 30 min at 37 °C, as described by Juárez et al. (2004) with modifications (Juárez et al., 2004). The fragments were then washed with 100 mM ammonium bicarbonate and dehydrated with acetonitrile. Finally, samples were subjected to digestion with 20 μL of trypsin (20 μg/μL in 40 mM ammonium bicarbonate, containing 9% of acetonitrile) at 37 °C for 16 h. Triplicates of the samples were made and desalted using C18 pipette tip columns (ZipTips, Millipore).

2.2.3. Mass spectrometry and database search

Desalted peptide samples (1 μL) were mixed with 0.5 μL of matrix solution (1% α-cyano-4hydroxycinnamic acid in 0.1% TFA, 50% ACN) and applied to a MALDI target plate. Matrix Assisted Laser Desorption/Ionization time of flight (MALDI-TOF) was used to determine masses, using a Bruker Autoflex III Smartbeam MALDI-TOF/TOF instrument in positive reflector mode, controlled by the FlexControl™ and FlexAnalyses™ softwares.

Calibration was performed using a mixture of Peptide Calibration Standard II (bradykinin m/z = 757.39; angiotensin II m/z = 1046.54; angiotensin I m/z = 1296.68; substance P m/z = 1347.73; bombesin m/z = 1619.82; ACTH (1–17) m/z = 2093.08; ACTH (18–39) m/z = 2465.19 and somatostatin (28) m/z = 3147.47).

The mass spectra of digested proteins (MS and MS/MS) were combined by BioTools software and searched against SwissProt-Database using MASCOT search engine (www.matrixscience.com). The parameters for database search were: Carbamidomethylation of the cysteine residues, as fixed modification, and the oxidation of methionine (M), as variable modification. For all searches, trypsin was specified as the used

Fig. 1. Distribution of *B. brazili* in Peru. The area in blue represents the Peruvian Departments where the pitviper has been reported. Snake photo was taken from the site: (https://www.tropicalherping.com/science/books/reptiles/viperidae.html).

Author's Personal Copy

enzyme and two missed enzymatic cleavages were allowed. A fragment mass tolerance of 0.80 Da and a peptide mass tolerance of 0,8% were permitted. If the sequences did not match with deposited proteins, their spectra were manually inspected and their sequences were subjected to protein-BLAST-program at NCBI (National Center for Biotechnology Information). All searches were restricted to the Snakes (taxid: 8570).

2.2.4. Relative protein quantification

The relative protein quantification was performed according to Eichberg et al. (2015). Briefly, the relative abundances of the venom protein families were estimated by dividing the total percentage of proteins from the same family by the whole area of the venom protein peaks in the chromatogram. For fractions composed of more than one peak, the sum of the peak areas was considered for quantification. If the peak was composed of more than one gel band, their proportions were estimated according to the densitometry analysis (ImageLab software).

2.3. Enzymatic activities

2.3.1. Hyaluronidase (Hyal) activity

Hyal activity was quantified using a *BbV* serial dilution (80–0.625 µg) incubated in 0.2 M sodium acetate-acetic acid and 0.15 M NaCl buffer with 12.5 µg of hyaluronic acid (HA), a control curve was made with 5 points of known HA concentrations as previously described (Guerra-Duarte et al., 2015).

2.3.2. Phospholipase A_2 (PLA_2) activity

PLA_2 activity was analyzed using an EnzChek® PLA_2 Assay Kit (Life Technologies). The experiment was performed according to the Kit protocol, with 2 µg of *BbV* as described in Rodrigues et al. (2018). As positive control, a solution of reaction buffer (PLA_2 10 U/mL in 1 × PLA_2) was used. The same buffer without PLA_2 was utilized as negative control. The assays were carried out in duplicates. Means of the results from three different experiments were analyzed and plotted as activity percentage, relative to the positive control.

2.3.3. L-amino acid oxidase (LAAO) activity

LAAO activity was assessed according to Costal-Oliveira et al (2019), with modifications (Costal-Oliveira et al., 2019). 2 µg of *BbV* were utilized and the same amount of venom from *B. atrox* was used as positive control. The venom was incubated in microtiter plates, at 37 °C, with substrate solution (L-leucine as, 2 mM OPD and 5U/mLperoxidase in 100 mM Tris- HCl buffer pH 8.5). After 1 h of incubation, 50 µL of 2 M H_2SO_4 was added to stop the reaction. The activity was measured using a microplate reader (BioRad Model 680) at 492 nm. Three different assays were performed in duplicates. The results were expressed as ΔA492 nm/min related to protein concentration (mg).

2.3.4. Proteolytic activity

2.3.4.1. Metalloproteinase (SVMP) and serine proteinase (SVSP) activities. Metalloproteinases activity of *BbV* and *B. atrox* venom was measured according to Souza et al. (2015), using an Abz-LVEALYQ-EDDnp peptide previously developed by our group (Schneider et al., 2014). Two micrograms of either *BbV* or *B. atrox* venom were incubated with 20 µL of the peptide diluted in 60 µL of 100 nM Tris-HCl/50 nM NaCl buffer for 30 min. The plate was read at 340 nm (excitation) and 440 nm (emission) in a fluorometer (Synergy, Bio-Tek). Results were expressed as means of three different experiments and plotted as relative fluorescence units.

Serine proteinase activity was quantified in a similar way, using another peptide, Abz-FLPRSFRQ-EDDnp, also produced by Schneider et al. (2014), as previously described (Souza et al., 2015).

2.3.4.2. Fibrinogenolytic activity. Fibrinogenolytic activity was assessed using 3 µg of either *BbV* or *B. atrox* venom diluted in phosphate buffered saline (PBS), Fifty microliters of 2.5 mg/mL bovine fibrinogen in 0.1 M Tris-HCl buffer, pH 8.0 were added to the samples, as described by Rodrigues et al. (2018). In order to determine the metalloproteinase contribution to this activity, the venom was pre-incubated for 1 h at 37 °C with 2 mM EDTA. Electrophoresis was carried out in a 10% acrylamide gel using 15 µL of the final solution. Gels were stained by Coomassie Blue R250.

2.3.5. Hemorrhagic activity

Hemorrhagic activity of 10 µg of either *BbV* or *B. atrox* venom was evaluated by subcutaneous (s.c.) injections into mice dorsal skin (Swiss, n = 4), as previously described (Molina et al., 2018) with modifications. Saline solution (50 µL) was used as negative control. After 2 h, animals were anesthetized [ketamine 10% (0.05 mL/kg) + xylazine 2% (0.025 mL/kg)] and euthanized. Skins were removed, and the hemorrhagic halo was measured.

2.4. Cytotoxicity

Venom cytotoxicity was determined by Alamar Blue™ assay, conducted according to Costal-Oliveira et al. (2019), using VERO cell line (epithelial cells from normal African green monkey kidney) (Costal-Oliveira et al., 2019). Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) with 10% fetal bovine serum (Thermo Scientific-HyClone) and 0.2% gentamicin (Gibco by Life Technologies) was used to culture the cells. The cells were maintained in a 10% CO_2 incubator at 37 °C. The Alamar Blue™ assay was performed as described by Damico et al. (2007), with modifications, to evaluate venom cytotoxicity (Damico et al., 2007). Approximately 1×10^4 cells were added to microtiter plates. After a 24 h incubation at 37 °C and 10% CO_2, a dilution curve of BbV from 80 µg/mL to 1.25 µg/mL was added to the cells for 24 h in the same incubation conditions described above. A 10% v/v solution of Alamar Blue in DMEM was prepared and 100 µL of this solution were added to each well. After 3 h, the plates were measured in a fluorometer Synergy 2 (Bio-Tek) at 540 nm (excitation) and 590 nm (emission). The amount of venom capable of reducing cell viability in 50% (CD_{50}) was calculated using the GraphPad Prism 5 software. Three different experiments were performed in duplicates.

2.5. Immunoassays

2.5.1. ELISA

A solution consisting of 0.5 µg/well of *BbV* in carbonate buffer, pH 9.8 was added to microtiter plates, which were incubated at 4 °C overnight. The plate was washed, blocked with 3% skimmed milk in PBS and incubated at 37 °C for 1 h. After washing, plates were incubated with either P-BAV, B-BAV or pre-immune horse serum (PI) diluted from 1:8000 to 1:1024000 in 0.01% milk-PBS for 1 h at 37 °C. Plates were washed and incubated with anti-horse peroxidase secondary antibody (1:6000 in incubation buffer) for another 1 h at 37 °C. SigmaFast OPD tablets were used to detect the reaction, following the manufacturer protocol. After incubation in the dark for 20 min, the reactions were stopped with 2 M H_2SO_4. Absorbance was measured at 490 nm, using a BioRad microplate reader (Model 680).

2.5.2. SDS-PAGE (1-DE and 2-DE) and Western Blotting

Non-reducing sample buffer was used to dilute 20 µg of *BbV*. Electrophoresis (1-DE) was carried out as above mentioned. The proteins in the gels were either stained with Coomassie Blue R-250 or transferred to nitrocellulose membranes for Western Blotting (Towbin et al., 1992).

Two-dimensional electrophoresis (2-DE) was performed using 200 µg of *BbV* diluted in 125 µL of a buffer with rehydration solution (7 M urea, 2 M thiourea, 2% Triton X-100, IPG buffer, 0,002% bromophenol blue), 50 mM DTT, 0.5% of IPG buffer pH 4–7 and 1% of protease

Author's Personal Copy

inhibitor. A 7 cm IPG gel strip containing a linear pH gradient from 4 to 7 (GE Healthcare) was used. Isoelectric focussing (IEF) was performed on Ettan™ IPGphor™ 3 (GE Healthcare Life), following the manufacturer manual. A 5-step electrophoresis program was used for isofocalization: 100 V for 1 h, 200 V for 1 h, 500 V for 1 h, 1000 V for 1 h, 3500 V for 3:30 h, 3500 V for 30 min and 500 V for 30 min. An equilibration buffer was then added to the strips and the proteins were reduced and alkalinized by the solutions: 1% SDS, 30% glycerol, 0.04 M Tris-HCl, pH 6.8 (equilibration buffer) with 4 mg/mL DTT followed by 40 mg/mL iodoacetamide in equilibration buffer. Electrophoresis was carried out on a 12% polyacrylamide gel at 200 V. Protein spots were visualized by Coomassie Blue R-250 staining.

Western Blotting was performed as previously described (Rodrigues et al., 2018), using 20 μg of either *Bb*V and *B. atrox* venom. After an overnight wet-transfer, the membrane was blocked for 1 h using PBS-Tween 0.3%. The membrane was washed with PBS-Tween 0.05% and incubated with either PI, P-BAV or B-BAV (1:8000) for 1 h. The reaction was detected using goat anti-horse IgG conjugated to peroxidase (Sigma, 1:5000). Recognition was observed using DAB/-chloronaphthol as substrate.

2.6. Statistical analysis

For statistical analysis of the data, quantitative variables were represented as means ± standard deviation. All statistics were performed with GraphPad Prism 6 software. One-way analysis of variance (ANOVA) with Bonferroni post-test was used for hemorrhagic activity and cell viability. The level of significance was set as $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

3. Results

3.1. RP-HPLC, SDS-PAGE and mass spectrometry analysis

A total of 14 fractions were obtained by RP-HPLC *Bb*V fractionation (Fig. 2A). These 14 fractions were submitted to SDS-PAGE. The gel bands were excised (Fig. 2B), subjected to tryptic digestion and analyzed by MALDI-TOF/TOF.

MALDI-TOF/TOF analysis was carried out in order to identify *Bb*V main proteins. The identification of these proteins was carried by the search against protein database using MASCOT or BLAST programs. Their relative concentration was estimated based on the area of the chromatogram at 215 nm, along with the densitometry analysis of 1-DE SDS-PAGE gels (Supplementary material, Table 1). Fig. 3 shows that *Bb*V contains large amounts of snake venom metalloproteinases (SVMPs, 33.05%), followed by snake venom serine proteinases (SVSPs, 26.11%), phospholipases A_2 (PLA_2, 25.57%) snake C-type lectins (CTLs, 9.61%), L-amino acid oxidase (LAAO, 3.80%) cysteine-rich secretory proteins (CRISPs, 1.67%) and Bradykinin-potentiating peptide (BPP, 0.20%).

As PII and PIII SVMP proteolytic processing can release disintegrins and disintegrins-like domains, respectively (Kini and Evans, 1992), we included fragments that matched disintegrins in our database analysis within the metalloproteinase family rather than in a distinct family. This method has been previously employed (Junqueira-de-Azevedo and Ho, 2002) since a tryptic peptide reported as disintegrin could be a product of SVMP processing.

3.2. *In vitro* and *in vivo* activities of *Bb*V

Diverse enzymatic activities of *Bb*V were performed. Table 1 compares our results with previous studies that used the same methodology (Guerra-Duarte et al., 2015; Rodrigues et al., 2018). Compared to *B. jararaca*, *B. atrox*, *B. barnetti*, *B. pictus* and *Bothriopsis bilineata* venoms, *Bb*V presented high SVMP, SVSP and LAAO activities and low Hyal and PLA_2 activities.

Cell viability after incubation with different concentrations of *Bb*V was also tested and the results are shown in Fig. 4. *Bb*V seems to impair VERO cells viability (Fig. 4). The use of this primary cell line allows the evaluation of direct venom toxicity to the kidneys cells. Eight dilutions were tested and the dilution of 80 μg/mL was able to reduce cell viability significantly.

In addition to toxicity, another important aspect of bothropic envenomation is the haemostatic disturbances that it may cause. Therefore, fibrinogenolytic assays were performed in order to analyse the

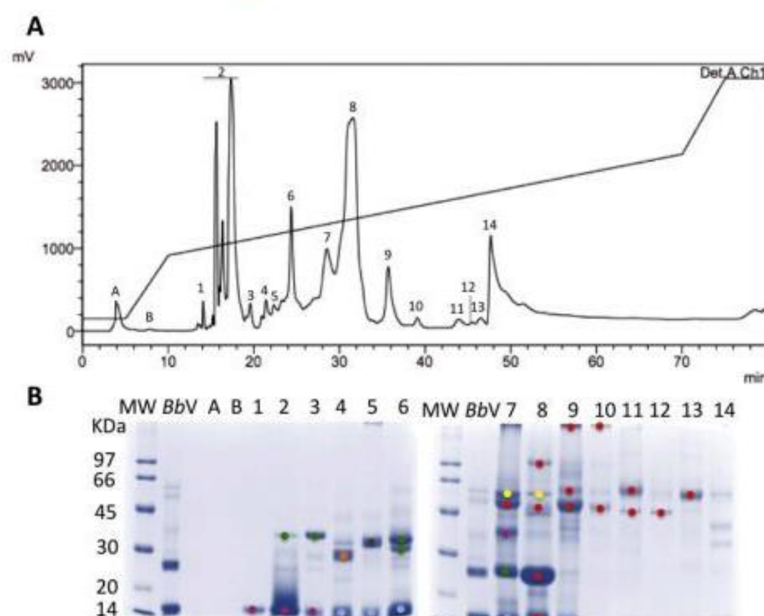


Fig. 2. *Bb*V reversed phase-high performance liquid chromatography. RP-HPLC fractionation of 2 mg of *Bb*V. The collected peaks were subjected to electrophoresis and the protein content of the brands was analyzed by MALDI-TOF/TOF. (A) RP-HPLC of *Bb*V. Proteins were fractionated on a C8 column. (B) SDS-PAGE analysis of *Bb*V fractions separated by RP-HPLC. Spots are color-coded to indicate their corresponded family. Red SVMP; green SVSP; pink PLA_2; blue CTL; orange CRISP; yellow LAAO and brown BPP. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Author's Personal Copy

Table 1

Comparison between the enzymatic activities of *BbV* and other *Bothrops* venoms activities. *BbV* activities were contrasted with different *Bothrops* venoms activities. Data were obtained as described in Material and Methods and represented as means of independent experiments. Hyal (hyaluronidase); PLA_2 (phospholipase A_2); LAAO (L-amino acid oxidase); SVMP (snake venom metalloproteinase); SVSP (snake venom serine proteinase); CD_{50} (median cytotoxic dose). [a]Data from (Guerra-Duarte et al., 2015). [b]Data from (Rodrigues et al., 2018).

Venom	Hyal (TRU)	PLA_2 (% activity)	LAAO (U/mg/min)	SVMP (RFU)	SVSP (RFU)	Cytotox (CD_{50})
B. brazili	**107.8**	**5.75**	**12.79**	**16841.16**	**10463.66**	**55.01**
B. jararaca[a]	48.39	54.19	3.48	7739.88	4350.17	18.78
B. atrox[a]	61.03	87.93	10.31	5698.75	2571.5	12.12
B. barnetti[a]	45.04	11.02	1.39	5391.92	578.17	16.01
B. pictus[a]	45.01	80.03	3.22	2797.42	5501.67	19.6
B. bilineata[b]	58.7	7.47	4.74	5263.5	16859.33	29.55

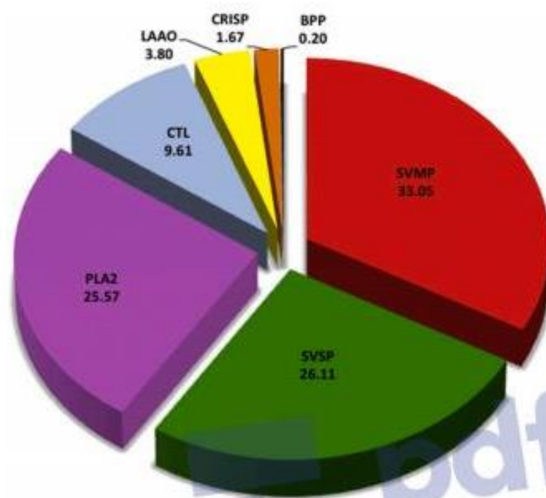


Fig. 3. Relative abundance of the proteins identified by proteomic analysis. SVMP Snake venom metalloproteinase; SVSP Snake venom serine protease; PLA_2 Phospholipase A_2; CTL C-type lectin; LAAO L-amino acid oxidase; CRISP Cysteine rich secretory protein; BPP Bradykinin-potentiating peptide.

effect of *BbV* on fibrinogen. Results showed that *BbV* was able to cleave two out of three fibrinogen chains (Aα and Bβ), while *B. atrox* venom, used as control, cleaved only the Aα chain (Fig. 5). Furthermore, fibrinogenolytic activity was inhibited by EDTA incubation (Fig. 5B).

To further evaluate how the venom can affect hemostasis, *BbV* capacity of inducing hemorrhage was assessed *in vivo*. After 2 h of *BbV* injection in mice dorsal skin, it was possible to observe hemorrhage halos, as shown in Fig. 6.

3.3. *Antigenic analysis and electrophoretic patterns of* BbV

ELISA and Western Blotting techniques were used to analyse two antivenoms, P-BAV and B-BAV, immune recognition of *BbV* proteins. Fig. 7 shows ELISA reactivity at different sera dilutions. B-BAV and P-BAV were cross-reactive against *BbV*. 1-DE and Western Blotting results are shown in Fig. 8. Both P-BAV and B-BAV reacted against most of the bands in Western Blotting assays (Fig. 7B). No reactivity was observed using PI serum.

In order to further analyse *BbV* electrophoretic pattern, 2-DE was carried out. *BbV* proteins (200 μg) were applied to a 4–7 IPG strip and electrophoresis was performed on 12% polyacrylamide gels (Fig. 9). The 2-DE analysis shows the *Bbv* proteins complexity.

4. Discussion

4.1. *Toxic activities and proteomic profile of BbV*

In Peru, *Bothrops brazili* is considered a highly venomous snake ("WHO - Health Systems and Services," 2010) and its venom composes the antigenic pool used to produce bothropic antivenom in this country. However, there are not many studies aiming to characterize its venom. Studying venom composition is important to better understand envenomation pathophysiological mechanisms, to improve treatment protocols and to detect novel molecules with potential biotechnological application.

In the present work, we combined proteomic analysis, *in vitro* and *in vivo* assays to disclose *BbV* venom composition and activities. Results show that in comparison to *B. jararaca*, *B. atrox*, *B. barnetti*, *B. pictus* and

pdfelement

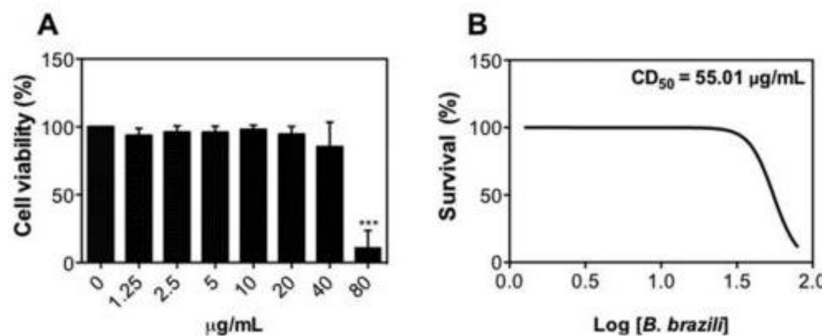


Fig. 4. VERO cells viability (1 × 10^4/well). (A) Cell viability measured after incubation with a serial dilution of *BbV* (80–1.25 μg/mL) for 24h. The Alamar Blue® assay was used to assess cell viability. Data were shown as the means ± S.D. ***$p < 0.001$ vs. control. (B) The concentration that reduces 50% of cell viability (EC_{50}) was calculated using Graph Pad Prism software. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Author's Personal Copy

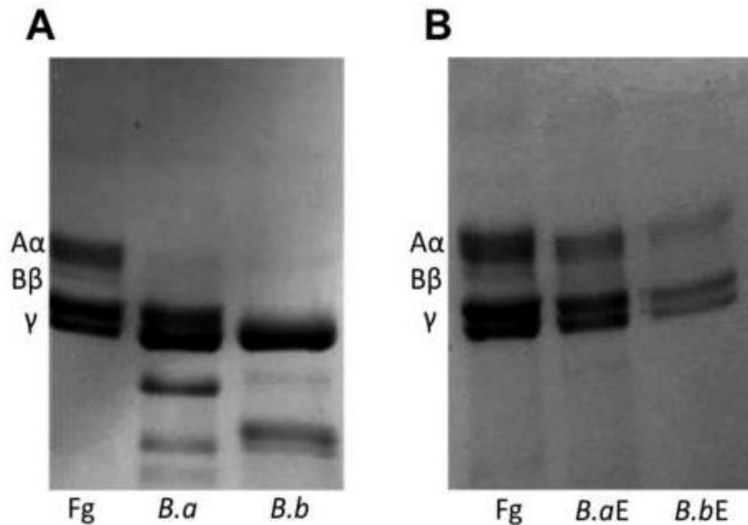


Fig. 5. Fibrinogenolytic activity of *Bb*V. Samples were loaded into 10% acrylamide gel and stained with Coomassie blue. Fibrinogen is composed of two sets of three different polypeptide chains Aα (66 kDa), Bβ (54 kDa) and γ (48 kDa). (A) Fibrinogen digestion by 3 μg of either *Bb*V or *B. atrox* venom (used as positive control). Fibrinogen alone (no *Bb*V or *B. atrox* venom) was used as negative control. (B) Effects of EDTA pre-incubation on fibrinogen digestion. *B.a* 3 μg of *B. atrox*. *B.b* 3 μg of *Bb*V. *B.a*E 3 μg of *B. atrox* incubated with EDTA. *B.b*E 3 μg of *Bb*V incubated with EDTA. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

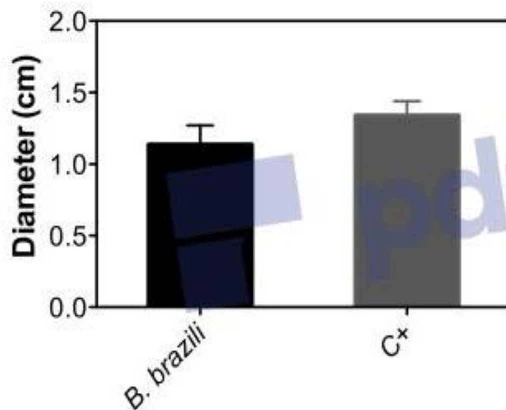


Fig. 6. Hemorrhagic activity of *Bb*V. Hemorrhagic halo diameter caused by the subcutaneous injection of 10 μg of *Bb*V. *B. atrox* was used as positive control (C+).

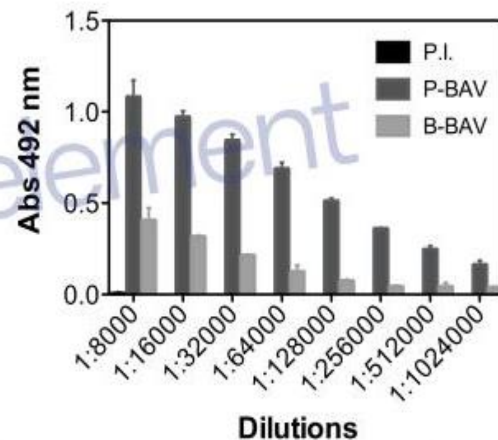


Fig. 7. B-BAV and P-BAV cross-reactivity with *Bb*V. ELISA of B-BAV and P-BAV immune recognition of *Bb*V. Data is shown as the recorded absorbance at 492 nm plotted against antivenom dilution (1:1000–1:128000).

B. bilineata venoms, *Bb*V has the lowest Hyal activity (Table 1). The proteomic analysis did not detect Hyal in *Bb*V, which could be explained by its low expression level in venom glands (Tasoulis and Isbister, 2017), hindering its elution from HPLC columns. Hyals are present in most animal venoms, including snakes (Kudo and Tu, 2001), scorpions (Ramanaiah et al., 1990), bees (Gmachl and Kreil, 1993), hornets (Lu et al., 1995), spiders (Wright et al., 1973) and caterpillars (da C B Gouveia et al., 2005). Despite being known as a non-toxic enzyme, Hyals are responsible for hyaluronic acid degradation, helping toxins to spread from the bite site into circulation (Fox, 2013). Furthermore, Hyal, along with other proinflammatory toxins, can cause local edema (Kemparaju and Girish, 2006). Hyal activity in bothropic venoms has already been associated with their lethal activity (Souza et al., 2015). Queiroz et al. (2008) compared the venom composition and toxicity of several Brazilian snakes from *Bothrops* genus. The authors reported that the median lethal dose (LD_{50}) for *Bb*V was the second highest when compared to all the other analyzed species, indicating the direct relation betweeen Hyal activity and lethality (Queiroz et al., 2008). Similar results were obtained by Girish and Kemparaju (2006), utilizing the venom from a different snake genus, *Naja*. In this study, the authors used both a polyclonal antiserum against Hyal (Anti-NNH1) and aristolochic acid to inhibit Hyal activity. When mice were injected with *Naja naja* whole venom pre incubated with Anti-NNH1/aristolochic acid, there was an increase in mice survival time, compared to animals injected only with venom. When the Anti-NNH1/aristolochic acid was administrated 10 min after the injection of the venom, a more moderate increase in survival was observed, showing the importance of Hyal in snake venom toxicity (Kemparaju and Girish, 2006).

Contrary to what was found for Hyal, enzymatic tests showed higher levels of SVMP and SVSP activities in *Bb*V when compared to the other venoms (Table 1). Another assay used for the detection of proteolytic enzymes is the fibrinogenolytic assay, which also indicated the presence of this type of proteins in *Bb*V. Fibrinogen consists of three pairs of polypeptide chains Aα, Bβ and γ, with molecular masses of 66, 54 and 48 kDa, respectively. *Bb*V was able to cleave Aα and Bβ chains of fibrinogen, while γ chain remained undamaged (Fig. 5A). Moreover, after incubation with EDTA, a chelating agent, fibrinogenolytic activity was

Author's Personal Copy

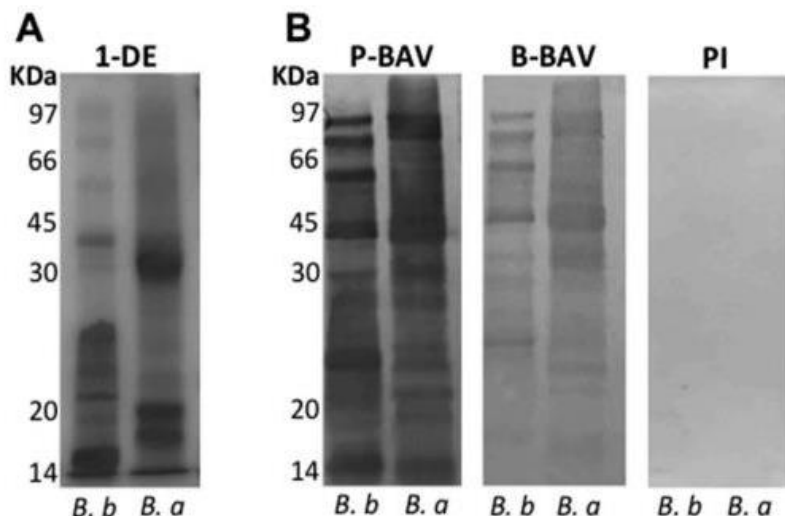


Fig. 8. *BbV* cross-reactivity against P-PAV and B-BAV. (A) 1-DE of *BbV*. 20 μg of *B. brazili* venom were applied to 12% acrylamide gel. (B) Western Blotting of 1-DE gels with both B-BAV and P-BAV. Sample was wet-transferred from gel to nitrocellulose membranes, tested against B-BAV, P-BAV or PI (1:8000) and incubated with the secondary antibody (anti-horse IgG conjugated to peroxidase). Recognition was observed using DAB/chloronaphthol as substrate. *B.b Bothrops brazili* venom, *B.a Bothrops atrox* venom.

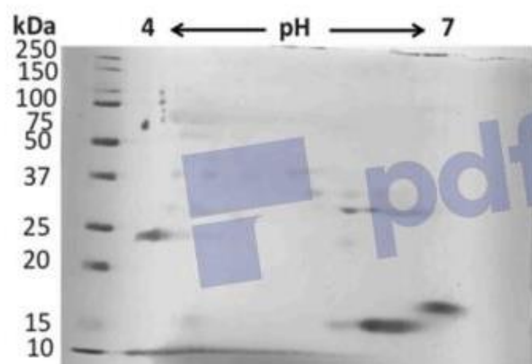


Fig. 9. 2-DE of *BbV* proteins. *BbV* proteins (250 μg) were submitted to isoelectric focussing in a 4–7 IPG strip and electrophoresis was carried out on 12% acrylamide gel.

inhibited, endorsing SVMP specific activity (Fig. 5B).

SVMPs and SVSPs are extensively distributed in bothropic venoms and have different substrate specificity (Juliana L. Bernardoni et al., 2014a,b; Swenson and Markland, 2005). These enzymes are involved in several biological activities, such as modifications in the platelet function, endothelial cells, blood coagulation cascade, and fibrinolytic and kallikrein-kinin system (Juliana L Bernardoni et al., 2014a,b; Bjarnason and Fox, 1994; Lu et al., 2005; Zhou et al., 1995). SVMPs are abundant in snake venoms, especially in the Viperidae family. This class of enzymes can cause alterations in the basal membrane, resulting in hemorrhage, which is the most significant symptom of *Bothrops* envenomation (Sajevic et al., 2011). After *BbV* inoculation under mice dorsal skin, hemorrhage halos were observed (Fig. 6), validating the presence of SVMPs in the venom.

Both SVMPs and SVSPs have been purified from *BbV*. Kayano and collaborators 2015 isolated a metalloproteinase named BbMP-1 that was considered weakly hemorrhagic in *in vivo* assays but was also myotoxic and edematogenic. As demonstrated in the present work for whole *BbV*, the purified BbMP-1 was also capable of degrading both Aα and Bβ chains of fibrinogen. Interestingly, BbMP-1 showed anti-malarial activity, highlighting a potential biotechnological application for *BbV* (Kayano et al., 2015).

Two different SVSPs were also purified from *BbV* and partially characterized. Zaqueo et al. (2016) isolated a thrombin-like SVSP of 36 kDa that was named BbrzSP-32. Bhat et al., also in 2016 studied another SVSP purified from *BbV*. In addition to its amidolytic, fibrinogenolytic and gelatinolytic activities, this SVSP showed pro-angiogenic effect, activating the signalling cascade of PI3K/Akt (Bhat et al., 2016).

LAAOs are another group of enzymes largely present in notably hemorrhagic venoms (Du and Clemetson, 2002). LAAOs are flavoenzymes that cause stereospecific oxidative deamination of L-amino acids, producing α-keto acid, H_2O_2 and ammonia. The H_2O_2 is associated with LAAO multifunctionality, being responsible for actions such as myotoxicity, hemorrhage, edema, cytotoxicity, and hemolysis (Ciscotto et al., 2009; Pišlar et al., 2016; Stábeli et al., 2004; Wei et al., 2009, 2007). *BbV* presented the highest LAAO activity when compared to *B. jararaca, B. atrox, B. barnetti, B. pictus* and *B. bilineata* venoms (Table 1). Bothropic LAAOs have already been correlated with cytotoxicity (Souza et al., 2015). When tested on VERO cells *BbV* impaired cell viability in a concentration of 80 μg/mL (Fig. 4). However, comparing the obtained CD_{50} with those from other *Bothrops* species, *BbV* appear to be less toxic to these cells (Table 1).

In addition to LAAO, another enzyme involved in cytotoxicity and edema formation is PLA_2. Low PLA_2 activity was detected in our *in vitro* enzymatic test (Table 1), while the proteomic findings show high quantities of PLA_2 when compared to other *Bothrops* venoms (Fig. 3) (Kohlhoff et al., 2012). Snake venom PLA_2s are divided into three groups: I, II and III, according to the snake family that they belong (Arni and Ward, 1996). PLA_2s from the Viperidae family are placed in class II and are subdivided into other two groups: (1) Asp49 PLA_2s, which have an Asp residue at position 49 and present high catalytic activity against artificial substrates; (2) Lys49 PLA_2s, which displays a Lys residue at position 49 and present low or no catalytic activity. The probable presence of both types of PLA_2s could explain why the results show low PLA_2 activity in the enzymatic assays.

Indeed, both types of PLA_2 have been isolated from *BbV*. Costa et al. (2008) purified two PLA_2 from *BbV*, one belonging to Asp49 group named MTX-I; and the other to Lys49 group named MTX-II. Both molecules showed myotoxicity and were edematogenic in mice. These PLA_2s also showed a possible biotechnological value, as they were toxic to

Author's Personal Copy

T-cell leukemia cells (JURKAT) lines and to *Escherichia coli*, *Candida albicans* and *Leishmania sp.* Huancahuire-Vega et al. (2009) characterized other two PLA_2s from *Bb*V, named BbTxII and BbTxIII. They showed edema-forming and myotoxic activities, in addition to *in vitro* neuromuscular blockage. These two purified toxins are also from the Lys49 group, therefore with low enzymatic activity. More recently, Sobrinho et al. (2018) purified another two PLA_2 from *Bb*V, but from Asp49 group and with acidic characteristics, called Braziliases I and II. They did not cause myonecrosis and had only discrete edematogenic activity. On the other hand, they were very effective in inhibiting platelet aggregation, highlighting the functional diversity of *Bb*V PLA_2s.

Working with *Bb*V collected from Brazilian specimens, Sales and Santoro (2008) demonstrated that this venom presented remarkably high ATPase, ADPase and 5-nucleotidase activities, as well as high phosphodiesterase and DNase activities, when compared with 28 other venoms from various snakes (Sales and Santoro, 2008). However, in our proteomic analysis these enzymes were not identified. This can be due to regional differences among venoms from individuals of distinct geographic locations or to the limitations of our methods, but this matter remains to be further investigated.

South America is home to many pitviper species (Campbell and Lamar, 2004). Various *Bothrops* species co-exist and display diverse venom contents, particularly in the Amazonian region. Their venoms are also differently neutralized by the available antivenoms. With this in mind, it is of great interest for public health to access the potential of antivenoms, produced in different countries, of recognizing and inhibiting the venoms from snakes present in these regions. Both P-BAV and B-BAV showed immune recognition of *Bb*V when tested by ELISA and Western Blotting (Fig. 7 and 8). As *Bb*V is only included in the pool of venoms used to produce P-BAV, but not B-BAV, data suggest that the *Bothrops* spp. venoms have similar proteins, indicating that venom antigens can share resembling epitopes. These findings could initially support the use of either antivenoms in accidents involving this snake, but more studies testing their *in vivo* neutralization should be conducted in order to confirm it.

5. Final remarks

This work shows the protein composition profile and enzymatic activities of the Peruvian snake *Bb*V. They were analyzed in order to determine their function in envenoming pathophysiology and to better comprehend this venom. *Bb*V has a predominance of proteases, SVMPs and SVSPs, which together account for more than 50% of the total venom proteins. Proteome data correlated well with *in vitro* enzymatic activities and cytotoxicity. Although *Bb*V is not included in the pool of venoms used to produce B-BAV, the presence of cross-reactive antibodies in both B-BAV and P-BAV suggests that *Bothrops* venom antigens share similar epitopes (Estevao-Costa et al., 2016).

Declaration of competing interest

The authors state no conflict of interest.

CRediT authorship contribution statement

Carolina Rego Rodrigues: Investigation, Methodology, Writing - original draft. **Denis Alexis Molina Molina:** Investigation, Methodology. **Thamyres C. Silva de Assis:** Investigation, Methodology. **Camila Liberato:** Investigation, Methodology. **Marcella N. Melo-Braga:** Methodology, Writing - review & editing. **César Bonilla Ferreyra:** Resources, Validation. **Javier Cárdenas:** Resources, Validation. **Fernanda Costal-Oliveira:** Validation, Writing - review & editing. **Clara Guerra-Duarte:** Validation, Formal analysis, Writing - review & editing. **Carlos Chávez-Olórtegui:** Funding acquisition, Project administration, Writing - review & editing, Validation.

Acknowledgments

The authors acknowldge the "Laboratório Multiusuário de Proteômica (LMProt) – Centro de Laboratórios Multiusuários (CELAM), ICB-UFMG" for technical support in the mass spectrometry analysis.

This research was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil (CNPq) (Process: 406163/2018-9), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil - CAPES (Program COFECUB; Process: 88881.191812/2018-01) and by Fundação de Amparo à Pesquisa do Estado de Minas Gerais, Brazil (FAPEMIG; Process: PPM-00270-16).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.toxicon.2020.05.016.

References

Arni, R.K., Ward, R.J., 1996. Phospholipase A2-A Structural Review 34.

Bernardoni, J.L., Sousa, L.F., Wermelinger, L.S., Lopes, A.S., Prezoto, B.C., Serrano, S.M. T., Zingali, R.B., Moura-da-Silva, A.M., 2014a. Functional variability of snake venom metalloproteinases: adaptive advantages in targeting different prey and implications for human envenomation. PloS One 9. https://doi.org/10.1371/journal.pone.0109651.

Bernardoni, J.L., Sousa, L.F., Wermelinger, L.S., Lopes, A.S., Prezoto, B.C., Serrano, S.M. T., Zingali, R.B., Moura-da-Silva, A.M., 2014b. Functional variability of snake venom metalloproteinases: adaptive advantages in targeting different prey and implications for human envenomation. PloS One 9, e109651.

Bhat, S.K., Joshi, M.B., Ullah, A., Masood, R., Biligiri, S.G., Arni, R.K., Satyamoorthy, K., 2016. Serine proteinases from Bothrops snake venom activates PI3K/Akt mediated angiogenesis. Toxicon 124, 63–72. https://doi.org/10.1016/j.toxicon.2016.11.001.

Bjarnason, J.B., Fox, J.W., 1994. Hemorrhagic metalloproteinases from snake venoms. Pharmacol. Ther. 62, 325–372. https://doi.org/10.1016/0163-7258(94)90049-3.

Campbell, J.A., Lamar, W.W., 2004. The Venomous Reptiles from the Western Hemisphere, 2 vol. Cornell University Press, Ithaca.

Ciscotto, P., Machado de Avila, R.A., Coelho, E.A.F., Oliveira, J., Diniz, C.G., Farias, L.M., Carvalho, M.A.R. De, Maria, W.S., Sanchez, E.F., Borges, A., Cha, C., 2009. Antigenic , microbicidal and antiparasitic properties of an L -amino acid oxidase isolated from Bothrops jararaca snake venom. Toxicon 53, 330–341. https://doi.org/10.1016/j.toxicon.2008.12.004.

Costa, T.R., Menaldo, D.L., Oliveira, C.Z., Santos-Filho, N.A., Teixeira, S.S., Nomizo, A., Fuly, A.L., Monteiro, M.C., de Souza, B.M., Palma, M.S., Stabeli, R.G., Sampaio, S.V., Soares, A.M., 2008. Myotoxic phospholipases A(2) isolated from Bothrops brazili snake venom and synthetic peptides derived from their C-terminal region: cytotoxic effect on microorganism and tumor cells. Peptides 29, 1645–1656. https://doi.org/10.1016/j.peptides.2008.05.021.

Costal-Oliveira, F., Stransky, S., Guerra-Duarte, C., Naves de Souza, D.L., Vivas-Ruiz, D. E., Yarlequé, A., Sanchez, E.F., Chávez-Olórtegui, C., Braga, V.M.M., 2019. L-amino acid oxidase from Bothrops atrox snake venom triggers autophagy, apoptosis and necrosis in normal human keratinocytes. Sci. Rep. 9, 781. https://doi.org/10.1038/s41598-018-37435-4.

da C B Gouveia, A.I., da Silveira, R.B., Nader, H.B., Dietrich, C.P., Gremski, W., Veiga, S. S., 2005. Identification and partial characterisation of hyaluronidases in Lonomia obliqua venom. Toxicon 45, 403–410. https://doi.org/10.1016/j.toxicon.2004.11.008.

Damico, D.C.S., Nascimento, J.M., Lomonte, B., Ponce-Soto, L.A., Joazeiro, P.P., Novello, J.C., Marangoni, S., Collares-Buzato, C.B., 2007. Cytotoxicity of Lachesis muta muta snake (bushmaster) venom and its purified basic phospholipase A2 (LmTX-I) in cultured cells. Toxicon 49, 678–692. https://doi.org/10.1016/j.toxicon.2006.11.014.

Du, X.Y., Clemetson, K.J., 2002. Snake venom L-amino acid oxidases. Toxicon 40, 659–665. https://doi.org/10.1016/S0041-0101(02)00102-2.

Eichberg, S., Sanz, L., Calvete, J.J., Pla, D., 2015. Constructing comprehensive venom proteome reference maps for integrative venomics. Expert Rev. Proteomics 9450. https://doi.org/10.1586/14789450.2015.1073590.

Estevao-Costa, M.I., Gontijo, S.S., Correia, B.L., Yarleque, A., Vivas-Ruiz, D., Rodrigues, E., Chávez-Olortegui, C., Oliveira, L.S., Sanchez, E.F., 2016. Neutralization of toxicological activities of medically-relevant Bothrops snake venoms and relevant toxins by two polyvalent bothropic antivenoms produced in Peru and Brazil. Toxicon 122, 67–77. https://doi.org/10.1016/j.toxicon.2016.09.010.

Fox, J.W., 2013. A brief review of the scientific history of several lesser-known snake venom proteins: L-amino acid oxidases, hyaluronidases and phosphodiesterases. Toxicon 62, 75–82. https://doi.org/10.1016/j.toxicon.2012.09.009.

Gmachl, M., Kreil, G., 1993. Bee venom hyaluronidase is homologous to a membrane protein of mammalian sperm. Proc. Natl. Acad. Sci. U.S.A. 90, 3569–3573. https://doi.org/10.1073/pnas.90.8.3569.

Guerra-Duarte, C., Lopes-Peixoto, J., Fonseca-de-Souza, B.R., Stransky, S., Oliveira, D., Schneider, F.S., Lopes-de-Souza, L., Bonilla, C., Silva, W., Tintaya, B., Yarleque, A.,

Author's Personal Copy

Chávez-Olórtegui, C., 2015. Partial in vitro analysis of toxic and antigenic activities of eleven Peruvian pitviper snake venoms. Toxicon. https://doi.org/10.1016/j.toxicon.2015.09.007.

Hoge, A.R., 1953. A new Bothrops from Brazil, Bothrops brazili, sp. nov. Mem. Inst. Butantan (Sao Paulo) 25, 15–21.

Huancahuire-Vega, S., Ponce-Soto, L.A., Martins-de-Souza, D., Marangoni, S., 2009. Structural and functional characterization of brazilitoxins II and III (BbTX-II and -III), two myotoxins from the venom of Bothrops brazili snake. Toxicon 54, 818–827. https://doi.org/10.1016/j.toxicon.2009.06.008.

Juárez, P., Sanz, L., Calvete, J.J., 2004. Snake venomics: characterization of protein families in Sistrurus barbouri venom by cysteine mapping, N-terminal sequencing, and tandem mass spectrometry analysis. Proteomics 4, 327–338. https://doi.org/10.1002/pmic.200300628.

Junqueira-de-Azevedo, I.L., Ho, P.L., 2002. A survey of gene expression and diversity in the venom glands of the pitviper snake Bothrops insularis through the generation of expressed sequence tags (ESTs). Gene 299, 279–291.

Kayano, A.M., Simoes-Silva, R., Medeiros, P.S.M., Maltarollo, V.G., Honorio, K.M., Oliveira, E., Albericio, F., da Silva, S.L., Aguiar, A.C.C., Krettli, A.U., Fernandes, C.F. C., Zuliani, J.P., Calderon, L.A., Stabeli, R.G., Soares, A.M., 2015. BbMP-1, a new metalloproteinase isolated from Bothrops brazili snake venom with in vitro antiplasmodial properties. Toxicon 106, 30–41. https://doi.org/10.1016/j.toxicon.2015.09.005.

Kemparaju, K., Girish, K.S., 2006. Snake venom hyaluronidase : a therapeutic target 52, 7–12.

Kini, R.M., Evans, H.J., 1992. Structural domains in venom proteins: evidence that metalloproteinases and nonenzymatic platelet aggregation inhibitors (disintegrins) from snake venoms are derived by proteolysis from a common precursor. Toxicon 30, 265–293.

Kohlhoff, M., Borges, M.H., Yarleque, A., Cabezas, C., Richardson, M., Sanchez, E.F., 2012. Exploring the proteomes of the venoms of the Peruvian pit vipers Bothrops atrox, B. barnetti and B. pictus. J. Proteomics 75, 2181–2195. https://doi.org/10.1016/j.jprot.2012.01.020.

Kudo, K., Tu, A.T., 2001. Characterization of hyaluronidase isolated from Agkistrodon contortrix contortrix (Southern Copperhead) venom. Arch. Biochem. Biophys. 386, 154–162. https://doi.org/10.1006/abbi.2000.2204.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680.

Laing, G., Yarleque, A., Marcelo, A., Rodriguez, E., Warrell, D., Theakston, R., 2004. Preclinical testing of three south American antivenoms against the venoms of five medically-important Peruvian snake venoms. Toxicon 44, 103–106. https://doi.org/10.1016/j.toxicon.2004.03.020.

Lu, G., Kochoumian, L., King, T.P., 1995. Sequence identity and antigenic cross-reactivity of white face hornet venom allergen, also a hyaluronidase, with other proteins. J. Biol. Chem. 270, 4457–4465. https://doi.org/10.1074/jbc.270.9.4457.

Lu, Q., Clemetson, J.M., Clemetson, K.J., 2005. Snake venoms and hemostasis. J. Thromb. Haemostasis 3, 1791–1799. https://doi.org/10.1111/j.1538-7836.2005.01358.x.

Ministerio de salud, P., 2005. Norma técnica sobre prevención y tratamiento de accidentes por animales ponzoñosos.

Molina, D.A., Guerra-duarte, C., Naves, D.L., Souza, D., Costal-oliveira, F., Reis, G., Ávila, D., Soccol, V.T., Machado-de-ávila, R.A., Chávez-olórtegui, C., 2018. Identification of a linear B-cell epitope in the catalytic domain of bothropasin, a metalloproteinase from Bothrops jararaca snake venom. Mol. Immunol. 104, 20–26. https://doi.org/10.1016/j.molimm.2018.10.019.

Pišlar, A., Sabotič, J., Šlenc, J., Brzin, J., Kos, J., 2016. Cytotoxic L-amino-acid oxidases from Amanita phalloides and Clitocybe geotropa induce caspase-dependent apoptosis. Cell Death Dis. 2, 16021.

Queiroz, G.P., Pessoa, L.A., Portaro, F.C.V., Furtado, M. de F.D., Tambourgi, D.V., 2008. Interspecific variation in venom composition and toxicity of Brazilian snakes from Bothrops genus. Toxicon 52, 842–851. https://doi.org/10.1016/j.toxicon.2008.10.002.

Ramanaiah, M., Parthasarathy, P.R., Venkaiah, B., 1990. Isolation and characterization of hyaluronidase from scorpion (Heterometrus fulvipes) venom. Biochem. Int. 20, 301–310.

Rodrigues, C., Teixeira-ferreira, A., Vargas, F., Guerra-duarte, C., Costal-oliveira, F., Stransky, S., Lopes-de-souza, L., Dutra, A., Yarlequé, A., Bonilla, C., Flores, E., Perales, J., Chávez-olórtegui, C., 2018. Proteomic profile , biological activities and antigenic analysis of the venom from Bothriopsis bilineata smaragdina (" loro machaco "), a pitviper snake from Peru. J. Proteomics 187, 171–181. https://doi.org/10.1016/j.jprot.2018.07.016.

Sajevic, T., Leonardi, A., Križaj, I., 2011. Haemostatically active proteins in snake venoms. Toxicon 57, 627–645. https://doi.org/10.1016/j.toxicon.2011.01.006.

Sales, P.B.V., Santoro, M.L., 2008. Nucleotidase and DNase activities in Brazilian snake venoms. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 147, 85–95. https://doi.org/10.1016/j.cbpc.2007.08.003.

Schneider, F.S., Le Nguyen, D., Castro, K.L., Cobo, S., Machado de Avila, R.A., Ferreira, N. de A., Sanchez, E.F., Nguyen, C., Granier, C., Galéa, P., Chávez-Olortegui, C., Molina, F., 2014. Use of a synthetic biosensor for neutralizing activity-biased selection of monoclonal antibodies against atroxlysin-I, an hemorrhagic metalloproteinase from Bothrops atrox snake venom. PLoS Neglected Trop. Dis. 8, e2826.

Sobrinho, J.C., Kayano, A.M., Simoes-Silva, R., Alfonso, J.J., Gomez, A.F., Gomez, M.C. V., Zanchi, F.B., Moura, L.A., Souza, V.R., Fuly, A.L., de Oliveira, E., da Silva, S.L., Almeida, J.R., Zuliani, J.P., Soares, A.M., 2018. Anti-platelet aggregation activity of two novel acidic Asp49-phospholipases A2 from Bothrops brazili snake venom. Int. J. Biol. Macromol. 107, 1014–1022. https://doi.org/10.1016/j.ijbiomac.2017.09.069.

Souza, L.L. de, Stransky, S., Guerra-duarte, C., Flor-sá, A., Schneider, F.S., Kalapothakis, E., Chávez-olórtegui, C., 2015. Determination of toxic activities in Bothrops spp. snake venoms using animal-free approaches: correlation between in vitro versus in vivo assays. Toxicol. Sci. 147, 458–465. https://doi.org/10.1093/toxsci/kfv140.

Stábeli, R.G., Marcussi, S., Carlos, G.B., Pietro, R.C.L.R., Selistre-De-Araújo, H.S., Giglio, J.R., Oliveira, E.B., Soares, A.M., 2004. Platelet aggregation and antibacterial effects of an L-amino acid oxidase purified from Bothrops alternatus snake venom. Bioorg. Med. Chem. 12, 2881–2886. https://doi.org/10.1016/j.bmc.2004.03.049.

Swenson, S., Markland, F.S., 2005. Snake venom fibrin(ogen)olytic enzymes. Toxicon 45, 1021–1039. https://doi.org/10.1016/j.toxicon.2005.02.027.

Tasoulis, T., Isbister, G.K., 2017. A review and database of snake venom proteomes. Toxins 9, 290. https://doi.org/10.3390/toxins9090290.

Towbin, H., Staehelint, T., Gordon, J., 1992. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets : procedure and some applications. Biotechnology 24, 145–149, 1979.

Wei, J., Yang, H., Wei, X., Qiao, L., Wang, W., He, S., 2009. Purification , characterization and biological activities of the L -amino acid oxidase from Bungarus fasciatus snake venom. Toxicon 54, 262–271. https://doi.org/10.1016/j.toxicon.2009.04.017.

Wei, X., Wei, J., Li, T., Qiao, L., Liu, Y., 2007. Purification, characterization and potent lung lesion activity of an L -amino acid oxidase from Agkistrodon blomhoffii ussurensis snake venom. Toxicon 50, 1126–1139. https://doi.org/10.1016/j.toxicon.2007.07.022.

WHO - health systems and Services [WWW document], 3.20.20. http://apps.who.int/bloodproducts/snakeantivenoms/database/.

Wright, R.P., Elgert, K.D., Campbell, B.J., Barrett, J.T., 1973. Hyaluronidase and esterase activities of the venom of the poisonous brown recluse spider. Arch. Biochem. Biophys. 159, 415–426.

Zaqueo, K.D., Kayano, A.M., Domingos, T.F.S., Moura, L.A., Fuly, A.L., da Silva, S.L., Acosta, G., Oliveira, E., Albericio, F., Zanchi, F.B., Zuliani, J.P., Calderon, L.A., Stabeli, R.G., Soares, A.M., 2016. BbrzSP-32, the first serine protease isolated from Bothrops brazili venom: purification and characterization. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 195, 15–25. https://doi.org/10.1016/j.cbpa.2016.01.021.

Zavaleta, A., Campos, S.M., 1992. Estimación de la cantidad individual de veneno producida por serpientes venenosas peruanas. Rev. Méd. Hered. 3.

Zhou, Q., Smith, J.B., Grossman, M.H., 1995. Molecular cloning and expression of catrocollastatin, a snake-venom protein from Crotalus atrox (western diamondback rattlesnake) which inhibits platelet adhesion to collagen. Biochem. J. 307 (Pt 2), 411–417.

4. DISCUSSÃO

Neste capítulo é apresentada uma discussão geral integrando os artigos já apresentados no capítulo anterior.

O veneno produzido por serpentes é um composto tóxico capaz de matar a maior parte dos seres vivos, é formado por uma complexa mistura de componentes tóxicos e não-tóxicos que possuem variados efeitos fisiológicos em tecidos, sistema cardiovascular, permeabilidade vascular, função renal e homeostase (ESTÊVÃO-COSTA et al., 2000).

Na área da toxinologia encontramos espécies de serpentes que não possuem seus venenos caraterizados a nível proteômico. Esta caracterização é de grande importância para fornecer informação para a análise das variações da composição dos venenos inter e intraespécie. As serpentes do gênero *Bothrops* são as serpentes mais amplamente distribuídas em Sul América, o acidente ofídico com estas serpentes é caracterizado por efeitos locais, como edema, dor e equimose, e efeitos sistêmicos, como hipotensão arterial, hemorragia e choque (MINISTÉRIO DA SAÚDE DO BRASIL, 2001). Estes efeitos estão relacionados à composição do veneno, que pode variar entre as espécies, o que leva a necessidade de conhecer a composição de proteínas e toxinas que compor os venenos, estudando o proteoma do veneno, já que este tipo informação nos permite identificar cada uma das toxinas que gera um determinado efeito toxico, desse modo pode ser direcionada a abordagem dos pacientes em casos de acidentes ofídicos, e também é de vital importância conhecer a sequencias de aminoácidos das proteínas do veneno, dado que esta informação é fundamental no estudo de mapeamento de epítopos que leva a identificação das regiões antigênicas das toxinas o que nos permitiria direcionar a produção de anticorpos neutralizantes específicos contra as toxinas de maior importância medica nos venenos.

Bothrops brazili é uma das principais espécies responsáveis por acidentes ofídicos no Peru e seu veneno compõe o reservatório antigênico utilizado para produzir antiveneno botrópico no país. No entanto, não existem muitos estudos visando caracterizar seu veneno. O estudo da composição do veneno é importante para melhor compreender os mecanismos fisiopatológicos do envenenamento, melhorar os protocolos de tratamento e detectar novas moléculas com potencial aplicação biotecnológica.

No artigo 3 – "Proteomic and toxinological characterization of Peruvian pitviper *Bothrops brazili* ("jergón shushupe"), venom", é mostrado a abundância relativa das proteínas que compõem o veneno de *Bothrops brazili* e suas atividades enzimáticas. A composição de *B. brazili* é semelhante à de outros venenos botrópicos. No entanto, a quantificação relativa de proteínas por contagem espectral revelou que a proporção das famílias de proteínas deste veneno é diferente da que foi previamente relatada para outros venenos botrópicos. Também apresentou atividades enzimáticas diferentes, como é mostrado na Tabela 1 do artigo mencionado anteriormente.

A Tabela 1 do paper 3, compara os resultados enzimáticos obtidos por diferentes autores para os venenos de *B. jararaca, B. atrox, B. barnetti, B. pictus* e *B. bilineata* aos obtidos para *B brazili*. Em comparação a esses venenos, *B. brazili* tem a menor atividade de Hialuronidase. A análise proteômica não detectou Hialuronidase, isto pode ser dado pela baixa concentração desta proteína nos venenos de serpentes o que dificulta sua detecção (TASOULIS; ISBISTER, 2017), a Hialuronidase são toxinas presentes na maioria dos venenos de animais, incluindo serpentes (KUDO; TU, 2001), escorpiões (RAMANAIAH et al., 1990), abelhas (GMACHL; KREIL, 1993), vespas (LU et al., 1995), aranhas (GREMSKI et al., 2014) e lagartas (DA C B GOUVEIA et al., 2005). A Hialuronidase é uma enzima comum de venenos de serpentes e indiretamente inicia a

toxicidade do veneno, são enzimas muito variantes que clivam naturalmente o ácido hialurônico, que é um glicosaminoglicano de alta massa molecular, principal componente da matriz extracelular de vertebrados que conecta as células do tecido conjuntivo, filamentos de proteínas e fibras de colágeno (BALA et al., 2018; LAURENT; FRASER, 1992). É conhecida como fator de disseminação e, portanto, facilita a disseminaçãoda toxinas durante o envenenamento por degeneração do ácido hialurônico na matriz extracelular dos tecidos locais que é o evento chave. A difusão de toxinas específicas para o alvo na circulação sistêmica e nos agentes que promovem esse processo é chamada de "fatores de disseminação". As enzimas hidrolíticas, como as hialuronidases e as metaloproteases hemorrágicas, causam danos ao tecido local, como a degradação da matriz extracelular e do tecido conjuntivo ao redor dos vasos sanguíneos. Essas proteínas degradam levam à perda de integridade estrutural e facilitam a difusão da toxina (BALA et al., 2018).

Por outro lado o veneno de *B. brazili* apresentou uma alta atividade proteolítica dada pelas Metaloproteases e Serinopreteases, o que coincide muito bem com a alta presencia destas proteínas no veneno determinadas no analise proteômico, um dos testes utilizado para detecção de enzimas proteolíticas foi o ensaio fibrinogenolítico, que também indicou a presença desse tipo de proteínas no veneno de *B. brazili*. O fibrinogênio consiste em três pares de cadeias polipeptídicas Aα, Bβ e γ, com massas moleculares de 66, 54 e 48 kDa, respectivamente. O veneno de *B. brazili* foi capaz de clivar as cadeias Aα e Bβ do fibrinogênio, enquanto a cadeia γ permaneceu sem danos. Além disso, após incubação com EDTA, um agente quelante, a atividade fibrinogenolítica foi inibida, endossando a atividade específica de SVMP (Fig. 5A e 5B do paper 3), a pesar da atividade fibrinogenolítica tanto as metaloproteases prócoagulantes quanto as que não induzem coagulação são capazes de induzir a

defibrinogenação em animais experimentais, caraterizado pelo consumo do fibrinogênio *in vivo* (DE MORAIS et al., 2012; TORRES et al., 2012), pelo que realizar ensaios de atividade coagulantes de frações coletadas por cromatografia do veneno de *B. brazili* seria ideal para identificar a presença de toxinas pró coagulantes.

Outra atividade avaliada no veneno de *B. brazili* foi a citotoxicidade, que é relacionada as LAAOs e PLA_2 (Souza et al., 2015), no analise de viabilidade celular testado em células VERO, o veneno de *B. brazili,* apresentou uma baixa citotoxicidade comparado com outros venenos botrópico, parece ser menos tóxico para essas células (Tabela 1).

Baixa atividade de PLA_2 foi detectada no teste enzimático realizado (Tabela 1, paper 3), enquanto os achados proteômicos mostram quantidades elevadas de PLA_2 quando comparadas a outros venenos de *Bothrops* (Fig. 3, paper 3) (KOHLHOFF et al., 2012). Pesquisas anteriores mostraram que espécies de *Bothrops* que possuem uma ampla faixa de distribuição geográfica podem apresentar variação intraespecífica de veneno, com diferentes proporções de toxinas, principalmente a PLA_2 (CALVETE et al., 2011; NÚÑEZ et al., 2009).

As PLA_{2s} dos venenos de serpentes são divididas em dois grupos: I e II (SIX; DENNIS, 2000), encontradas na peçonha de serpentes, compreendendo proteínas estáveis, versáteis, relativamente pequenas (~13-17 kDa). As PLA_{2s} da família Viperidae são colocadas na classe II e subdivididas em outros dois grupos: (1) Asp49 PLA_{2s}, que possuem resíduo Asp na posição 49 e apresentam alta atividade catalítica contra substratos artificiais; (2) Lys49 PLA_{2s}, que apresenta um resíduo Lys na posição 49 e apresenta baixa ou nenhuma atividade catalítica. A provável presença de ambos os

tipos de PLA_{2s} poderia explicar porque os resultados mostram baixa atividade de PLA_2 nos ensaios enzimáticos.

O estudo da composição do veneno de serpentes *Bothrops* pode auxiliar no entendimento da variabilidade dentro desses táxons, bem como no desenvolvimento de protocolos de medicação aprimorados em caso de acidentes, uma vez que o conteúdo de toxinas do veneno pode estar relacionado às características clínicas do envenenamento (GUERRA-DUARTE et al., 2015; QUEIROZ et al., 2008).

A América do Sul é o lar de muitas espécies de serpentes (AUERBACH, 2005). Várias espécies de *Bothrops* coexistem e exibem diversos conteúdos de veneno, principalmente na região amazônica. Seus venenos também são neutralizados de forma diferente pelos antivenenos disponíveis. Diante disso, é de grande interesse para a saúde pública acessar o potencial dos antivenenos, produzidos em diversos países, de reconhecer e inibir os venenos de serpentes presentes nessas regiões. Os dados do analise da caraterização do veneno de *B. brazili* sugerem que os venenos botrópico têm proteínas semelhantes, indicando que os antígenos do veneno podem compartilhar epítopos semelhantes. Essas descobertas poderiam inicialmente apoiar o uso de qualquer um dos antivenenos em acidentes envolvendo esse tipo de serpente, mas mais estudos testando sua neutralização *in vivo* devem ser realizados para confirmá-la.

Uma vez identificadas às proteínas dos venenos e caraterizadas suas atividades tóxicas, se pode abordar novos estúdios que nos permita desenvolve novas metodologias, que nos ajudem a compreender melhor o mecanismo de ação dos venenos de serpentes. Um de estes abordagens é a identificação de epítopos de cada proteína que compor o veneno. O mapeamento de epítopos é fundamental no desenvolvimento de vacinas, e uns dos principais desafios é de predizer e mapear epítopos conformacionais

e lineais para células B em um antígeno (ANDERSEN; NIELSEN; LUND, 2006; ANSARI; RAGHAVA, 2010; CASTRO et al., 2015), Os métodos identificação de epítopos lineais baseados nas sequências de proteínas tem sido eficazes, uma das principais técnicas utilizadas no mapeamento de epítopos lineais é *Spot Synthesis* é uma técnica fácil e muito flexível para a síntese química paralela simultânea em suportes de membrana. Esta técnica tem sido amplamente utilizada no mapeamento de epítopos, como por exemplo, no mapeamento de epítopos de toxinas do veneno de *Micrurus corallinus* e no mapeamento de um epítopo linear na atroxlisina-I entre outros trabalhos (CASTRO et al., 2015; SCHNEIDER et al., 2016). Este método permite acesso rápido e de baixo custo a um grande número de peptídeos, tanto como produtos ligados à fase sólida quanto em fase de solução para análise sistemática de epítopos.

O uso de peptídeos sintetizados em sua forma solúvel é amplamente utilizado em diferentes áreas da ciência entre os que podemos citar: na imunização de cabras com um peptídeo sintético englobando o sítio antigênico G5 da glicoproteína de superfície do vírus da raiva induz uma forte resposta imune humoral na ausência de uma proteína transportadora (NIEDERHÄUSER et al., 2008), os peptídeos também têm sido usados em ensaios de ELISA na detecção de anticorpos específicos do vírus influenza A H1N1 2009 no soro e na saliva (AVILA et al., 2020), atualmente uma abordagem imunoinformática mostrou um avanço significativo no mapeamento de epítopos baseados em peptídeos no qual foram identificados 15 peptídeos antigênicos na glicoproteína de superfície do SARS-CoV-2, poderiam ser benéfico para uma rápida progressão do desenho da vacina. No entanto, este estudo necessita de validação experimental por *in vitro* e *in vivo* (KHAN et al., 2020). O que mostra uma amplia potencial do uso de peptídeos sintéticos, nosso trabalho Identification of a linear B-cell epitope in the catalytic domain of bothropasin, metalloproteinase from *Bothrops*

jararaca snake venom, o epítopo nomeado BotEp1 mostro ser conservado e imunodominante, sendo reconhecido por os três soros anti-botrópico diferentes de origem equino. O BotEp1 foi sintetizado como um peptídeo solúvel que mimetiza um epítopo linear identificado na bothropasina e ao ser utilizado em imunizações de camundongos e coelhos foi capaz de estimular células imunes para gerar um anticorpo específicos que foram capazes de neutralizar a atividade hemorragica causada pela bothropasina.

A sequência exata de aminoácidos de BotEp1 foi encontrada dentro da sequência do PIII-SVMP BITM06 A (UNIPROT: Q8QG88) do veneno de *Bothrops insularis*. Notavelmente, entre os vinte e um resíduos que conforma o BotEp1, apenas Leu15 é substituído por Phe, nesta região imunodominante em Jararhagin e Batroxrhagin, (do veneno de *B. atrox*). Os dados mostram que essas proteínas compartilham um possível epítopo comum e imunodominante. Curiosamente, esta região é altamente conservada entre as SVMPs P-III, quando comparada com as SVMPs P-I e P-II.

Por conseguinte, nossos resultados demonstram os benefícios da utilização de peptídeos sintéticos, porque eles podem ser sintetizados como reagentes precisos em imunoensaios, além do fato de que estes componentes são facilmente obtidos e ao custo relativamente baixo. A identificação de regiões comuns nas SVMPs é de grande importância no desenvolvimento de imunógenos que possam produzir uma resposta imune com ampla reatividade cruzada, capaz de neutralizar a atividade hemorrágica causada por venenos de diferentes serpentes do gênero *Bothrops*.

Como foi descrito, o envenenamento por *C. d. terrificus*, leva ao desenvolvimento de paralisia muscular e insuficiência renal aguda. A crotoxina é encontrada em alta concentração no veneno da *C. d. terrificus* e possui um papel muito

importante no seu efeito tóxico em vítimas de acidentes com serpentes do gênero crotálico na América do Sul (BEGHINI et al., 2000). Dessa forma, a inibição da crotoxina do veneno de serpentes é importante para o prognóstico em vítimas acidentadas.

Em nosso trabalho: Engineered protein containing Crotoxin epitopes induces neutralizing antibodies in immunized rabbits, foi realizado com a perspectiva de induzir anticorpos específicos contra os epítopos da crotoxina, utilizou-se uma proteína multiepitópica recombinante para imunização de animais, visando uma possível neutralização do efeito desta toxina pelos anticorpos produzidos. Epítopos identificados tem sido expressados como proteínas recombinantes, com a finalidade de expressar diferentes epítopos como uma proteína única, este tipo de abordagem já tem sido utilizado na geração de uma proteína quimérica recombinante (rCpLi) que consiste em epítopos de células B de uma proteína dermonecrótica do veneno de aranha *Loxosceles intermedia* (MENDES et al., 2013), demostrando que a proteína quimérica gerada é não tóxica gerando anticorpos protetores da toxina dermonecrótica. Uma abordagem semelhante foi feito na produção de uma proteína recombinante contendo epítopos de células B de diferentes toxinas de aranha de *Loxosceles*, esta proteína foi capaz de induzir a produção de anticorpos protetores em coelhos imunizados (LIMA et al., 2018).

A falha de implantes é uma das principais preocupações na área de biomateriais. Vários fatores têm sido relacionados à falha, mas em geral esses biomateriais não apresentam propriedades físicas, químicas ou biológicas comparáveis aos tecidos naturais e, em última análise, esses dispositivos podem levar à inflamação crônica e reações de corpo estranho. Materiais e compósitos biodegradáveis são promissores para uma ampla gama de aplicações biomédicas (MARQUES; REIS; HUNT, 2005). Materiais implantados por via subcutânea ativam o sistema imunológico inato do

hospedeiro, resultando na reação de corpo estranho (KASTELLORIZIOS; TIPNIS; BURGESS, 2015). A reação de corpo estranho é um fenômeno complexo e ainda não é totalmente compreendido.

O método de mapeamento de epítopos de células B foi realizado, avaliando a capacidade dos implantes subcutâneos, feitos de peptídeos ligados à celulose, para gerar anticorpos capazes de reconhecer crotoxina, este método foi capaz de induzir uma resposta imune contra o peptídeo presente no implante. A reatividade dos anticorpos de camundongos eliciados pelos implantes contra o veneno crotálico foi avaliada por ELISA, identificando regiões imunorreativas que diferem das encontradas no outro método de mapeamento de epítopos realizado, ensaio SPOT. Além disso, como os adjuvantes tradicionais não são necessários com este sistema, as respostas inflamatórias graves e o desconforto animal associado são reduzidos. Outra vantagem desse método é o aumento da resposta imune, uma vez que a membrana de celulose que compor o implante ajuda a reter o antígeno no animal e, portanto, atua como adjuvante (AMERO; JAMES; ELGIN, 1994), também é útil quando apenas pequenas quantidades de proteína estão disponíveis.

Para a produção de anti-soros contra a crotoxina do veneno da serpentes *C. d. terrificus*. Epítopos foram identificados por dois métodos de mapeamento de epítopos da crotoxina, uma proteína neurotóxica, principal componente letal do veneno das serpentes da espécie *Crotalus durrisus.* Muitos motivos justificam o uso de peptídeos em vez de proteínas inteiras para a produção de antivenenos. Em primeiro lugar, ao contrário dos antígenos tóxicos, as formulações de peptídeos não são prejudiciais ao animal imunizado. Em segundo lugar, a apresentação de formulações contendo proteínas quiméricas constituídas por epítopos da crotoxina ao sistema imunológico de animais imunizados pode aumentar o sucesso de protocolos de imunização. Finalmente,

a produção de proteínas multiepitópicas projetadas é geralmente mais fácil e seguro do que obter e preparar o veneno bruto de serpentes.

No presente estudo, apresentamos o desenvolvimento de um imunógeno proteico multipetitópico rMEPCtx, que contem epítopos mapeados da crotoxina do veneno da C. d. terrificus, a espécie de serpente mais letal no Brasil. Em nossa primeira tentativa, a construção de proteína multipetitópica que expressa 5 epítopos foi gerada por clonagem do respectivo gene sintéticos no vetor pET 28a. Demonstramos que uma proteína não tóxica expressando epítopos de crotoxina pode ser gerada.

Os ensaios de ELISA e Immunoblot revelaram que a rMEPCtx exibia atividade antigênica contra o soro anticrotálico. A resposta de anticorpos dos coelhos imunizados com a rMEPCtx foi avaliada por ELISA. Através desses ensaios, fomos capazes de detectar uma boa resposta de anticorpos nos coelhos imunizados. Os anticorpos dos coelhos imunizados com a rMEPCtx reagiram significativamente aos venenos de *C. d. terrificus, C. d. ruruima, C. durissus* do Peru e *B. jararaca*. Este achado não é surpreendente, pois proteínas PLA_2 são encontradas nos venenos crotálico e botrópico (CALVETE et al., 2009; GONÇALVES-MACHADO et al., 2016; SOUSA et al., 2013).

A capacidade de inibição da atividade letal da crotoxina em camundongos virgens foi avaliada por pré-incubação com anticorpos anti-rMEPCtx e crotoxina, estes anticorpos forneceram boa proteção, evitando a letalidade causada por duas DL_{50} de crotoxina. Portanto, esta proteína projetada pode ser um candidato promissor para o desenvolvimento de soro terapêutico ou vacinação no futuro.

Estes resultados mostram que mapear epítopos e usá-los para compor um imunógeno multi-epítopo recombinante para obter anticorpos anti-epítopos específicos

é uma estratégia eficiente para atingir a neutralização do veneno. Esta abordagem pode ser considerada para a preparação direta de antivenenos, diminuindo ou mesmo dispensando o uso de veneno bruto. O uso de peptídeos ou de proteína multiepitópica recombinante são moléculas atóxica e, portanto, representa um imunógeno mais seguro em relação ao bem-estar animal na produção de antivenenos. Além disso, tem flexibilidade para ser produzido em grande escala e pode ser modificado usando abordagens simples para incorporar novos conhecimentos sobre a toxicidade do veneno e refino de epítopos.

O tratamento recomendado pelo Ministério da Saúde para casos de acidentes ofídicos é o uso dos soros heterólogos antivenenos de origem equina. Estes soros são capazes de impedir a ação de alguns componentes do veneno e, consequentemente, seus efeitos patológicos. Entretanto, este tratamento pode ser arriscado devido às reações de hipersensibilidade que podem ocorrer durante a administração do antiveneno e nas duas horas subsequentes, a pesquisa apresentada nesta tese busca e demostra novas abordagens que podem ser utilizados no melhoramento da produção de antivenenos.

5. CONCLUSÕES

- A técnica de *Spot synthesis* e de mapeamento por implantes subcutâneos foram eficientes para o mapeamento de epítopos lineares da bothropasina e da crotoxina.
- O uso do BotEp1 foi capaz em induzir a produção de anticorpos neutralizantes da atividade hemorrágica induzida pela bothropasina.
- O uso de uma proteína multiepitopica rMEPCtx, mostrou ser eficaz em induzir anticorpos neutralizantes da atividade letal causada pela crotoxina.
- O uso de peptídeos e proteínas multiepitópicas mostrou ser uma alternativa eficiente na produção de soros direcionados contra toxinas especificas encontrada nos venenos ofídicos.
- O perfil de composição proteica e atividades enzimáticas do veneno da serpente peruana *B. brazili*, foram analisados e apresentou predominância de proteases, SVMPs e SVSPs, que juntas representam mais de 50% do total de proteínas do veneno. Os dados do proteoma estão bem correlacionados com as atividades enzimáticas e citotoxicidade *in vitro*.

6. PERSPECTIVAS

- Avaliar a capacidade do anticorpo anti-BotEp1 de neutralizar outras metaloproteases de diferentes espécies de serpentes assim como das diferentes classes de metaloproteases do tipo PI, PII e PIII.
- Avaliar quais epítopos da crotoxina são reconhecidos pelo soro anti- rMEPCtx.
- Produzir novas proteínas multiepitopicas contendo epítopos já identificados de outras toxinas de venenos como LAAO, Crotamina, Serinoproteases, e avaliar sua capacidade induzir anticorpos neutralizantes.

7. REFERENCIAS

AKRAM, A.; INMAN, R. D. Immunodominance : A pivotal principle in host response to viral infections. **Clinical Immunology**, v. 143, n. 2, p. 99–115, 2012.

ALVARENGA, L. et al. Design of antibody-reactive peptides from discontinuous parts of scorpion toxins. **Vaccine**, v. 28, n. 4, p. 970–980, jan. 2010.

AMERO, S. A.; JAMES, T. C.; ELGIN, S. C. Production of antibodies using proteins in gel bands. **Methods in molecular biology (Clifton, N.J.)**, v. 32, p. 401–406, 1994.

AMORIM, F. G. et al. Characterization of Bothrops moojeni Snake Venom : Influence of Snake Gender on Venom Composition. **Toxins**, v. 10, n. 5, p. 1–18, 2018.

ANDERSEN, P. H.; NIELSEN, M.; LUND, O. L. E. Prediction of residues in discontinuous B-cell epitopes using protein 3D structures. p. 2558–2567, 2006.

ANSARI, H. R.; RAGHAVA, G. P. S. Identification of conformational B-cell Epitopes in an antigen from its primary sequence. **Immunome Research**, v. 6, n. 1, p. 6, 2010.

ASSAKURA, M. T. et al. Molecular cloning and expression of structural domains of bothropasin, a P-III metalloproteinase from the venom of Bothrops jararaca. **Toxicon**, v. 41, n. 2, p. 217–227, 2003.

AUERBACH, P. The Venomous Reptiles of the Western Hemisphere. **Wilderness & Environmental Medicine**, v. 16, 2005.

AVILA, G. et al. Influenza A H1N1 Virus 2009 Synthetic Hemagglutinin and Neuraminidase Peptides for Antibody Detection. **Archives of Medical Research**, v. 51, n. 5, p. 436–443, 2020.

BALA, E. et al. A biological overview of Hyaluronidase: A venom enzyme and its inhibition with plants materials. **Materials Today: Proceedings**, v. 5, n. 2, Part 1, p. 6406–6412, 2018.

BATTELLINO, C. et al. Assessment of efficacy of bothropic antivenom therapy on microcirculatory effects induced by Bothrops jararaca snake venom. **Toxicon : official journal of the International Society on Toxinology**, v. 41, n. 5, p. 583–593, abr. 2003.

BEGHINI, D. G. et al. Enzymatic Characterization of a Novel Phospholipase A 2 from Crotalus durissus cascavella Rattlesnake (Maracambóia) Venom. **Journal of protein Chemistry**, v. 19, n. 7, p. 603–607, 2000.

BERNARDE, P. S.; NEGREIROS DE ALMEIDA, M. R. The Brazil's Lancehead (Bothops brazili): An Uncommon Pit Viper of the Amazonia. **Wilderness & Environmental Medicine**, v. 31, n. 1, p. 126–127, 2020.

BERNHEIMER, A. W. et al. Isolation and characterization of a phospholipase B from venom of Collett's snake, Pseudechis colletti. **Toxicon : official journal of the International Society on Toxinology**, v. 25, n. 5, p. 547–554, 1987.

BOLDRINI-FRANÇA, J. et al. Snake venomics and antivenomics of Crotalus durissus subspecies from Brazil: Assessment of geographic variation and its implication on snakebite management. **Journal of Proteomics**, v. 73, n. 9, p. 1758–1776, 2010.

BRIGATTE, P. et al. Walker 256 Tumor Growth Suppression by Crotoxin Involves Formyl Peptide Receptors and Lipoxin A_4. **Mediators of inflammation**, v. 2016, p. 2457532, 2016.

BRINKHOUS, K. M. et al. Botrocetin (venom coagglutinin): reaction with a broad spectrum of multimeric forms of factor VIII macromolecular complex. **Proceedings of**

the National Academy of Sciences of the United States of America, v. 80, n. 5, p. 1463–1466, mar. 1983.

BROWN, R. L. et al. Pseudechetoxin binds to the pore turret of cyclic nucleotide-gated ion channels. **The Journal of general physiology**, v. 122, n. 6, p. 749–760, dez. 2003.

CALDERON, L. A. et al. Antitumoral activity of snake venom proteins: new trends in cancer therapy. **BioMed research international**, v. 2014, p. 203639, 2014.

CALVETE, J. J. et al. Venoms, venomics, antivenomics. **FEBS Lett**, v. 583, p. 1736–1743, 2009.

CALVETE, J. J. et al. Snake Venomics of the Central American Rattlesnake *Crotalus simus* and the South American *Crotalus durissus* Complex Points to Neurotoxicity as an Adaptive Paedomorphic Trend along *Crotalus* Dispersal in South America. **Journal of Proteome Research**, v. 9, n. 1, p. 528–544, 2010a.

CALVETE, J. J. et al. Snake Venomics of the Central American Rattlesnake Crotalus simus and the South American Crotalus durissus Complex Points to Neurotoxicity as an Adaptive Paedomorphic Trend along Crotalus Dispersal in South America research articles. p. 528–544, 2010b.

CALVETE, J. J. et al. Snake population venomics and antivenomics of Bothrops atrox: Paedomorphism along its transamazonian dispersal and implications of geographic venom variability on snakebite management. **Journal of proteomics**, v. 74, n. 4, p. 510–527, abr. 2011.

CALVETE, J. J. Snake venomics: from the inventory of toxins to biology. **Toxicon : official journal of the International Society on Toxinology**, v. 75, p. 44–62, dez. 2013.

CALVETE, J. J.; JU, P.; SANZ, L. Snake venomics . Strategy and applications. **Journal of Mass Spectrometry**, n. July, p. 1405–1414, 2007.

CARRASCO, P. A. et al. Nomenclatural instability in the venomous snakes of the Bothrops complex: Implications in toxinology and public health. **Toxicon**, v. 119, p. 122–128, 2016.

CASTRO, K. L. et al. Toxicon Identi fi cation and characterization of B-cell epitopes of 3FTx and PLA 2 toxins from Micrurus corallinus snake venom. **Toxicon**, v. 93, p. 51–60, 2015.

CHERNYSHENKO, V. et al. Fibrin(ogen)olytic and platelet modulating activity of a novel protease from the Echis multisquamatis snake venom. **Biochimie**, v. 105, p. 76–83, 2014.

CHISARI, A. et al. A phospholipase A2-related snake venom (from Crotalus durissus terrificus) stimulates neuroendocrine and immune functions: determination of different sites of action. **Endocrinology**, v. 139, n. 2, p. 617–625, fev. 1998.

CORONADO, M.; GEORGIEVA, DESSISLAVA. GABDOULKHAKOV, A. H.; ANWAR, ULLAH. SPENCER, PATRICK. ARNI, K. BETZEL, C. crystallization communications Purification , crystallization and preliminary X-ray diffraction analysis of crotamine , a myotoxic polypeptide from the Brazilian snake Crotalus durissus terrificus crystallization communications. **crystallization communications Acta**, n. July, p. 1052–1054, 2012.

DA C B GOUVEIA, A. I. et al. Identification and partial characterisation of hyaluronidases in Lonomia obliqua venom. **Toxicon : official journal of the International Society on Toxinology**, v. 45, n. 4, p. 403–410, mar. 2005.

DAMICO, D. C. S. et al. Cytotoxicity of Lachesis muta muta snake (bushmaster) venom and its purified basic phospholipase A2 (LmTX-I) in cultured cells. **Toxicon**, v. 49, n. 5, p. 678–692, 2007.

DE MORAIS, N. C. G. et al. Isolation and characterization of moojenin, an acid-active, anticoagulant metalloproteinase from Bothrops moojeni venom. **Toxicon : official journal of the International Society on Toxinology**, v. 60, n. 7, p. 1251–1258, dez. 2012.

ESPINO-SOLIS, G. P. et al. Antidotes against venomous animals: State of the art and prospectives. **Journal of Proteomics**, v. 72, n. 2, p. 183–199, 2009.

ESTEVAO-COSTA, M. I. et al. Neutralization of toxicological activities of medically-relevant Bothrops snake venoms and relevant toxins by two polyvalent bothropic antivenoms produced in Peru and Brazil. **Toxicon**, v. 122, p. 67–77, 2016.

ESTÊVÃO-COSTA, M. I. et al. Action of metalloproteinases mutalysin I and II on several components of the hemostatic and fibrinolytic systems. **Thrombosis research**, v. 99, n. 4, p. 363–376, ago. 2000.

FAURE, G.; XU, H.; SAUL, F. A. Crystal structure of crotoxin reveals key residues involved in the stability and toxicity of this potent heterodimeric β-neurotoxin. **Journal of Molecular Biology**, v. 412, n. 2, p. 176–191, 2011.

FRY, B. G. et al. Early evolution of the venom system in lizards and snakes. **Nature**, v. 439, n. 7076, p. 584–588, 2006.

FRY, B. G. et al. The toxicogenomic multiverse: convergent recruitment of proteins into animal venoms. **Annual review of genomics and human genetics**, v. 10, p. 483–511, 2009.

FRY, B. G.; WÜSTER, W. Assembling an arsenal: origin and evolution of the snake venom proteome inferred from phylogenetic analysis of toxin sequences. **Molecular biology and evolution**, v. 21, n. 5, p. 870–883, maio 2004.

GERSHONI, J. M. et al. Epitope mapping: the first step in developing epitope-based vaccines. **BioDrugs : clinical immunotherapeutics, biopharmaceuticals and gene therapy**, v. 21, n. 3, p. 145–156, 2007.

GEYSEN, H. M.; MASON, T. J.; RODDA, S. J. Cognitive features of continuous antigenic determinants. **Journal of Molecular Recognition**, v. 1, n. 1, p. 32–41, 1988.

GMACHL, M.; KREIL, G. Bee venom hyaluronidase is homologous to a membrane protein of mammalian sperm. **Proceedings of the National Academy of Sciences of the United States of America**, v. 90, n. 8, p. 3569–3573, abr. 1993.

GONÇALVES-MACHADO, L. et al. Combined venomics, venom gland transcriptomics, bioactivities, and antivenomics of two Bothrops jararaca populations from geographic isolated regions within the Brazilian Atlantic rainforest. **Journal of Proteomics**, v. 135, p. 73–89, 2016.

GREMSKI, L. H. et al. Recent advances in the understanding of brown spider venoms: From the biology of spiders to the molecular mechanisms of toxins. **Toxicon : official journal of the International Society on Toxinology**, v. 83, p. 91–120, jun. 2014.

GUERRA-DUARTE, C. et al. Partial in vitro analysis of toxic and antigenic activities of eleven Peruvian pitviper snake venoms. **Toxicon**, v. 108, p. 86–96, set. 2015.

GUTIÉRREZ, J. M.; THEAKSTON, R. D. G.; WARRELL, D. A. Confronting the neglected problem of snake bite envenoming: the need for a global partnership. **PLoS medicine**, v. 3, n. 6, p. e150, jun. 2006.

HARRISON, R. A. et al. Snake envenoming: A disease of poverty. **PLoS Neglected Tropical Diseases**, v. 3, n. 12, 2009.

HARRISON, R. A. et al. Research strategies to improve snakebite treatment: Challenges and progress. **Journal of Proteomics**, v. 74, n. 9, p. 1768–1780, 2011.

HUANG, T.-F.; HSU, C.-C.; KUO, Y.-J. Anti-thrombotic agents derived from snake venom proteins. **Thrombosis journal**, v. 14, n. Suppl 1, p. 18, 2016.

KANG, T. et al. Enzymatic toxins from snake venom : structural characterization and mechanism of catalysis. **FEBS Journal**, v. 278, p. 4544–4576, 2011.

KASTELLORIZIOS, M.; TIPNIS, N.; BURGESS, D. J. Foreign Body Reaction to Subcutaneous Implants. **Advances in experimental medicine and biology**, v. 865, p. 93–108, 2015.

KHAN, A. et al. Design of an Epitope-Based Peptide Vaccine against the Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2): A Vaccine Informatics Approach. **bioRxiv**, p. 2020.05.03.074930, 1 jan. 2020.

KINI, R. M. Anticoagulant proteins from snake venoms: structure, function and mechanism. **The Biochemical journal**, v. 397, n. 3, p. 377–387, ago. 2006.

KOHLHOFF, M. et al. Exploring the proteomes of the venoms of the Peruvian pit vipers Bothrops atrox , B . barnetti and B . pictus. **Journal of Proteomics**, v. 75, n. 7, p. 2181–2195, 2012.

KUDO, K.; TU, A. T. Characterization of hyaluronidase isolated from Agkistrodon contortrix contortrix (Southern Copperhead) venom. **Archives of Biochemistry and Biophysics**, v. 386, n. 2, p. 154–162, 2001.

LAING, G. D. et al. Preclinical testing of three south American antivenoms against the venoms of five medically-important Peruvian snake venoms. **Toxicon**, v. 44, p. 103–106, 2004.

LAURE, C. J. [The primary structure of crotamine (author's transl)]. **Hoppe-Seyler's Zeitschrift fur physiologische Chemie**, v. 356, n. 2, p. 213–215, fev. 1975.

LAURENT, T. C.; FRASER, J. R. Hyaluronan. **FASEB journal : official publication of the Federation of American Societies for Experimental Biology**, v. 6, n. 7, p. 2397–2404, abr. 1992.

LIMA, S. DE A. et al. Recombinant Protein Containing B-Cell Epitopes of Different Loxosceles Spider Toxins Generates Neutralizing Antibodies in Immunized Rabbits. **Frontiers in Immunology**, v. 9, n. April, abr. 2018.

LIRA-DA-SILVA, R. M. et al. Serpentes de importância médica do nordeste do Brasil. **Gazeta Médica da Bahia**, v. 79, p. 7–20, 2009.

LOMEO, S. et al. Toxicon Crotoxin from Crotalus durissus terri fi cus snake venom induces the release of glutamate from cerebrocortical synaptosomes via N and P / Q calcium channels. **Toxicon**, v. 85, p. 5–16, 2014.

LU, G.; KOCHOUMIAN, L.; KING, T. P. Sequence identity and antigenic cross-reactivity of white face hornet venom allergen, also a hyaluronidase, with other proteins. **The Journal of biological chemistry**, v. 270, n. 9, p. 4457–4465, mar. 1995.

LU, Q.; CLEMETSON, J. M.; CLEMETSON, K. J. Snake venoms and hemostasis. **Journal of Thrombosis and Haemostasis**, v. 3, n. 8, p. 1791–1799, ago. 2005.

MAMILLAPALLI, R. et al. Enhancement and inhibition of snake venom

phosphodiesterase activity by lysophospholipids. **FEBS letters**, v. 436, n. 2, p. 256–258, out. 1998.

MANDELBAUM, FAJGA R. REICHEL, ANTONIA P. ASSAKURA, M. T. ISOLATION AND CHARACTERIZATION OF A PROTEOLYTIC ENZYME FROM THE VENOM OF THE. **Toxicon**, v. 20, n. 6, p. 955–972, 1982.

MARKLAND, F. S. Snake venoms and the hemostatic system. **Toxicon : official journal of the International Society on Toxinology**, v. 36, n. 12, p. 1749–1800, dez. 1998.

MARQUES, A. P.; REIS, R. L.; HUNT, J. A. An in vivo study of the host response to starch-based polymers and composites subcutaneously implanted in rats. **Macromolecular bioscience**, v. 5, n. 8, p. 775–785, ago. 2005.

MARSH, N.; WILLIAMS, V. Practical applications of snake venom toxins in haemostasis. **Toxicon : official journal of the International Society on Toxinology**, v. 45, n. 8, p. 1171–1181, jun. 2005.

MARTINS, M.; MARQUES, O.; SAZIMA, I. Ecological and phylogenetic correlates of feeding habits in Neotropical pitvipers of the genus Bothrops. In: **Biology of the Vipers**. [s.l: s.n.]. p. 307–328.

MATAVEL, A. C. S. et al. Tension generation and increase in voltage-activated Na+ current by crotamine. **European journal of pharmacology**, v. 348, p. 167–173, 1998.

MENDES, T. M. et al. Generation and characterization of a recombinant chimeric protein (rCpLi) consisting of B-cell epitopes of a dermonecrotic protein from Loxosceles intermedia spider venom. **Vaccine**, v. 31, n. 25, p. 2749–2755, 2013.

MINISTÉRIO DA SAÚDE. **Guia de vigilância epidemiológica**. 7 edição ed. Brasilia/DF: Ministerio da saúde, 2009.

MINISTÉRIO DA SAÚDE DO BRASIL. **Manual de diagnóstico e tratamento de acidentes por animais peçonhentos.** 2ª Edição ed. Brasilia: [s.n.].

MUNIZ, E. G. et al. Neutralizing potency of horse antibothropic Brazilian antivenom against Bothrops snake venoms from the Amazonian rain forest. **Toxicon : official journal of the International Society on Toxinology**, v. 38, n. 12, p. 1859–1863, dez. 2000.

MUNIZ, R. C. et al. Toxicon The three-dimensional structure of bothropasin , the main hemorrhagic factor from Bothrops jararaca venom : Insights for a new classification of snake venom metalloprotease subgroups. v. 52, p. 807–816, 2008.

NICASTRO, G. et al. Solution structure of crotamine, a Na+ channel affecting toxin from Crotalus durissus terrificus venom. **European Journal of Biochemistry**, v. 270, n. 9, p. 1969–1979, 2003.

NIEDERHÄUSER, S. et al. A synthetic peptide encompassing the G5 antigenic region of the rabies virus induces high avidity but poorly neutralizing antibody in immunized animals. **Vaccine**, v. 26, n. 52, p. 6749–6753, 2008.

NÚÑEZ, V. et al. Snake venomics and antivenomics of Bothrops atrox venoms from Colombia and the Amazon regions of Brazil, Perú and Ecuador suggest the occurrence of geographic variation of venom phenotype by a trend towards paedomorphism. **Journal of proteomics**, v. 73, n. 1, p. 57–78, nov. 2009.

OGUIURA, N. BONI-MITAKE, M.; RADIS, G. New view on crotamine , a small basic polypeptide myotoxin from South American rattlesnake venom. **Toxicon**, v. 46, p. 363–

370, 2005.

OTERO-PATIÑO, R. Epidemiological, clinical and therapeutic aspects of Bothrops asper bites. **Toxicon : official journal of the International Society on Toxinology**, v. 54, n. 7, p. 998–1011, dez. 2009.

OYAMA, E.; TAKAHASHI, H. Structures and Functions of Snake Venom Metalloproteinases (SVMP) from Protobothrops venom Collected in Japan. n. C, 2017.

PINHO, F. M. O.; PEREIRA, I. D. Ofidismo. **Revista da Associação Médica Brasileira**, v. 47, n. 1, p. 24–29, 2001.

QUEIROZ, G. P. et al. Interspecific variation in venom composition and toxicity of Brazilian snakes from Bothrops genus. **Toxicon : official journal of the International Society on Toxinology**, v. 52, n. 8, p. 842–851, dez. 2008.

RAMANAIAH, M.; PARTHASARATHY, P. R.; VENKAIAH, B. Isolation and characterization of hyaluronidase from scorpion (Heterometrus fulvipes) venom. **Biochemistry international**, v. 20, n. 2, p. 301–310, 1990.

RAWLINGS, N. D.; BARRETT, A. J.; FINN, R. Twenty years of the MEROPS database of proteolytic enzymes , their substrates and inhibitors. v. 44, n. November 2015, p. 343–350, 2016.

RAWLINGS, N. D.; TOLLE, D. P.; BARRETT, A. J. MEROPS: the peptidase database. **Nucleic acids research**, v. 32, n. Database issue, p. D160-4, jan. 2004.

REGENMORTEL, V. Antigenicity and Immunogenicity of Synthetic Peptides. p. 209–213, 2001.

RIBEIRO, P. H. et al. Mechanism of the cytotoxic effect of l-amino acid oxidase

isolated from Bothrops alternatus snake venom. **International Journal of Biological Macromolecules**, v. 92, p. 329–337, 2016.

SAKURAI, Y. et al. Anticoagulant activity of M-LAO, L-amino acid oxidase purified from Agkistrodon halys blomhoffii, through selective inhibition of factor IX. **Biochimica et biophysica acta**, v. 1649, n. 1, p. 51–57, jun. 2003.

SALDARRIAGA-CÓRDOBA, M. et al. Phylogeography of the Central American lancehead Bothrops asper (SERPENTES: VIPERIDAE). **PLoS ONE**, v. 12, n. 11, p. 1–20, 2017.

SAMPAIO, S. C. et al. Crotoxin: Novel activities for a classic β-neurotoxin. **Toxicon**, v. 55, n. 6, p. 1045–1060, 2010.

SANT'ANA, C. D. et al. Molecular characterization of BjussuSP-I, a new thrombin-like enzyme with procoagulant and kallikrein-like activity isolated from Bothrops jararacussu snake venom. **Biochimie**, v. 90, n. 3, p. 500–507, 2008.

SANTOS-FILHO, N. A. et al. A new acidic myotoxic, anti-platelet and prostaglandin I2 inductor phospholipase A2 isolated from Bothrops moojeni snake venom. **Toxicon : official journal of the International Society on Toxinology**, v. 52, n. 8, p. 908–917, dez. 2008.

SANTOS., M. F. L.; FARANI., M. C.; ROCHA., P. N. Insuficiência renal aguda em acidentes ofídicos por Bothrops sp . e Crotalus sp .: revisão e análise crítica da literatura. **Jornal Brasileiro de Nefrologia2**, v. 31, n. 2, p. 132–138, 2009.

SANZ, L. et al. Venomics and antivenomics of the poorly studied Brazil's lancehead, Bothrops brazili (Hoge, 1954), from the Brazilian State of Pará. **The journal of venomous animals and toxins including tropical diseases**, v. 26, p. e20190103, 2020.

SCHNEIDER, F. S. et al. Identification of protective B-cell epitopes of Atroxlysin-I : A metalloproteinase from Bothrops atrox snake venom. **Vaccine**, v. 34, n. 14, p. 1680–1687, 2016.

SERRANO, S. M. T. The long road of research on snake venom serine proteinases. **Toxicon**, v. 62, p. 19–26, 2013.

SERRANO, S. M. T.; MAROUN, R. C. Snake venom serine proteinases: sequence homology vs. substrate specificity, a paradox to be solved. **Toxicon : official journal of the International Society on Toxinology**, v. 45, n. 8, p. 1115–1132, jun. 2005.

SILVA, CRSITIANE C F. MENEZES, MILENE C. PALOMINO, MIRYAM. OLIVEIRA, ANA K. IWAI, LEO K. FARIA, MARCELLA. PORTARO, FERNANDA V. Toxicon Peptides derived from plasma proteins released by bothropasin , a metalloprotease present in the Bothrops jararaca venom. **Toxicon**, v. 137, p. 65–72, 2017.

SILVA, A. M. DA et al. Extractivism of palm tree fruits: A risky activity because of snakebites in the state of Acre, Western Brazilian Amazon. **Revista da Sociedade Brasileira de Medicina Tropical**, v. 52, p. e20180195, mar. 2019.

SIX, D. A.; DENNIS, E. A. The expanding superfamily of phospholipase A2 enzymes: classification and characterization. **Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids**, v. 1488, n. 1, p. 1–19, 2000.

SOUSA, L. et al. Coagulotoxicity of Bothrops (Lancehead Pit-Vipers) Venoms from Brazil: Differential Biochemistry and Antivenom Efficacy Resulting from Prey-Driven Venom Variation. **Toxins**, v. 10, n. 10, p. 411, 2018.

SOUSA, L. F. et al. Comparison of Phylogeny , Venom Composition and Neutralization

by Antivenom in Diverse Species of Bothrops Complex. **PLoS ONE**, v. 7, n. 9, p. 1–16, 2013.

STÁBELI, R. G. et al. Platelet aggregation and antibacterial effects of an L-amino acid oxidase purified from Bothrops alternatus snake venom. **Bioorganic and Medicinal Chemistry**, v. 12, n. 11, p. 2881–2886, 2004.

SUHR, S. M.; KIM, D. S. Comparison of the apoptotic pathways induced by L-amino acid oxidase and hydrogen peroxide. **Journal of biochemistry**, v. 125, n. 2, p. 305–309, fev. 1999.

TASOULIS, T.; ISBISTER, G. K. A Review and Database of Snake Venom Proteomes. **Toxins**, v. 9, n. 9, p. 290, set. 2017.

TEMPONE, A. G. et al. Bothrops moojeni venom kills Leishmania spp. with hydrogen peroxide generated by its L-amino acid oxidase. **Biochemical and biophysical research communications**, v. 280, n. 3, p. 620–624, jan. 2001.

THEAKSTON, R. D. G.; WARRELL, D. A.; GRIFFITHS, E. Report of a WHO workshop on the standardization and control of antivenoms. **Toxicon**, v. 41, n. 5, p. 541–557, 2003.

TORRES, F. S. et al. Bmoo FIBMP-I: A New Fibrinogenolytic Metalloproteinase from Bothrops moojeni Snake Venom. **ISRN toxicology**, v. 2012, p. 673941, 2012.

TRIER, N. H.; HANSEN, P. R.; HOUEN, G. Production and characterization of peptide antibodies. **Methods**, v. 56, n. 2, p. 136–144, 2012.

VALENTA, J. **Venomous Snakes-Envenoming, Therapy**. 2nd. ed. New York: Nova Science Publishers, Inc., 2010. v. 347

WALDEZ, FABIANO AND VOGT, R. C. Aspectos ecológicos e epidemiológicos de acidentes ofídicos em comunidades ribeirinhas do baixo rio Purus, Amazonas, Brasil. **Acta Amaz**, v. 39, p. 681–692, 2009.

WHITE, J. Snake venoms and coagulopathy. **Toxicon : official journal of the International Society on Toxinology**, v. 45, n. 8, p. 951–967, jun. 2005.

WHO. Post ECBS version EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION Geneva , 17 to 21 October 2016 WHO Guidelines for the Production , Control and Regulation of Snake Antivenom Immunoglobulins. **World Health Organization**, n. October, p. 1–138, 2016a.

WHO. **WHO Guidelines for the Production, Control and Regulation of Snake Antivenom Immunoglobulins**, 2016b.

WHO. Snakebite Envenoming - Epidemiology. 2019.

YANG, C.-A. et al. Identification of antibacterial mechanism of L-amino acid oxidase derived from Trichoderma harzianum ETS 323. **The FEBS journal**, v. 278, n. 18, p. 3381–3394, set. 2011.

ZAMUNÉR, S. R. et al. Comparison of the neurotoxic and myotoxic effects of Brazilian Bothrops venoms and their neutralization by commercial antivenom. **Toxicon : official journal of the International Society on Toxinology**, v. 44, n. 3, p. 259–271, set. 2004.

ZHANG, Y.-J. et al. Molecular characterization of Trimeresurus stejnegeri venom L-amino acid oxidase with potential anti-HIV activity. **Biochemical and biophysical research communications**, v. 309, n. 3, p. 598–604, set. 2003.