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Modificação estrutural de PLA2 de *Crotalus durissus ruruima* e *Crotalus durissus cumanensis* com p-bromofenacil e cumarinas sintéticas – Caracterização bioquímica e biológica. Estudo da agregação plaquetária e efeito edematogênico

Tese apresentada ao Instituto de Biologia da Universidade Estadual de Campinas para obtenção do título de Doutora em Biologia Funcional e Molecular – Área de Bioquímica

Orientador: Prof. Dr. Marcos Hikari Toyama

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"Para realizar grandes conquistas, devemos não apenas agir, mas também sonhar; não apenas planejar, mas também acreditar."
Anatole France

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ABREVIAÇÕES

AA – Ácido araquidônico

ADP – adenosina difosfato

ACD-C- Citrato de glicose ácido (acid citrate dextrose)

BSA- albumina soro bovino

C.d.cumanenses – Cdcu- Crotalus durissus cumanenses

C.d ruruima – Cdru- Crotalus durissus ruruima

CEMIB - Centro Multidisciplinar para Investigação Biológica na Área da Ciência em Animais de Laboratório

CK- creatina quinase

Da / KDa – Dalton / Kilodalton

DMSO - dimetilsulfóxido

DTT - ditiotreitol

EDTA - ácido etilenodiaminotetraácido

EOCC – etil 2-oxo-2H- chromen-3-carboxilato

FAD – flavina adenina dinucleotídeo

FMN – flavina mononucleotídeo

GPIIb/IIIa – glicoproteína IIb/IIIa

GP-Ib – glicoproteína Ib

GP-IIIa – glicoproteína IIIa

G25 – Sephadex G25 - Coluna de exclusão molecular com matriz composta de dextrano

HCL- ácido clorídrico

HPLC- cromatografia líquida de alta eficiência

H_2O_2 – água oxigenada

IBSBF – Instituto Biológico Sessão Bacteriológico Fitopatológico

IL-6 – interleucina-6

LAO – L – aminoácido oxidase

mA – miliampere

MALDI-TOF- Espectômetro de massa (Matrix Assisted Laser Desorption/Ionization Time of Flight)

NBSF- Nitrobencenosulfonil fluoride

NOBA - 4-nitro-3-(octanoyloxy)- benzóico

PAGE-SDS – eletroforese em gel de poliacrilamida/ duodecil sulfato de sódio

PBS – Tampão fosfato salina

p – BPB – *p*ara-brometo de bromofenacil

PL - plaqueta lavada

PLA2- Fosfolipase A2

sPLA2ru1 - fosfolipase A2 ruruima isoforma 1

sPLA2ru2 – fosfolipase A2 ruruima isoforma 2

pI – ponto isoelétrico

PRP – Plasma rico em plaqueta

PTC- fenilisotiocianato

PTH- Phenil Tio Hidantoina

RGD – domínio com seqüência de aminoácidos arginina-glicina-ácido aspártico

Superdex 75 – gel de filtração composto por matriz de dextrano e agarose

TFA- Ácido trifluoracético

Tampão A TFA 0,1% utilizado para cromatografia de HPLC-RP

Tampão B Acetonitrila 66% utilizado em cromatografia de HPLC-RP

Tris-HCL- hidroximetil aminometano

µ-bondapack C-18- Coluna de HPLC com n-octadecyl como fase da base estacionária

UFC- unidades formadoras de colônia

v/v – volume por volume

V máx – velocidade máxima

ABREVIAÇÕES PARA AMINOÁCIDOS

AMINOÁCIDOS	ABREVIAÇÕES (3 LETRAS)	SÍMBOLO
Alanina	Ala	A
Arginina	Arg	R
Asparagina	Asn	N
Ácido aspártico	Asp	D
Cisteína	Cys	C
Glutamina	Gln	Q
Ácido glutâmico	Glu	E
Glicina	Gly	G
Histidina	Hys	H
Isoleucina	Iso	I
Leucina	Leu	L
Lisina	Lys	K
Metionina	Met	M
Fenilalanina	Phe	F
Prolina	Pro	P
Serina	Ser	S
Treonina	Thr	T
Triptofano	Trp	W
Tirosina	Tyr	Y
Valina	Val	V

Nomenclatura IUPAC

RESUMO

No Brasil a espécie predominante de serpente peçonhenta do gênero *Crotalus* é a *Crotalus durissus*, que é composta por várias subespécies encontradas em todo território nacional, as subespécies alvos de nosso estudo são a *Crotalus durissus ruruima* e a *Crotalus durissus cumanenses,* presentes na região amazônica e na Venezuela respectivamente, mas que ainda não foram tão bem estudadas quanto a *Crotalus durissus terrificus*.

As principais frações já caracterizadas para o veneno total de *Crotalus* são: convulxina, giroxina, crotoxina e crotamina. Através de uma combinação de varias metodologias em HPLC como exclusão molecular e em fase reversa conseguimos isolar os principais constituintes da crotoxina ($PLA_{2,}$ crotapotina e as proteínas "trombina-like") dos venenos totais. Durante o fracionamento do veneno total em coluna de HPLC de exclusão molecular foi detectado com atividade fosfolipásica nas frações crotoxínicas de *Crotalus durissus ruruima* e *Crotalus durissus cumanenses* sendo confirmado através de eletroforese PAGE-SDS e espectrometria de massa MALDI-TOF. As PLA_2 das duas subespécies alvo apresentaram alta homegeneidade com massa molecular de aproximadamente 14kDa e mostraram uma maior quantidade de aminoácidos básicos; lisina e arginina para um total de 122 aminoácidos. O N-terminal da seqüência de aminoácidos de PLA_2 mostrou homologia com outras proteínas PLA_2 do gênero crotálico.

A fosfolipase (PLA_2) constitui o maior componente presente no veneno de serpente e tem sido extensivamente investigado não só por ser mais abundante, mas por exibir uma ampla gama de efeitos biológicos, incluindo neurotoxidade, miotoxicidade, citotoxidade, efeito edematogênico, anti-coagulante, agregação plaquetária, hipotensivo, bactericida, anti-HIV, anti- tumor e anti-parasitário. Devido a esta diversidade funcional, estas proteínas estruturalmente semelhantes despertaram o interesse de muitos pesquisadores como modelos moleculares para o estudo das relações estrutura-função.

No presente trabalho foi nosso propósito purificar o veneno total de *Crotalus dirussus ruruima* e *Crotalus durissus cumanenses*, isolar a fração fosfolipásica e verificar que as atividades biológicas e bioquímicas foram afetadas após prévias modificações estruturais com p-bromofenacil brometo e cumarinas sintéticas.

Os resultados apresentados neste trabalho proporcionam a obtenção de uma ferramenta bioquímica útil para acrescentar novos conhecimentos à relação entre sítios farmacológicos, região catalítica nas fosfolipases A2 e função.

ABSTRACT

In Brazil the predominant species of poisonous snake of the genus *Crotalus* is the *Crotalus durissus*, which is composed of several subspecies found throughout the country. The sub-targets of our study is the *Crotalus durissus ruruima* and *Crotalus durissus cumanensis* present in the Amazon region and Venezuela respectively, that haven't been as well studied as the *Crotalus durissus terrificus*.

The main fractions we know for the total of Crotalus poison were convulxin, giroxin, crotoxin and crotalin. Through a combination of many methodologies such as HPLC molecular exclusion and in a reverse phase we managed isolate the principal components of crotoxin (PLA2, crotapotin and proteins "thrombin-like") of the total poison. During the division of the total poison in column HPLC the molecular exclusion was detected a phospholipase activity in the fractions crotoxin *Crotalus durissus ruruima* and *Crotalus durissus cumanensis* that confirmed through SDS-PAGE and electrophoresis of the mass MALDI-TOF. The two subspecies of PLA2 show us they are highly homogeneous with the molecular mass of almost 14kDa and a great amount of basic amino acids, lysine and arginine for a total of 122 amino acids. The N-terminal amino acid sequence of PLA_2 showed homology with other proteins of the kind *Crotalus* PLA_2.

The phospholipase (PLA2) is the major component present in snake poison and has been extensively investigated not only because it is more abundant, but it shows a wide range of biological effects, including neurotoxicity, myotoxicity, cytotoxicity, edematogenic effect, anti-coagulant, platelet aggregation, hypotensive, bactericidal, anti-HIV, anti-tumor and anti-parasitic. Because of this functional diversity, these proteins structurally similar aroused the interest of many researchers as molecular models for the study of structure-function relationships.

In this study our purpose was to purify the total poison of *Crotalus durissus ruruima* and *Crotalus durissus cumanensis* and isolate the fraction of phospholipase and found that the biochemical and biological activities were affected after previous structural changes with p-bromofenacil bromide and synthetic coumarins.

The results presented in this work allow them to obtain a useful biochemical tool to add new knowledge to the relation between pharmacological sites, the catalytic region in phospholipases A2 and function.

1. INTRODUÇÃO

1.1. Ofidismo

As serpentes são répteis que pertencem ao mesmo Filo Chordata das tartarugas, dos crocodilos e dos jacarés e a linhagem Sauropsida. Evoluíram dos lagartos há mais de 130 milhões de anos e exercem importante função no ecossistema terrestre, uma vez que, controlam naturalmente a população de roedores que além de serem transmissores de diversas patologias também atacam os produtos armazenados gerando grandes perdas econômicas (de Oliveira, 1999).

Evolutivamente, as serpentes alteraram suas características morfológicas e fisiológicas propiciando sua adaptação aos mais variados ambientes, como, as árvores, as águas e as superfícies superiores e inferiores do solo. Dificilmente são encontradas em algumas ilhas, devido ao isolamento, e em locais de alta latitude (as calotas polares) ou acima de 3000 m de altitude, devido ao frio intenso. A quantidade de espécies é maior nas regiões tropicais e reduz-se conforme aumenta a distância à linha do equador, pois a temperatura ambiente ideal para as serpentes é de 30 graus centígrados. (Matsui et al., 2000).

Segundo a literatura vigente foram catalogadas aproximadamente 3.315 espécies de serpentes sendo que 200 são consideradas peçonhentas. As serpentes dividem-se em vinte-uma famílias sendo que no Brasil são encontradas apenas dez: Anomalepididae, Leptotyphopidae, Typhlopidae, Analiidae, Boidae, Colubridae, Dpsadidae, Tropidophiidae, Elapidae e Viperidae. No Brasil ocorrem 371 espécies de serpentes (SBH 2008), dessas 55 são peçonhentas. São quatro grupos de serpentes que podem causar acidentes ofídicos no Brasil (Melgarejo, 2003): Grupo I: *Bothrops* (Viperidae); Grupo II: *Crotalus* (Viperidae); Grupo III: *Lachesis* (Viperidae); Grupo IV: *Micrurus* e *Leptomicrurus* (Elapidade).

1.2 Epidemiologia

Estimativas mundiais demonstraram que a incidência de acidentes ofídicos é maior no continente asiático e em diversas regiões da América Latina, enquanto que, na América do Sul, os países com maiores episódios anuais foram Brasil (20.000 casos), Peru (4.500 casos), Venezuela (3.000 casos), Colômbia (2.675 casos), Equador (aproximadamente 1.300 casos) e Argentina (aproximadamente 1.200 casos) (Warrel, 2002).

Apesar das estimativas, acredita-se que esses índices estejam desatualizados, uma vez que, em regiões pobres como nos países tropicais e subtropicais, este fato não recebe a devida atenção e muitas vezes não são registrados, notificados ou informados adequadamente aos órgãos competentes (Pinho et al., 2001).

No Brasil no ano de 2002, foram registrados pela FUNASA (Fundação Nacional de Saúde – Ministério da Saúde) 158.495 casos de acidentes ofídicos. O gênero *Bothrops* apresenta os maiores índices de noticação, porém o gênero que apresenta maior taxa de letalidade é o gênero *Crotalus* (**Tabela 1**). Desta maneira, o veneno produzido pelas serpentes do gênero *Crotalus*, por ser mais letal, torna-se importante epidemiologicamente e é o principal foco de estudo deste trabalho.

Tabela 1: Letalidade dos acidentes ofídicos, divididos por gênero (FUNASA 2002)

Gênero	**N Casos**	**N Óbitos**	**Letalidade (%)**
Bothrops	119.238	369	0,31
Crotalus	10.143	189	1,87
Lachesis	1878	18	0,95
Micrurus	558	6	0,36
Não informado	26.678	138	0,52
Total	**158.495**	**720**	**0,45**

1.3 *Crotalus durissus sp.*

Para o gênero *Crotalus* é descrita apenas uma espécie *Crotalus durissus* que se subdivide em 7 subespécies sendo encontrada em toda América Latina (**Tabela 2**) (Sorensen, 1990)

Tabela 2: Principais subspécies de *Crotalus* encontradas na América Latina e sua localização geográfica.

Subspécie de Crotalus	Nome Popular	Localização Geográfica na América Latina
Crotalus durissus terrificus	Cascavel	Regiões altas e secas desde Minas Gerais até Rio Grande do Sul
Crotalus durissus cascavella	Maracambóia	Áreas de Caatinga do Nordeste, desde Maranhão até norte do estado de Minas Gerais
Crotalus durissus collilineatus		Regiões secas do Centro-Oeste, desde Mato Grosso do Sul, Goías e Minas Gerais até Norte do Estado de São Paulo
Crotalus durissus marajoensis		Ilha de Marajó e em toda a América Equatorial
Crotalus durissus ruruima		Algumas regiões de Roraima, Amapá, Pará, Amazonas e Rondônia, assim como também na América Equatorial
Crotalus durissus trigonicus		Roraima
Crotalus durissus cumaneneses		Todo território venezuelano Região de contatocom a espécie *Crotalus durissus ruruima*

De forma geral, as viperídeas medem aproximadamente 180 centímetros de comprimento e possuem características em comum como, presença de pequenas escamas em forma de quilha, fosseta loreal e glândulas produtoras de veneno localizadas na cabeça (**Figura 1A**). Na ponta da cauda um guizo ou chocalho alerta sua presa de seu bote. Alimentam-se principalmente de mamíferos e répteis menores (Alves, 1991).

As serpentes da família Viperidae apresentam dentição solenóglifa. Nesta dentição encontramos presas que possuem um canal central que se comunica com o canal excretor da glândula de veneno **(Figura 1B)** (Guimarães, 1974; Leitão-de-Araújo & Alves, 1976; Rosenfeld, 1961). Durante o mecanismo do bote, quando a serpente abre a boca, estas presas se deslocam para frente e no ato da mordida inoculam o veneno em sua vítima (Buononato, 2001).

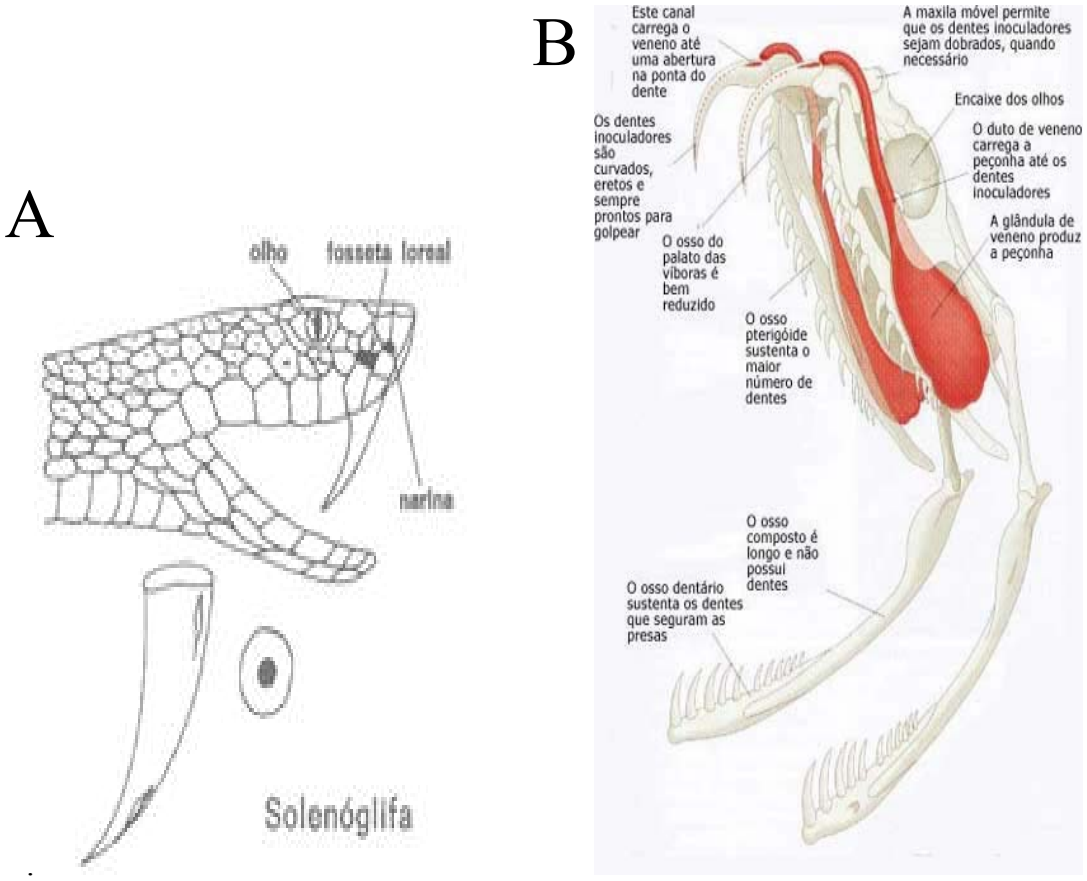

Figura 1: A) Dentição solenóglifa. B) Tipo de aparato venenoso das serpentes (Guimarães, B, 1974).

As serpentes pertencentes à espécie *Crotalus sp.* são responsáveis por aproximadamente 9% do número total de acidentes ofídicos e, sua picada é caracterizada por um discreto edema local seguido ou não por dor acentuada. A discreta reação local não

espelha a gravidade do acidente crotálico, uma vez que, o veneno possui ação neurotóxica e hemolítica que desencadeia o quadro clínico descrito na **Tabela 3** (Amaral et al., 1991).

Tabela 3: Provável Quadro Clínico apresentado por paciente envenenado por picada de cobra *Crotalus sp* (Caderno Técnico Escola Medicina Veterinária UFMG, 1999)

<table>
<tr><th>Ação do Veneno</th><th>Sintomas e sinais precoces
(até 6 horas após o acidente)</th><th>Sintomas e sinais tardios
(de 6 a 12 horas após o acidente)</th></tr>
<tr><td>Neurotóxica</td><td colspan="2">Ptose palpebral (fáceis miastênicas), diplopia, oftalmoplegia e
visão turva por dificuldade de acomodação visual.
Relatos de insuficiência respiratória aguda, em casos graves</td></tr>
<tr><td>Miotóxica</td><td>Dor muscular generalizada,
Urina avermelhada ou marrom.
Edema discreto no local da picada</td><td rowspan="2">Urina avermelhada ou marrom escura
(hemoglobinúria e miobinúria).
Insuficiência Renal aguda</td></tr>
<tr><td>Hemolítica</td><td>Urina avermelhada</td></tr>
<tr><td>Coagulante</td><td colspan="2">Aumento do tempo de coagulação.
Raramente ocorrem hemorragias</td></tr>
</table>

Apesar do grande número de subespécies de *Crotalus durissus* a mais bem estudada é a *Crotalus durissus terrificus* (*C. d. terrificus*). Embora seja a mais estudada, este trabalho tem como foco principal o estudo das serpentes *Crotalus durissus cumanensis* (*C. d. cumanensis*) e *Crotalus durissus ruruima* (*C. d. ruruima*) pois ambas são endêmicas do território nacional e seus venenos possuem diversos tipos de atividades que nunca antes foram estudados para os objetivos propostos (Toyama et al., 2003).

Desta maneira, a *C. d. cumanensis* é uma das espécies mais comuns da Venezuela sendo encontrada em todo território venezuelano desde o nível do mar até 2.500 metros de altitude (Kornacker, 1999). É responsável pela maioria dos casos de envenenamento por cobras neste país (Roze, 1966). Embora seja uma serpente endêmica da Venezuela, ela também é encontrada em uma região no estado de Roraima onde a *C.d. ruruima* também habita. Seu veneno é composto principalmente por um complexo protéico de atividade

neurotóxica denominado crotoxina. Este complexo protéico possui atividade hemolítica, proteolítica, esterolítica e miotóxica.

Enquanto que, a *C. d. ruruima* é comumente encontrada nos limites entre Brasil, Venezuela e Guiana (Hoge, 1965; Hoge & Romano, 1972; Dos-Santos, 2005; do Nascimento, 2000).

Assim como, a *C. d. terrificus*, o veneno da *C. d. ruruima* é classificado de acordo com a sua coloração em branco ou amarelo (Dos-Santos et al.,1993). Desta maneira, encontramos diferenças bioquímicas e biológicas nos diferentes tipos de veneno produzidos por um único animal, sendo que, o veneno branco mostrou atividades letal, coagulante, miotóxica, edematogênica e hemolítica, enquanto que, a variedade amarela, além destas, também apresenta atividades hemorrágica, necrotizante e caseinolítica (Dos-Santos, 1993).

1.4 O veneno crotálico

O veneno crotálico apresenta como principal função imobilizar e matar as presas (Karlsson, 1979). Ele é composto por uma mistura complexa de carboidratos, lipídios e íons metálicos. Além disso, possui proteínas e peptídeos que representam aproximadamente 90% de veneno total seco (Bieber, 1979). Quando fracionado encontramos como proteínas principais a convulxina, giroxina, crotamina e crotoxina, sendo que, a crotoxina representa aproximadamente 47% a 55% de todo o veneno em massa seca (Martins et al., 2002; Toyama et al., 2003).

1.4.1 Crotoxina

A crotoxina foi primeiramente isolada, com o uso de técnicas de precipitação em sulfato de amônio, em 1938 por Slotta e Frankel-Conrat. Ela tem sido descrita como uma

proteína heterodimérica, constituída de duas cadeias polipeptídicas: uma PLA_2, (subunidade básica), componente fracamente neurotóxico, quando usado de forma isolada em preparações de junção neuromuscular de ratos ou camundongos, com massa molecular ao redor de 14-15 kDa, com um ponto isoelétrico de 7,8-8,6. E a crotapotina (subunidade ácida), um polipeptídeo constituído por três cadeias denominadas de cadeias A, B e C, unidas tanto por pontes dissulfeto intracadeia como intercadeia, com massas moleculares ao redor de 4,0, 3,5 e 1,5 kDa, respectivamente (Toyama et al., 2003; Oliveira et al., 2003), que não mostra nenhuma atividade neurotóxica, com uma massa molecular ao redor de 9kDa.

Experimentos conduzidos por Hendon & Frankel-Conrat (1971) e Hawgood & Santana (1979), mostraram que, quando as duas frações são colocadas juntas em uma proporção eqüimolar, observam-se dois resultados: diminuição da atividade enzimática PLA_2 do componente básico e aumento da atividade farmacológica.

Experimentos realizados por Rübsamen et al. (1971) e por Breithaupt (1976), foram os primeiros a mostrar este efeito sinergista. A função da crotapotina parece ser a de impedir a ligação de fosfolipase A_2 em várias estruturas, orientado-a principalmente para as membranas pré e pós-sinápticas da junção neuromuscular (Bon et al., 1979 e Vital Brazil, 1982). Nakazone (1978) mostrou que a proporção de crotapotina para fosfolipase A_2 é de 1:1 em relação molar: uma molécula de PLA_2 para uma de crotapotina.

A crotoxina é a principal fração do veneno de *Crotalus durissus terrificus* e bloqueia a transmissão de impulsos nervosos na junção neuromuscular (Vital-Brazil, 1966) da qual decorrem as paralisias motoras apresentadas pelos pacientes. A junção neuromuscular é uma região especializada da fibra muscular, onde o impulso nervoso, ao atingir a terminação nervosa pré-sináptica, determina a sua despolarização e liberação do

neurotransmissor acetilcolina. A acetilcolina liberada combina-se com os receptores nicotínicos pós-sinápticos. Esta ligação está relacionada ao potencial da placa terminal, que induz a contração muscular, ao atingir o limiar de excitabilidade, desencadeia o potencial de ação muscular que se propaga ao longo dos túbulos "T". Para explicar a inibição da contração muscular pela crotoxina tem sido proposto um mecanismo de ação molecular envolvendo atividade pré-sináptica (Vital Brazil, 1966) e pós-sináptica (Bon et al., 1979). Na ação pré-sináptica, a crotoxina genericamente inibe a liberação do neurotransmissor acetilcolina (Vital Brazil & Excell, 1971; Chang & Lee, 1977). Na ação pós-sináptica atua bloqueando a resposta a acetilcolina através da estabilização do receptor de acetilcolina a um estado inativo (Bon et al., 1979). Existem atualmente dois modelos para explicar a interação da crotoxina com membranas pré-sinápticas: o modelo da dissociação e o do complexo ternário transiente. No primeiro modelo, quando a crotoxina se aproxima do seu receptor na membrana celular, a crotopatina se dissocia da PLA_2 e esta última se liga então ao receptor. No segundo modelo, a crotoxina primeiramente se liga ao seu receptor de membrana e só então ocorre a dissociação de seus componentes com liberação da crotapotina, enquanto a PLA_2 permanece ligada ao receptor (Delót & Bon, 1993). O termo "molécula chaperone" ou simplesmente "chaperon" foi primeiramente empregado por Faure & Bon. (1988). Para eles a crotapotina teria a função de prevenir a ligação não específica da PLA_2 com outros receptores biologicamente importantes e que não estão envolvidas em suas atividades fisiológicas bem como farmacológicas (Bon et al., 1979).

A crotoxina isolada por Slotta & Fraenkel-Conrat (1938), apesar de mostrar-se homogênea e constituída por uma mistura de PLA_2 e crotopatina é, na realidade, composta por uma mistura de várias isoformas, as quais estão presentes em diferentes lotes de venenos coletados de várias serpentes. A presença destas isoformas de crotoxina resulta das

diferentes combinações de suas subunidades. Estudos eletroforéticos e cromatográficos demonstraram a existência de muitas isoformas de crotoxina. A presença de várias isoformas no veneno de uma única espécie indica a existência de vários genes codificantes para essas fosfolipases neurotóxicas. Essas isoformas parecem ser resultantes ou de modificações pós-traducionais, que ocorrem sobre uma única forma de RNA mensageiro, ou na expressão de diferentes RNAs mensageiros (Faure & Bon, 1988; Faure et al., 1994).

Recentemente com uso de técnicas de purificação mais eficientes, Oliveira et al. (2003) e Toyama et al. (2003) mostraram que as crotoxinas de *Crotalus durissus terrificus* são constituídas por uma mistura de duas ou mais isoformas de crotapotinas e três a quatro isoformas de PLA_2, que foram identificadas por técnicas enzimáticas e de seqüenciamento de proteínas. Análises mais acuradas destes resultados mostraram que, além das crotapotinas e PLA_2, a crotoxina é constituída por uma mistura heterogênea de frações, que devido a sua baixa concentração na fração crotoxínica, ainda não foram caracterizadas. Toyama et al. (2003) também mostraram que a PLA_2 isolada da crotapotina é capaz experimentalmente de induzir uma atividade neurotóxica em preparações isoladas de junção neuromuscular de "biventer cervis" de pintainho. Desta forma, a PLA_2 de fato é o componente responsável pela atividade neurotóxica da crotoxina, mas a crotapotina não teria neste caso nenhuma atividade, uma vez que, tanto na presença como na ausência da crotapotina a resposta neurotóxica é a mesma (Toyama et al., 2003).

Landucci et al. (1995) mostraram que, em condições experimentais, de aplicação *in vivo* através da injeção intrapertitoneal ou por via oral, a crotopatina também possui atividade antiinflamatória. Embora esta não aconteça devido à liberação de corticosteróides endógenos ou inibição da atividade de ciclo-oxigenase. Acredita-se que a crotapotina poderia interagir com PLA_2 extracelulares gerados durante o processo inflamatório, levando

a uma redução da atividade hidrolítica destes últimos. Além de ter um efeito neurotóxico, a crotoxina é também miotóxica e afeta diretamente as células do músculo esquelético (Gopalakrishnakone et al., 1981). Evidências clínicas claramente indicam que o envenenamento por *Crotalus durissus terrificus* é caracterizado por uma miotoxicidade sistêmica, demonstrada pela presença de mioglobinúria, mioglobinemia e por um drástico aumento no nível sérico de creatina quinase, lactato desidrogenase e aspartato aminotransferase (Azevedo-Marques et al., 1982). Os sinais clínicos evidentes da ação miotóxica são dores musculares generalizadas (mialgias), urina que apresenta uma cor avermelhada ou de tonalidade mais escura (mioglobinúria) - manifestação clínica mais evidente da necrose de musculatura esquelética (rabdomiólise) - Desde que a crotoxina representa uma grande porcentagem deste veneno, é provável que essa miotoxicidade se dê pela ação da crotoxina nas células musculares. A PLA_2 induz danos nos músculos de ratos, enquanto a crotapotina é destituída de efeito miotóxico (Kouyoumdjian et al., 1986), mas ela potencializa a atividade miotóxica da PLA_2. A patogênese do dano causado ao músculo esquelético depois da injeção intramuscular de crotoxina foi estudada por Gopalakrishnakone et al. (1981), que constataram que, quatro horas depois da administração da toxina, há evidências histológicas e ultraestruturais de mionecroses; entre 24 e 48 horas, há um proeminente infiltrado inflamatório composto principalmente de macrófagos localizados dentro do espaço demarcado pela membrana das células musculares necrosadas.

A crotoxina (15 a 50 μg/ml) produz agregação de plaquetas humanas lavadas de modo dose-dependente e irreversível, e induz a liberação de tromoboxano B_2. (Laducci et al., 1994), Cardoso & Mota (1997).

Estudando o efeito da crotoxina no sistema imune, verificaram que o veneno da *Crotalus durissus terrificus* bem como seu componente crotoxina tem um efeito inibitório na resposta humoral, mas não na resposta celular imune. Pouco se sabe sobre o efeito do veneno de serpentes no sistema imune. Estudos estão sendo feitos para verificar como o veneno total ou seu principal componente poderiam modular a supressão da resposta imune.

Apesar de bem caracterizada, a crotoxina apresenta-se como uma fração de natureza polifuncional, constituída por várias frações biologicamente ativas e por várias isoformas.

1.4.1.1 Fosfolipase A_2

São enzimas largamente distribuídas na natureza, podendo ser encontradas em bactéria, plantas, tecidos de mamíferos (pulmão, fígado, baço, coração, eritrócitos, plaquetas e leucócitos polimorfonucleares). No entanto, as mais conhecidas e estudadas são aquelas encontradas nos tecidos pancreáticos de mamíferos e nos venenos de serpentes e insetos (Verheij, et al (1980); Van der Bosch, 1980). Todas as PLA_2 de venenos contêm cerca de 120-135 aminoácidos, várias pontes de sulfeto e uma alta conservação da estrutura tridimensional, sendo extremamente estáveis ao tratamento com calor e ácidos, apresentando uma homologia seqüencial entre si e especialmente na região do sítio catalítico (Chang et al., 1977). Além disso, a maioria dessas PLA_2 têm atividade catalítica, mas a caracterização sistemática destas tem conduzido para a identificação de várias variantes de PLA_2 como as PLA_2–like que são cataliticamente inativas (Gutierrez et al., 1995)

A unidade catalítica das fosfolipases (PLA_2) é constituída pelos resíduos His-48, Asp-99 e uma molécula de água. No mecanismo de catálise proposto (Verheij et al., 1980) um próton na posição 3 do anel imidazólico do aminoácido His-48 está envolvido em uma forte interação com o grupo carboxílico da Asp-99, impedindo que ocorra uma rotação no anel imidazol (que está envolvido com a catálise), deixando o nitrogênio da posição 1 deste anel na posição espacial própria.

Uma molécula de água promove um ataque nucleofílico ao carbono do grupo éster do substrato e nesse momento o anel imidazol da His-48 recebe um próton da molécula de água facilitando a reação. Logo após ocorre a hidrólise da união acil-éster na posição Sn-2 do fosfoglicerídeo (substrato), este próton é doado pelo anel imidazol para o oxigênio que forma então um grupo álcool de lisofosfolipídeo a ser liberado (Verheij et al., 1980).

O sítio para união do cálcio, importante para a catálise, apresenta um cátion ligado pelo oxigênio do grupo carbônico dos resíduos Tyr-28, Gly-30, Gly-32 e por oxigênio da cadeia lateral do resíduo Asp - 49. No mecanismo de catálise, o cálcio tem dupla função: primeiro: fixar o fosfato e segundo: estabilizar a carga negativa do oxigênio do grupo carbônico da ligação éster na posição Sn-2 do substrato (Yang, 1994).

As fosfolipases A_2 (EC 3.1.1.4) portanto são enzimas que catalisam especificamente a hidrólise da ligação acil-éster na posição Sn-2 do 1,2-diacil-3-sn fosfoglicerídeo em uma reação dependente de cálcio (Ami & Ward, 1996). Consistindo em uma superfamília de proteínas que têm sido caracterizadas como uma importante família de enzimas transdutoras de sinal, atuando como proteínas que interagem de forma específica com receptores denominados do tipo M e N ou como efetores de atividades biológicas intracelulares (Dennis, 1997). **(Figura 2).**

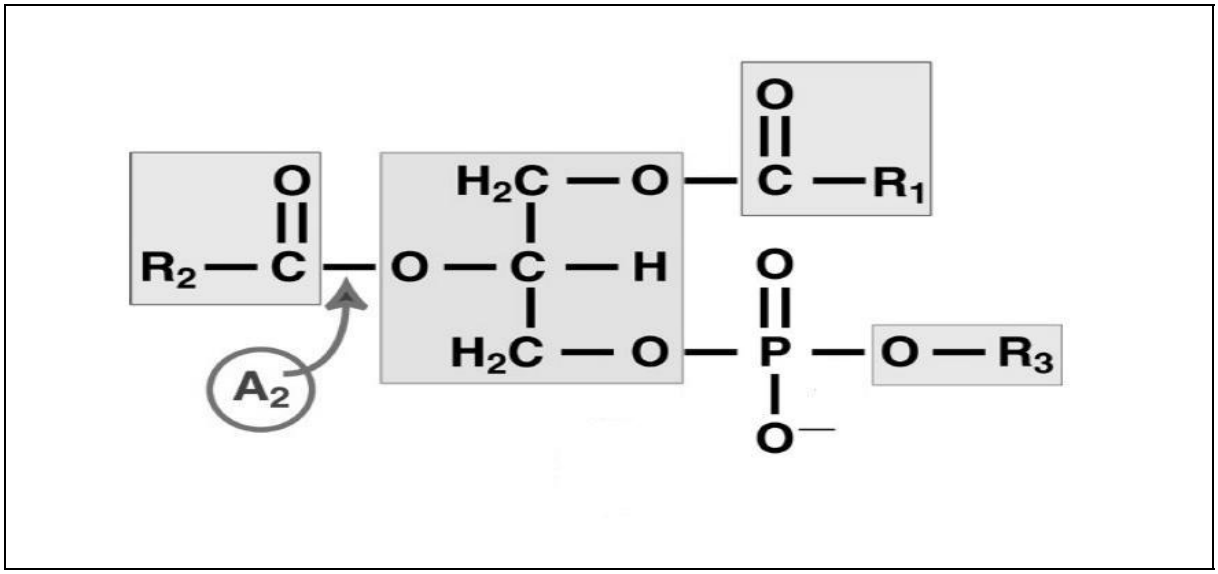

Figura 2: Sítio de hidrólise (Kini, 1997).

Classificação das Fosfolipases

As fosfolipases são classificadas dependendo de sua estrutura e de seu mecanismo de catálise. De acordo com seu sítio de hidrólise sobre o triacilglicerol se dividem: em fosfolipase A_1, fosfolipase A_2 (PLA_2), fosfolipase B, fosfolipase C, e fosfolipase D. As PLA_2s são as mais abundantes lípases do nosso organismo e são capazes de metabolizar vários tipos de lipídios tais como: fosfatidilcolina, fosfatidileatolamina, fosfatidilinositol, fosfatidilglicerol, plasminogênio.

As fosfolipases A_2 foram as primeiras fosfolipases a serem reconhecidas, sua descoberta foi baseada na observação da ação do suco pancreático e do veneno elapídico na hidrólise da fosfatidilcolina (Wittcoff, 1951).

As fosfolipases A_2 (PLA_2) também podem ser classificadas de acordo com a sua estrutura em dois grupos: um de alta massa molecular, intracelular e um de baixa massa molecular, extracelular. As PLA_2 de alto peso molecular estão bem caracterizadas e podem

ser subdivididas do ponto de vista catalítico, em dois grandes subgrupos: as que necessitam de cálcio para sua atividade, com uma massa molecular que vai de 140 a 400 KDa, e as que não necessitam, apresentando massa molecular que vai de 85 a 100 KDa (Kini, 1997).

De acordo com Kini (1997) as PLA_2 de baixa massa molecular relativa podem ser subdivididas em quatro subgrupos: Grupo I, Grupo II, Grupo III e Grupo IV **(Figura 3)**.

As PLA_2 do grupo I englobam as isoladas dos venenos elapídicos, hidrofilídicos (Grupo IA) e a isolada do pâncreas de mamífero (Grupo IB). Estas enzimas contêm cerca de 115-120 resíduos de aminoácidos e sete pontes dissulfeto.

As fosfolipases A_2 do grupo II são encontradas nos venenos de serpentes crotálicas e viperídicas e em células humanas como as plaquetas. Estas enzimas possuem cerca de 120 a 125 resíduos de aminoácidos e sete pontes dissulfeto. Fazem parte deste grupo as enzimas PLA_2 miotóxicas Asp 49 (D49) e Lys 49 (K49), que possuem uma atividade enzimática baixa ou residual.

As PLA_2 do grupo III englobam aquelas isoladas de abelhas, diferentemente das outras classes de PLA_2 estas são glicoproteínas, contendo de 130 a 135 resíduos de aminoácidos. Estas possuem uma baixa homologia com as outras PLA_2.

As PLA_2 do grupo IV podem ser consideradas as mais recentes dentro da família das PLA_2, do ponto de vista estrutural, é expressa por quase todas as células de mamíferos (Dennis, 1994). Estas PLA_2 têm duas cadeias polipeptídicas: uma longa com 77 resíduos de aminoácidos e uma curta com 42 resíduos de aminoácidos, ligados por pontes dissulfeto intercadeias. Estas enzimas mostram dependência de cálcio para sua atividade (McIntosh, 1995).

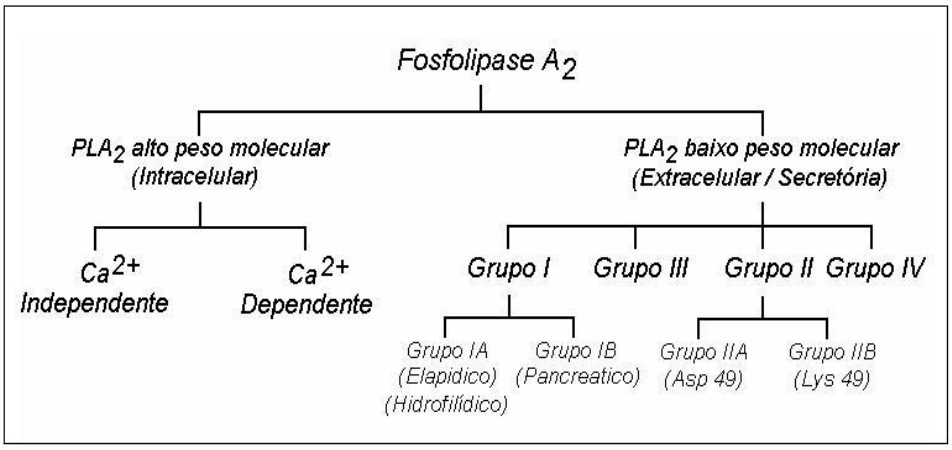

Figura 3: Classificação das PLA_2 (Kini, 1997).

Segundo Dennis (1994) as PLA_2 também podem ser classificadas de acordo com sua função, localização, regulação, mecanismo, seqüência de aminoácidos, estrutura e papel dos íons bivalentes.

Dentro do grupo de PLA_2 de alta massa molecular intracelular (Kini, 1997) podemos colocar as fosfolipases A_2 da classe IV assim como da classe V isolada do músculo cardíaco de cães, classificadas por Dennis, (1994).

Estudos subseqüentes mostraram que as fosfolipases A_2 são abundantemente encontradas em venenos de várias serpentes, e estas enzimas têm um papel importante na digestão de lipídios (Wittcoff, 1951, Dennis, 1994 e Harris, 1991).

Nestes últimos anos as pesquisas sobre fosfolipases A_2 têm sido impulsionadas principalmente pela ação destas em várias atividades biológicas: **Neurotoxicidade** (neurotoxinas pré-sinápticas e neurotoxinas pós-sinápticas); **Miotoxicidade** (mionecrose local e miotoxicidade sistêmica); **Cardiotoxicidade; Efeito anticoagulante; Iniciador da agregação plaquetária; Inibidor da agregação plaquetária; Atividade hemolítica;**

Hemorragia interna; Atividade anti-hemorrágica; Atividade convulsionante; **Atividade hipotensiva; Atividade edematogênica; Lesão de órgãos e tecidos.**

As fosfolipases A_2 também têm grande importância nos processos de fertilização (Frey et al., 1992); proliferação celular (Arita et al., 1991); contração da musculatura lisa (Nakajima et al., 1992; Vadas et al., 1993); hipersensibilização e processos inflamatórios crônicos (Vadas et al., 1986 e Vadas et al., 1993).

As fosfolipases têm um papel fundamental no metabolismo de lipídeos e estão intimamente relacionados com a liberação de ácido araquidônico que é um precursor comum de uma série de lipídios bioativos pertencentes a família dos eicosanóides tais como prostaglandinas, leucotrienos e tromboxanos e prostaciclinas, compostos de vida média curta, embora de resposta fisiológica importante.

Os efeitos farmacológicos, observados pela ação das fosfolipases A_2 (PLA_2) não são necessariamente gerados pela quebra de fosfolipídeos e pela ruptura das membranas biológicas. As fosfolipases A_2 parecem apresentar além do sítio catalítico característico, sítio (s) farmacológico (s) distinto (s) do catalítico (Yang, 1994) e são, portanto, mecanismos dependentes da atividade catalítica (Gutiérrez & Lomonte, 1995).

A presença do sítio específico distinguiria uma célula alvo de uma célula não alvo, isso determinaria a união de uma enzima com sua célula alvo (presença do sítio com alta especificidade) e da união com a célula não alvo (ausência do sítio e baixa especificidade, ocorrendo apenas quando há um excesso de enzima).

Os diferentes efeitos farmacológicos promovidos pelas fosfolipases A_2 podem ser explicados também pela presença de sítios alvos específicos, que estão localizados na superfície das células ou do tecido alvo. Estes sítios alvos seriam reconhecidos por sítios farmacológicos localizados na superfície das PLA_2 que de forma geral são independentes,

mas algumas vezes estes se sobrepõem com o catalítico (Kini, 1989). De acordo com esta hipótese haveria uma necessidade de complementaridade entre o (s) sítio (s) farmacológico (s) e o(s) sítio(s) alvo(s) em termos de equilíbrio de cargas, interações hidrofóbicas e interações de Van der Walls.

Segundo Kini & Evans (1989) a natureza química deste sítio de ligação entre as PLA_2 e as células ou tecidos alvos poderia estar entre as PLA_2 e um lipídio ou uma proteína (glicoproteínas). Devido à alta especificidade das interações pode-se supor que as glicoproteínas podem estar diretamente relacionadas com o reconhecimento, contudo lipídios próximos ao sítio de ligação também podem contribuir para o aumento da especificidade. Muitos dos receptores para estas PLA_2 já foram identificados e caracterizados tanto funcionalmente como estruturalmente. Um dos mais bem caracterizados e conhecidos é a proteína do canal de potássio (Kini, 1997).

Trabalhos recentes têm mostrado que algumas PLA_2 cataliticamente ativas podem ter um papel importante na morte de células bacterianas gram positivas, como a *Staphylococcus aureus* (Harwig, et al., 1995). Recentemente, um efeito bactericida de PLA_2 humanas não pancreáticas foi demonstrado sobre linhagens de *S.aureus*. De acordo com estes resultados, esta ação bactericida parece estar diretamente relacionada com a atividade catalítica das PLA_2 (Weinrauch, et al., 1996).

Modificações químicas

Várias metodologias, tais como modificações químicas, mutações sítio dirigidas, técnicas cristalográficas, espectrofotométricas e fluorométricas, assim como ressonância magnética nuclear e estudos da ação de inibidores e substratos naturais ou artificiais têm

sido utilizadas com objetivo de elucidar estruturalmente os sítios responsáveis pela toxicidade de várias PLA_2 provenientes de venenos de serpente.

As modificações químicas tradicionais têm sido uma das principais estratégias usadas para estudar suas relações estrutura-função (**Tabela 4**).

Tabela 4: Estratégias para modificações químicas de resíduos de aminoácidos específicos em PLA_2s de venenos de serpentes (Soares e Giglio, 2003)

Resíduo de Aminoácido	***Reagente/reação***
His	*p*-bromofenacil bromide/alquilação Metil-*p*-nitrobenzenosulfonato/metilação
Lys	Cianeto de potássio /carbamilação *o*-metilisourea/guanidinação Anidro acético/acetilação Trinitrobenzenosulfonato(TNBS)/trinitrofenilação
Tyr	Nitrobenzenosulfonil fluoride (NBSF)/sulfonação
Trp	Cloreto de nitrofenilsulfonil (NPSC)/sulfonação 2-Hidroxi-5-nitrobenzil bromide/alquilação
Met	Cloramine T/oxidação Ácido iodoacético/carboximetilação.

Quase todos os grupos funcionais presentes nas PLAs são modificados por uma variedade de reagentes muito específicos, a finalidade destes estudos é apontar com precisão os resíduos do sitio ativo para obter uma visão do mecanismo de ação da PLA_2.

As PLA_2s de veneno de serpente mostraram uma ampla variedade de atividades farmacológicas, tal diversidade funcional dentro desse grupo de proteínas estruturalmente similares cria interrogações referentes a relação entre sua estrutura e seu efeito. Tenta-se esclarecer a relação entre a atividade enzimática e a toxicidade através dos estudos de modificação seletiva ou preferencial dos grupos de cadeia lateral de aminoácidos na molécula de enzima PLA_2 e também comparam-se os efeitos destas modificações nestes dois parâmetros (Yang, 1997; Verheij, 1981).

Ao estudar a relação entre as propriedades enzimáticas e farmacológicas, verifica-se que as modificações químicas de certos resíduos de aminoácidos na PLA_2 podem diminuir seletivamente a atividade enzimática ou propriedades farmacológicas e letais.

Em algumas destas modificações concluiu-se que há perda quase total de atividade enzimática para o substrato presente, uma vez que o resíduo modificado participa do sitio ativo e também na potência letal intraventricular em ratas (Rosenberg, 1986).

A perda da atividade enzimática para os substratos agregados pode-se atribuir a incapacidade da PLA modificada em unir-se a interface lipídio-água ou alternativamente não se unir especificamente e assim evitar a formação de produtos, nestes casos os resíduos modificados são denominados de "resíduos do sitio ativo", tais resíduos diretamente envolvidos na ligação de substrato monomérico, envolvidos com o íon Ca^{2+} que é essencial e também aos resíduos que realizam o fracionamento real do enlace com o éster (Verheij, 1981)

Modificações químicas do resíduo de histidina.

Karlsson demonstrou através de um procedimento amplamente utilizado para avaliar a função da atividade enzimática na toxicidade que a fosfolipase pancreática de

porco pode ser completamente inativada por alquilação de um resíduo de histidina no sitio ativo com *p*-bromofenacil brometo (*p*-BPB). (Karlsson, 1980). Somente um resíduo de histidina foi modificado e o resíduo modificado era identificado como His-48 na seqüência segundo a análise de aminoácidos (Yang, 1980 a, b; Yang, 1991). Através dos estudos de modificações químicas de enzimas PLA_2 de diversas fontes verificou-se que o resíduo de histidina é crucial e foi potencialmente reagente para *p*-BPB, esta histidina foi localizada na mesma posição homóloga a outras fosfolipases. (Yang, 1980; Volwerk *et al*., 1974; Halpert *et al*., 1976; Viljoen *et al*., 1977). Assim, o resíduo His é conservado, pois participa na catálise (Verheij *et al.,* 1980, Verheij *et al.,* 1981 e Scott *et al.,* 1992). A alquilação do resíduo induz a perda da atividade hidrolítica e a diminuição de alguns efeitos farmacológicos (Yang & King, 1980a, Yang & King, 1980b, Díaz-Oreiro & Gutiérrez, 1997, Melo & Ownby, 1999 e Andrião-Escarso *et al.,* 2000). A primeira PLA_2 alquilada foi de pâncreas de mamífero com ρ-bromofenacil brometo, perdendo a sua atividade enzimática (Volwerk *et al.,* 1974), desde então a alquilação do resíduo His48 de PLA_2 procedentes de veneno de serpentes tem sido usado a fim de estudar a relação da atividade enzimática-tóxica (Halpert *et al.,* 1976, Roberts *et al.,* 1977, Yang & King, 1980a, Rosenberg, 1986 e Yang, 1994).

Durante os últimos 30 anos o reagente *ρ*-bromofenacil brometo (*p*-BPB) tem sido utilizado para os estudos de modificações químicas de PLA2 procedentes de veneno de serpentes tais como: *Naja* sp. (Roberts *et al.,* 1977, Yang & King, 1980a, Condrea *et al.,* 1981a, Condrea *et al.,* 1983a, Babu & Gowda, 1994), *Vipera* sp. (Babu & Gowda, 1994), *Bitis* sp. (Viljoen *et al.,* 1977), *Hemachatus* sp. (Yang & King, 1980b e Condrea *et al.,* 1981a), *Bungarus* sp. (Abe *et al.,* 1977 e Kondo *et al.,* 1978), *Calloselasma* sp. (Tsai *et al.,* 2000), *Trimeresurus* sp. (Wang & Teng, 1990), *Agkistrodon* sp. (Zhao *et al.,* 1998 e Melo

& Ownby, 1999), *Crotalus* sp. (Ownby *et al.,* 1999, Melo & Ownby, 1999), *Bothrops* sp. (Nisenbon *et al.,* 1988, Díaz *et al.,* 1993, Bultrón *et al.,* 1993, Díaz-Oreiro & Gutiérrez, 1997 e Andrião-Escarso *et al.,* 2000) e *Lachesis* sp. (Fuly *et al.,* 1997, Fuly *et al.,* 2000 e Fuly *et al.,* 2003).

As PLA_2 ácidas e básicas isoladas de *Naja nigricollis*, perderam suas atividades enzimáticas e tóxicas depois da modificação com *p*-BPB. As análises de aminoácidos mostram que um só resíduo, His48, foi modificado (Yang & King, 1980a e Yang *et al.,* 1981). A diminuição da atividade enzimática levou a uma diminuição da hemólise direta para a PLA_2 básica de *Naja nigricollis*, a hidrólise de fosfolipídios e o bloqueio dos potenciais de ação em enguia (Condrea *et al.,* 1981a), a fosfolipase A_2 histidina modificada perdeu atividade enzimática. O K_d para Ca^{2+} das enzimas PLA_2 histidina modificadas foi similar a àquela da PLA_2 nativa, sugerindo que a modificação do His-48 não altera a interação íon-proteína. É interessante notar que o Ca^{2+} tem um pronunciado efeito protetor no processo de inativação por *p*-BPB. Verheij *et al* (1980), observaram que a His-48 metilada em PLA_2 pancreática mostrou uma perda total da atividade enzimática, entretanto a união do Ca^{2+} permanece intacta. Assim His-48 não esta diretamente envolvida na união para o Ca^{2+}. A união a Ca^{2+} pode induzir a mudança conformacional que capacita a enzima a estar mais acessível ao substrato. (Verheij *et al.,* 1980 e Yang, 1997)

As PLA_2s dos venenos de viperídeos compreendem dois grandes grupos de proteínas, basicamente as Asp49 com uma alta atividade catalítica em substratos artificiais e as PLA_2 Lys49 com uma baixa ou nenhuma atividade enzimática (Gutiérrez & Lomonte, 1995 e Ownby *et al.,* 1999).

A primeira PLA_2 de *Bothrops* modificada quimicamente com *p*-BPB foi uma PLA_2 ácida de *Bothrops alternatus* (Nisenbon, *et al.,* 1988). Estes autores observaram dissociação

entre suas atividades enzimáticas e tóxicas desde que a PLA_2 foi alquilada, no entanto conservaram a sua atividade letal considerável. De outro lado a PLA_2 Asp49 ácida de *Bothrops jararaca*, *Bothrops jararacussu*, e *Lachesis muta*, foram capazes de perder suas atividades enzimáticas e farmacológicas depois de serem alquiladas com *p*-BPB, mostrando que os efeitos biológicos são dependentes da integridade catalítica (Serrano *et al.,* 1999, Fuly *et al.,* 2000 e Fuly *et al.,* 2003).

A alquilação de His48 da PLA_2 básica de *Bothrops asper* Basp-III, BthTX-II de *Bothrops jararacussu* e a PrTX-III de *Bothrops pirajai*, inibiram quase completamente a atividade catalítica, anticoagulante, miotóxica e citotóxica (Díaz-Oreiro & Gutiérrez, 1997, Andrião-Escarso *et al.,* 2000 e Soares *et al.,* 2001b), mostrando que a hidrólise de fosfolipídios nos efeitos farmacológicos são dependentes da atividade catalítica. No entanto, a indução de edema só estava reduzida em 50% e a ruptura de liposomas em 20%, levando a considerar a dissociação parcial de sítios catalíticos e farmacológicos. A substância *p*-BPB inibe a indução de edema e a degranulação de mastócitos *in vitro* da BthTX-I e II (Landucci *et al.,* 1998). Foi observado que o edema induzido pela BthTX-II foi abolido em 50% depois de ser alquilada , no entanto a PLA_2 Lys49 BthTX-I foi em menor grau. Foram informados também resultados para PrTX-I e PrTX-III de *Bothrops pirajai*, e crotoxina B Asp49 e *Crotalus durissus terrificus* (Soares *et al.,* 2001a e Soares *et al.,* 2001b). Alquilação de PLA2 miotoxicas Lys49 de *Botrhosp asper*, *Bothrops moojeni*, *Bothrops pirajai* e *Bothrops neuwiedi*, reduziram a miotoxicidade em 40 a 50% e a citotoxicidade em 80 a 85% sendo o edema induzido em 15 a 20%, sem anular a capacidade de destruir liposomas carregados negativamente (Díaz *et al.*, 1993, Rodrigues *et al.,* 1998, Toyama *et al.,* 1998). Estes resultados sugerem a presença de regiões moleculares distintas do sitio ativo, responsável pelo menos parcialmente, pelas atividades

farmacológicas destas toxinas foi proposta algumas hipóteses para explicar as mudanças das atividades farmacológicas das PLA_2 pela alquilação de His48 com *p*-BPB: a) A alquilação específica do resíduo no sítio ativo diminui a atividade farmacológica induzindo mudanças conformacionais nas regiões moleculares distinta do sítio ativo. b) O efeito tóxico das PLA_2 Asp49, pode se associar com a atividade catalítica. Nenhuma mudança conformacional significativa tem sido encontrada na PLA_2 ácida de *Agkristodon halys* depois de ser alquilada com *p*-BPB, a exceção de modificações no sitio catalítico onde a His-48 liga-se com o *p*-BPB covalentemente (Zhao, *et al.,* 1998). No entanto as miotoxinas PLA_2 básicas classificadas no grupo II têm uma região C terminal estendida. Não se sabe se o *p*-BPB induz uma mudança conformacional destas proteínas que poderiam afetar seu perfil farmacológico. O mecanismo exato pelo qual o *p*-BPB altera as propriedades tóxicas e farmacológicas das miotoxinas PLA_2 Lys49 permanece ainda indeterminado.

Além disso, o *p*-BPB não pode ser considerado um inibidor de PLA_2 específicas devido ao fato de agir reciprocamente de forma irreversível com outras enzimas, como também pode se ligar a grupos tioles livres (Blackwell & Flower, 1983).

Recentemente Zieler, *et al.,* (2001) demonstraram que PLA_2 de venenos crotálicos da *Crotalus adamanteus* e de outras *Crotalus* e de abelha, bloqueiam o desenvolvimento do intestino médio do mosquito parasita da malária, inibindo a associação do oocinete com a superfície do intestino médio.

A inibição ainda ocorre na presença de *p*-BPB, indicando que a atividade hidrolítica da enzima não é necessária para o efeito antiparasitário, este é um grande exemplo da utilidade da alquilação de PLA_2 no estudo entre as atividades enzimáticas e farmacológicas no veneno crotálico. Fenard. *et al*., (1999) mostraram que as PLA_2 da classe II inibida cataliticamente, atuam como um potente inibidor gerando um processo de citotoxicidade na

propagação do vírus de HIV através de um mecanismo totalmente independente da atividade catalítica.

Modificações químicas do resíduo de lisina.

A maioria das PLA_2s tóxicas são proteínas básicas que levam conteúdos altos de aminoácidos hidrofóbicos e catiônicos. Foram reportados vários estudos em modificações químicas de resíduos Lys e foram deduzidas as relações de suas atividades enzimáticas e farmacológicas (Yang & Chang, 1989; Takasaki *et al*., 1990; Babu & Gowda, 1994; Yang, 1997). A carbamilação de 9 dos 10 resíduos de Lys da PLA_2 de *Naja nigricollis* e 3 dos 5 em *Naja naja atra*, drasticamente foram reduzidos ou completamente anulados em sua letalidade, assim como os efeitos anticoagulante, hemolítico e neurotóxico, sem ter um efeito pronunciado em sua atividade enzimática; sugerindo que a toxicidade destas PLA_2 é devido principalmente a um efeito específico não mediado pela hidrólise de fosfolipídios. Díaz-Oreiro & Gutierrez (1997) mostraram acetilação de resíduos de Lys das PLA_2s Asp49 miotoxina I e III de *B. godmani* e *B. asper*, respectivamente, causando uma redução ao redor de 75% na atividade enzimática e uma perda total das atividades anti-coagulante e miotóxica. As miotoxinas acetiladas retiveram uma significativa atividade de quebra de liposomas, mostrando que esta PLA_2s podem ainda romper as bicamadas mesmo depois da modificação extensa das lisinas. Além disso, estes resultados com miotoxinas acetiladas demonstraram uma dissociação parcial entre atividades enzimáticas e farmacológicas (miotóxicas e anti-coagulantes), de acordo com estudos prévios com outras PLA_2s. (Condrea *et al*., 1981a–c, 1983a,b; Babu & Gowda, 1994). Estes estudos podem ser interpretados como evidências da existência de regiões moleculares, distintas do local catalítico que podem ser responsáveis por pelo menos algumas das propriedades

farmacológicas delas (Rosenberg, 1986; Kini & Evans, 1989; Díaz-Oreiro e Gutiérrez, 1997; Soares *et al*., 2000a, 2001a,b). Tem sido proposto que as regiões básicas de PLA_2 neurotóxicas e miotóxicas poderiam fornecer a interação destas enzimas com as membranas musculares e neuronais carregadas negativamente (Karlsson & Pongsawasdi, 1980 e Gutiérrez & Lomonte, 1995). A guanidinação de PLA_2 básicas de *Vipera russelli* (VRV-PL-VIIIa) não reduz a sua atividade enzimática, mas diminui significativamente a letalidade, a indução de edema e a atividade anticoagulante, sugerindo uma dissociação entre sítios enzimáticos e farmacológicos (Babu & Godwa, 1994).

Modificações químicas do resíduo de tirosina.

A seqüência completa de aminoácidos de PLA_2 de veneno de serpente evidencia que todos os venenos de serpente apresentam nove resíduos de Tyr conservados na mesma posição ou posições homólogas, sugerindo sua importância dentro deste grupo de proteínas (Verjeij *et al*., 1981; Soons *et al*,. 1986 e Andrião-Escarso *et al*., 2000). Este caráter altamente invariável sugere que eles cumpram alguma função crucial para a atividade enzimática, por conseguinte a função da tirosina foi comprometida na esperança de abrigar alguma luz no mecanismo catalítico de PLA_2 e a relação entre toxicidade e atividade enzimática. O p-nitrobenzenosulfonil fluoride (NBSF) é um reagente específico para a modificação química de resíduos Tyr nas proteínas. A Tyr69 em PLA_2 pancreática (homóloga a Tyr62 ou 63 de veneno de serpentes) é considerada parte do sítio de reconhecimento interfacial e se encontra envolvida na ligação micelar. A presença de dois resíduos Tyr de uma PLA_2 de *Naja nigricollis* e *Naja naja atra*, dos nove; são p-nitrobenzenosulfonados por NBSF e os outros sete se encontram dentro da estrutura conformacional da molécula. Tyr 3 e Tyr62 (63) de ambas enzimas, poderiam estar

envolvidas na ligação ao substrato no canal hidrofóbico da PLA_2. O Tyr3 e Tyr62(63) não são essenciais para o desenvolvimento das propriedades farmacológicas, assim sendo modificadas, as atividades farmacológicas permanecem, no entanto na maioria dos casos diminuem (Soons *et al.,* 1986).

A modificação química da Notexina de *Notechis scutatus scutatus*, com NBSF, permitiu isolar e identificar três derivados por HPLC (Yang & Chang, 1991). A seqüência de aminoácidos revelou que só Tyr7, Tyr70 e Tyr77 foram modificados. Quando a Tyr7 foi modificada perdeu de 30 a 80% de sua atividade enzimática e tóxica. Embora a modificação de Tyr77 induzisse um aumento de 80% na atividade enzimática, a letalidade diminuiu até 50%. Quando ambos resíduos foram modificados simultaneamente perderam 56 e 98% das atividades enzimáticas e tóxicas respectivamente. Estes dados mostram que as modificações dos resíduos Tyr de Notexina, poderiam causar efeitos diferentes em suas atividades enzimáticas e biológicas (Yang & Chang 1991). A modificação de Tyr de miotoxinas bothrópicas, afeta a letalidade, miotoxicidade, citotoxicidade e o efeito de bloqueio neuromuscular induzido por estas toxinas (Soares *et al.,* 2000 e Andrião-Escarso *et al.,* 2000). Além da função da Tyr na catálise, também encontram-se 3-4 residuos Tyr entre os resíduos 112 e 121, sendo característicos das PLA_2 miotóxicas. Assim desde que as miotoxinas PLA_2 Lys49 MjTX-I e II, BthTX-I, PrTX-I e II e BnSP-7, exibem estes resíduos Tyr, pode-se inferir que as modificações químicas nestas regiões, podem afetar suas propriedades farmacológicas. Os resíduos de Tyr52 e Tyr73 participam indiretamente na estabilização do sítio catalítico das PLA2 Asp49 e estes resíduos poderiam ser afetados pelo tratamento com NBSF, onde uma diminuição da atividade catalítica foi observada, sem a redução da atividade anticoagulante (Andrião-Escarso *et al.,* 2000).

Modificações químicas e benzopiranos.

Benzopiranos e seus principais metabólitos (cromenos) estão entre os mais importantes metabólitos secundários e são importantes para a produção de flavonóides, cumarinas e outros compostos polifenólicos encontrados na natureza. Novos derivados benzopiranos como dihidroquinolina, benzotiopiran e dihydronapthalene têm mostrado promissores efeitos antiinflamatórios e estes compostos parecem preferencialmente e seletivamente inibir a ciclooxigenase-2 sobre a ciclooxigenase-1 (Watzl, 2008). Entre os benzopiranos mais comuns estão os flavonóides que são compostos derivados, que foram caracterizados como inibidores de enzimas, incluindo oxidases, xantina redutase, fosfodiesterase, Ca (+2)-ATPase, lipoxigenase, ciclooxigenase, etc (Rathee et al., 2009) As atividades precisas destes compostos envolvem antioxidante de alguns resíduos de aminoácidos hidrofóbicos e a formação da ligação de hidrogênio ou de reações químicas de alguns aminoácidos (Rathee et al., 2009).

Alguns derivados cromeno podem ser intermediários úteis para a síntese de certas substâncias naturais com atividades biológicas e farmacológicas, tais como antimicrobianos (Conti & Desideri, 2009), antiinflamatórios (Chen et al., 2008) e agentes antifúngicos (Vasanthanathan et al., 2006). Alguns derivados cromeno inibem a produção e a secreção do Fator de Necrose Tumoral α em monócitos e macrófagos em resposta a estímulos inflamatórios (Cheng et al., 2003). Apesar da sua importância no metabolismo secundário, o efeito destes compostos sobre certas enzimas permanecem obscuros quando relacionadas a outros compostos.

Os efeitos de flavonóides e cumarinas contra sPLA2 do veneno de serpente revelam que cada um destes compostos diminui fortemente a atividade enzimática da *Crotalus durissus sp*. Cumarinas inibem fortemente o efeito farmacológico da sPLA2 enquanto

flavonóides não tem qualquer efeito (Iglesias et al. 2005, e Toyama et al., 2005). Além disso, há alguma evidência de que cumarinas sintéticas podem inibir o metabolismo do ácido araquidônico. Clorocromeno (8 monocloros dietilaminoetil-3-beta--4-metil-7-etoxi-cumarina carbonilmetoxi), um antitrombótico derivado dos cumarínicos, inibe a função das plaquetas e leucócitos e suprime a liberação do ácido araquidônico por interferir na ativação da PLA_2 (Ribaldi et al. 1996), embora o modo específico de ação ainda não ter sido determinado. Cumarínicos também pode reduzir o edema e a inflamação inibindo a síntese da prostaglandinas, pois envolvem ácidos graxos intermediários como hidroperóxidos (Hadjipavlou-litina et al., 2007). É possível que cromenos e seus derivados sintéticos possam inibir tanto PLA_2 secretórias quanto intracelulares, bem como outras enzimas da cascata do ácido araquidônico. Compostos naturais polifenólicos desempenham um papel importante na adaptação de plantas ao seu ambiente e são também uma fonte importante de produtos farmacêuticos.

1.5 Venenos de serpentes e Homeostase sangüínea.

Plaquetas ou trombócitos constituintes do sangue são células sanguíneas sem núcleo produzidas na medula óssea através de diversas divisões celulares e que são originadas a partir da fragmentação do citoplasma de um megacariócito (Sixma et al., 1995). Essas plaquetas são fragmentos celulares encontrados no sangue circulante, apresentando forma discóide com 2 a 3 μm de diâmetro por litro de sangue sendo sua vida média de aproximadamente 10 dias (Hawiger, 1992).

Quando um vaso sanguíneo sofre injúria, ocorre a exposição das fibras de colágeno, que combinadas com receptores específicos localizados na membrana plaquetária, provocam a liberação de íons Ca^{+2}. Isto faz com que haja uma mudança na conformação da

plaqueta, que adota um formato esférico (Sherry, 1992). O resultado desse mecanismo é a exposição da GPIb, que atua como uma proteína de adesão plaquetária ao endotélio lesado. O processo consiste na cobertura da região injuriada por uma camada de plaquetas é chamado de adesão plaquetária (Caen & Rosa, 1995). Os sangramentos ativam dois processos homeostáticos: o de coagulação do sangue e o de agregação plaquetária. Em suma, a homeostase mantém as propriedades hemodinâmicas do sangue.

1.5.1 Agregação Plaquetária e Venenos de Serpentes:

A agregação plaquetária propriamente dita consiste na formação de pontes intracelulares entre proteínas solúveis, consiste na formação de pontes intracelulares entre proteínas solúveis, principalmente fibrinogênio e fator VIII e as glicoproteínas insolúveis GP-Ib e GP-IIIa presentes na membrana plasmática. Esse processo é desencadeado quando a concentração de fibras expostas de colágeno é muito alta ou quando há presença de agonistas no local lesado (Matos, 1993; Weiss & Lages, 1997).

Estes agonistas podem ser compostos de baixo peso molecular tais como: ADP; ácido araquidônico (AA); serotonina e epinefrina; podem ser enzimas ionóforos como A23187. Eles iniciam uma série de eventos celulares, comprometendo as propriedades e funções das plaquetas pela formação de peseudópodes, mudança da forma discóide para esferocítica, aderência de uma célula à outra (agregação) ou pela liberação de várias substâncias (secreção) (Zucher, 1989).

Mudanças da função normal das plaquetas podem causar desordens trombóticas ou hemorrágicas. Na trombose, causas desconhecidas podem resultar na formação espontânea de agregados e suficientemente grandes para bloquear a circulação (Deranleau, 1987). Esse distúrbio pode ser evitado pela inibição da agregação plaquetária, seja pela diminuição da

síntese de agonistas ou pela ação de desintegrinas (Coller et al,, 1995; London et al., 1996). As plaquetas se agregam sob condições adversas ou dano vascular "in vivo', ou por agregação controlada "in vitro". As plaquetas são excelentes modelos para análise de interações célula-célula e célula-substrato (Kawasaki et al., 1996).

Os processos desencadeados pelos agonistas são acompanhados pela exposição de receptores a moléculas adesivas presentes na circulação sanguínea, tais como o fibrinogênio e o fator de von Willebrand (vWF). Estas podem levar à formação do tampão plaquetário, conhecido como trombo, reduzindo ou interrompendo a perda de sangue no local de injúria. As plaquetas contribuem significativamente para manter a integridade da parede vascular e ajudam no processo de recuperação da lesão vascular (Jamieson, 1998; Hawiger, 1989). Trombo e plaquetas, uma vez ativados, facilitam a ação dos fatores de coagulação. As plaquetas também constituem uma superfície necessária aos vários passos de ativação das redes de agregação (Tracy & Mann 1966 a/b; Ganz et al., 1986).

Venenos de serpentes contêm uma grande variedade de proteínas que afetam a homeostase. Metaloproteinases são exemplos deste tipo de proteínas com uma desintegrina e/ou um domínio rico em cisteína e/ou um domínio lectínico tipo C (Bjarnason & Fox, 1994 e Kamiguti et al., (1998). Em alguns casos, o efeito de enzimas presentes nos venenos sobre a homeostase é claro. Como exemplo nós temos as nucleotidases, que hidrolisam ADP para produzir adenosina e que se torna um seqüestrador de ADP e são potentes inibidores da agregação plaquetária (Ouyang & Teng, 1979 e Huang, 1999). De forma similar, algumas PLA_2, enzimas de venenos de serpentes que hidrolisam os fosfolipídios da membrana e que atua como um cofator do complexo proteinase e protrombinase (Kini & Evans, 1990), agem também como anticoagulantes (Boffa & Boffa, 1976).

Outras PLA_2 hidrolisam fosfolipídios da membrana de plaquetas e induzem a agregação plaquetária pela liberação de ácido araquidônico (AA) e/ou fator de agregação plaquetária. Muitas metaloproteases e serinoproteases do veneno de serpentes também têm mostrado serem capazes de afetar a homeostase sangüínea. Várias delas atuam degradando fatores de coagulação ou células do endotélio. Contudo, em outros casos, estas proteases, ativando fatores específicos da cascata de coagulação sangüínea ou na fibrinólise atuam seletivamente como as enzimas fisiologicamente envolvidas no processo de controle da homeostase. No veneno das serpentes as proteínas cataliticamente não ativas que afetam a homeostase são principalmente desintegrinas e lectinas tipo C. Desintegrinas são polipeptídeos de baixo peso molecular que inibem a agregação plaquetária. Estas proteínas possuem uma seqüência RGD e competem especificamente com integrinas endógenas, em particular os receptores GPIIb/IIIa de plaquetas (Niewiarowski et al., 1994).

Por outro lado, lectinas tipo C ligam-se seletivamente a proteínas da membrana de plaquetas ou fatores de coagulação (Kini & Evans, 1990). Estas proteínas têm mostrado serem boas ferramentas para investigação dos mecanismos de coagulação sangüínea, uma vez que muitas delas presentes no veneno de serpentes possuem uma estrutura muito parecida com as proteínas fisiologicamente envolvidas (Braud, et al., 2000). Além destas aplicações estas proteínas purificadas de venenos de serpentes também estão sendo investigadas quanto ao seu potencial no desenvolvimento de novos agentes terapêuticos e novas drogas e desenvolvimento de testes diagnósticos.

1.6 Fatores protéicos do veneno de serpentes e a Agregação Plaquetária

Os venenos de serpentes possuem um grande número de proteínas que podem afetar o processo normal de coagulação, bem como a agregação plaquetária. Estes componentes podem ser classificados nos seguintes grupos:

I. Componentes que coagulam fibrinogênio

II. Enzimas Fibrino(geno)líticas:

(1) α-Fibrinogenase, que especificamente digerem a cadeia α(A) do monômero de fibrinegênio.

(2) β-Fibrinogenase, que especificamente digerem a cadeia β(B) do monômero de fibrinogênio.

(3) γ-fibrinogenase, que especificamente atacam a cadeia γ do monômero de fibrinogênio.

III. Ativador de Plasminogênio

IV. Atividor de Protrombina

V. Inibidor do complexo protrombinase

(1) inibidores sem atividade enzimática não reconhecível

(2) Fosfolipase A_2.

VI. Ativadores de fator X.

VII. Indutores de agregação plaquetária

(1) Indutores de agregação sem atividade coagulante

(2) Indutores de agregação com atividade coagulante

VIII. Inibidores de agregação plaquetária

(1) α-fibrinogenase que digere especificamente a cadeia α da cadeia do monômero de fibrinogênio.

(2) 5'nucleotidase ou ADPase

(3) Antagonista do receptor de fibrinogênio

VIII. Fator de von Willebrand

1.7 Efeito Edematizante

Brain & Whittle (1977) mostraram que PLA_2s isoladas a partir de venenos de *Vipera russeli* e *Crotalus durissus terrifucus* induziam edema em patas de camundongos e liberavam histamina dos mastócitos *in vitro* comprovando a possibilidade de se administrar PLA_2 exogenamente para gerar resposta inflamatória local. Estudos sugeriram que além de histamina e serotonina, substâncias vasoativas tais como prostaglandinas poderiam mediar à formação de edema local em resposta a PLA_2 de veneno de serpente (Bonta et al., 1979).

A atividade edematizante e a liberação de histamina induzida por fosfolipases A_2 de venenos de *Naja naja* e *Crotalus durissus terrificus* foram correlacionadas com a atividade enzimática através de mecanismos cálcio dependentes, suportando então o papel da atividade catalítica e a liberação de mediadores lipídicos no desenvolvimento de reações inflamatórias locais induzidas por essas proteínas (Gutiérrez & Lomonte, 1995).

Também tem sido mostrado que PLA_2 homóloga K49 procedente de *Bothrops atrox,* é capaz de induzir tanto efeito edematizante quanto aumento de níveis de IL-6 (Nuñez et al., 2004).

A BthTX, uma PLA_2 Asp-49, isolada a partir de veneno de *Bothrops jararacussu* tem sido evidenciada por induzir edema de pata e de pele em camundongos bem como a liberação *in vitro* de serotonina a partir de mastócitos peritoniais (Landucci et al.,2000). Sugerindo que em algumas PLA_2s a atividade catalítica possui um papel importante, mas não determinante no efeito edematizante. Todavia a PLA_2 isolada de veneno de *Bothrops neuwiedi* induziu edema de pata na ausência de atividade catalítica, descartando o papel da atividade enzimática na formação de edema (Ownby et al., 1999).

Kini et al., (2003) estabeleceram que PLA_2 apresentam sítios farmacológicos além de região catalítica.

2. OBJETIVOS

Desta maneira, este trabalho tem como objetivo

1. Isolar e caracterizar bioquimicamente a PLA_2 presente na crotoxina do veneno total de *Crotalus durissus ruruima* e *Crotalus durissus cumanenses* responsável pela indução da agregação plaquetária e do efeito edematogênico.

2. Modificar quimicamente a PLA_2 isoladas do veneno total purificado de *Crotalus durissus ruruima* e *Crotalus durissus cumanenses* com:

2.1. ρ-bromofenacil

2.2. cumarina sintética

3. Analisar a ação de PLA_2 nativa e PLA_2 quimicamente modificada

3.1. Na indução da agregação de plasma rico em plaquetas (PRP)

3.2. No efeito edematogênico

3.3. No dicroísmo circular

3.4. Na atividade miotóxica

3.5. Na atividade antibacteriana contra

3.5.1. *Xanthomonas axonopodis pv. passiflorae*

3.5.2. *Michiganensis michiganensis*

3. JUSTICATIVA

As serpentes pertencentes à espécie *Crotalus sp.* são responsáveis por aproximadamente 9% do número total de acidentes ofídicos, sendo epidemiologicamente importantes devido a sua letalidade (Amaral et al., 1991).

O envenenamento crotálico caracteriza-se por uma discreta reação local, o que não espelha a sua gravidade, uma vez que possui, além da ação neurotóxica, também ação hemolítica.

Dentre as subespécies crotalíneas a *Crotalus durissus terrificus* é a mais bem estudada, mas neste trabalho o foco principal foi o estudo das serpentes *Crotalus durissus cumanensis* (*C. d. cumanensis*) e *Crotalus durissus ruruima* (*C. d. ruruima*), ambas são endêmicas do território nacional e seus venenos possuem diversos tipos de atividades que nunca antes foram estudadas (Toyama et al., 2003).

Segundo a literatura o veneno crotálico total quando fracionado apresenta como proteínas principais: a convulxina, a giroxina, a crotamina e a crotoxina, sendo que, a crotoxina representa aproximadamente 47% a 55% de toda a massa seca (Martins et al., 2002; Toyama et al., 2003).

A crotoxina foi descrita como uma proteína heterodimérica, constituída de duas cadeias polipeptídicas: uma PLA_2, (subunidade básica) e a crotapotina (subunidade ácida). Apesar de bem caracterizada, a crotoxina apresenta uma fração de natureza polifuncional, constituída por várias frações biologicamente ativas e por várias isoformas (Toyama et al., 2003; Oliveira et al., 2003),

Neste trabalho isolamos a PLA_2, uma enzima largamente distribuída na natureza, que pode ser encontrada tanto em bactérias, plantas, tecidos de mamíferos (pulmão, fígado,

baço, coração, eritrócitos, plaquetas e leucócitos polimorfonucleares) quanto em tecidos pancreáticos de mamíferos e nos venenos de serpentes e insetos.

As últimas pesquisas com fosfolipases foram impulsionadas principalmente devido à ação destas em várias atividades biológicas tais como: neurotoxicidade (neurotoxinas pré-sinápticas e neurotoxinas pós-sinápticas); miotoxicidade (mionecrose local e miotoxicidade sistêmica); cardiotoxicidade; efeito anticoagulante; iniciador da agregação plaquetária; inibidor da agregação plaquetária; atividade hemolítica; hemorragia interna; atividade anti-hemorrágica; atividade convulsionante; atividade hipotensiva; atividade edematogênica; lesão de órgãos e tecidos e as tentativas para elucidar estruturalmente os sítios responsáveis pela toxicidade das PLA_2 provenientes do veneno de serpentes envolvem desde metodologias como as modificações químicas, mutações sítio dirigidas, técnicas cristalográficas, espectrofotométricas e fluorométricas, ressonância magnética nuclear até a ação de inibidores e substratos naturais ou artificiais (Soares e Giglio, 2003).

No presente trabalho a modificação química da PLA_2 com p-bromofenacil, uma substância que promove alquilação seletiva ou preferencial do grupo histidina na cadeia lateral de aminoácidos da enzima PLA_2 e com a cumarina sintética, um polifenólico, foram as principais estratégias adotadas para elucidar as relações estrutura-função.

A relação entre a modificação estrutural, a atividade enzimática, a toxicidade e a variação dos efeitos biológicos tais como: agregação plaquetária, efeito edematogênico, miotoxicidade e atividade antibacteriana foram analisados. A redução dos efeitos biológicos após a prévia modificação química da PLA_2 servem de base para novos estudos na tentativa de promover um melhor estudo do mecanismo estrutura-função das PLA_2. E ainda, a utilização de compostos polifenólicos sintéticos em ações biológicas podem ter seus efeitos medicamentosos melhor explorados.

4. MATERIAIS E MÉTODOS

4.1. Animais

Os animais utilizados foram ratos machos Wistar (120-150g), adultos (45 dias) cedidos pelo Centro Multidisciplinar para Investigação Biológica Universidade Estadual de Campinas (CEMIB-UNICAMP).

4.2. Venenos e reagentes

O veneno total de serpentes crotálicas foi adquirido junto ao Núcleo Regional de Ofidismo – UFCE. Todos os solventes, produtos químicos e reagentes utilizados foram de grau HPLC, grau seqüência ou de alto grau de pureza, obtidos do SIGMA, Aldrich Chemicals, Applied Biosystem, Pierce, Merk e Bio Rad.

4.3. Purificação do veneno de *Crotalus durissus ruruima* e *Crotalus durissus cumanenses*

4.3.1. Cromatografia de exclusão molecular em HPLC

Aproximadamente 20 mg de veneno de *Crotalus durissus ruruima* e *Crotalus durissus cumanenses* foram dissolvidos em tampão Bicarbonato de amônio 1,0 M, pH 7,9 até a sua completa homogeneização, seguido pelo processo de clarificação em uma centrífuga (4500 x *g* por 2 min). O sobrenadante obtido foi coletado e aplicado em coluna de exclusão molecular Superdex 75 (1,0 x 60 cm, Pharmacia) acoplada em sistema HPLC (1 x 60 cm, Waters) previamente equilibrado com o mesmo tampão utilizado para a eluição do veneno. A principal fração do veneno foi purificada em fluxo constante de 0,2 ml/min. Frações foram coletadas em intervalos constantes de 2 min para cada tubo. Uma alíquota de 20 µl de cada tubo foi retirada para ensaios enzimáticos (PLA_2). O monitoramento da

corrida cromatográfica foi realizado a 280 nm e a crotoxina-like eluída foi coletada, liofilizada e armazenada a -20 ºC para posterior utilização.

4.3.2. Cromatografia de fase reversa em HPLC

A fração crotoxina foi submetida a um segundo passo de purificação usando coluna μ-bondapack C-18 (0,78 x 30 cm). A crotoxina (5,5 mg) foi dissolvida em 200 μl de solução aquosa de TFA 0,1% (tampão A) até completa dissolução. A solução obtida resultante foi clarificada por centrifugação a 4500 x *g* por 3 min. O sobrenadante foi aplicado na coluna de fase reversa em sistema HPLC e a eluição de PLA_2, crotapotina ou fração "trombina-like" foi realizada em gradiente linear (C.d.ru) e não-linear (C.d.cu) de tampão B (67% de acetonitrila em 0,2% de bicarbonato de amônio) com fluxo constante de 2,0 ml /min. O monitoramento da corrida cromatográfica foi realizado a 214 nm para *C.d ruruima* e 280 nm para *C.d cumanenses* e as frações obtidas foram liofilizadas a - 20 C.

As amostras de PLA_2 foram incubadas com ρ-bromofenacil brometo e cumarina sintética (3,0 mg dissolvidos em 1 ml de DMSO) e repurificadas em fase reversa coluna μ-bondapack C-18 (0,78 x 30 cm).

4.3.3. Eletroforese em PAGE-SDS

A eletroforese em gel de poliacrilamida foi realizada seguindo-se a metodologia descrita por Laemmli (1970). As placas de poliacrilamida foram feitas de modo descontínuo, apresentando um gel de concentração de 5% e um gel de corrida de 12,5%. As placas foram preparadas utilizando-se uma solução de acrilamida estoque (30%T, 0,8%C). O gel de concentração a 5% foi preparado utilizando-se o tampão Tris-HCl 0,5M, pH 6,8 e

o gel de corrida foi feito utilizando-se o tampão Tris-HCl 1,0M, pH 8,8. Em ambos os géis foram acrescentados 0,1% (v/v) de SDS 207%.

A eletroforese PAGE-SDS foi realizada em um sistema duplo de mini placas SE 250 Mighty Small II (Hoefer Scientific Instruments). As amostras e os marcadores de peso molecular foram dissolvidos em tampão de amostra (Tris-HCl, 0,075M, pH 6,8; 10% de glicerol; 4% de SDS; 0,001% de Azul de Bromofenol). A corrida eletroforética foi realizada a 40 mA. Os géis foram corados com solução de Coomassie Blue 0,05% a 37ºC e o excesso de corante foi removido com ácido acético 7%.

4.3.4. Análise por Espectrometria de Massas por Maldi-Tof (MS)

A. massa molecular da amostra de PLA2 foi analisada por Espectrometria de Massa, utilizando-se um Voyager DE PRO MALDI TOF mass spectrometry (Applied Biosystems, Foster City, CA, USA). 1µl da amostra em TFA 0,1% foi misturada em 2 µl da matriz. A matriz foi preparada com ácido α-ciano-4-hidroxi-cinamico (Sigma), 60% acetonitrila e 0,1% v/v TFA. E a massa foi analisada sob as seguintes condições: aceleração de voltagem 25KV, laser ajustado a 2890mJ/com^2 em 300ns e o modo de análise foi linear.

4.4. Caracterização Bioquímica

4.4.1. Ensaios enzimáticos - Atividade PLA_2

A medida da atividade enzimática foi efetuada de acordo com o método descrito por Holzer e Mackessy (1996) adaptado para placa de ELISA com 96 poços. A curva padrão do ensaio foi composta da adição de 200 µl de tampão (Tris-HCl 10 mM, $CaCl_2$ 10 mM, NaCl 100 mM, pH 8,0), 20 µl de substrato (ácido 4-nitro-3-octanoyloxy-benzóico; $4N_3OBA$), 20 µl de água e 20 µl de amostra para um volume final de 260 µl. No ensaio, a solução padrão

sem PLA_2 e a solução com PLA_2 foram incubadas separadamente por 20 minutos a uma temperatura constante de 37 ºC. Após este prévio tempo de incubação, aproximadamente 20 µl da solução padrão foram incubados na presença de um substrato para o monitoramento da velocidade enzimática (Vo) que foi efetuada a 425 nm a intervalos de 10 min, durante 30 min. Todos os ensaios foram conduzidos em triplicata e a absorbância foi mensurada a 425 nm, usando um multileitor de placas SpectraMax 340 (Molecular Devices, Sunnyvale, CA) e a atividade enzimática foi calculada baseada nas diferenças dos valores da absorbância após 20 min do tempo de incubação.

4.4.2. Análise de aminoácidos

A análise de aminoácidos foi realizada no analisador automático de aminoácidos PICO TAG (Sistema Waters) seguindo a metodologia descrita por Henrikson e Meredith (1984). Um ou dois nmol das amostras purificadas foram hidrolizados com HCl a 106ºC por 24 horas. As amostras hidrolisadas foram posteriormente derivatizadas com PTC (Fenilisotiocianato) por uma hora a temperatura ambiente. Após a derivatização pré-coluna das amostras os aminoácidos derivatizados (PTC aminoácidos) foram identificados, em uma coluna de fase reversa, de acordo com o tempo de retenção dos PTC-aminoácido padrão. Para a quantificação de cisteína e metionina, as amostras foram previamente oxidadas com ácido perfórmico. A hidrólise e a derivatização das amostras oxidadas seguiram a metodologia descrita acima.

A determinação do triptofano foi de acordo com metodologia descrita pela Waters. A hidrólise foi realizada com 4M de Ácido Metilsulfônico ao invés de HCl 6M e a temperatura de hidrólise foi de 110 ºC por 20 horas. A derivatização e a análise dos hidrolisados seguiram os procedimentos anteriormente descritos.

4.4.3. Determinação da seqüência N-terminal

Dois miligramas da proteína purificada de acordo com a metodologia anteriormente descrita foram dissolvidas em 6M de guanidina contendo 0,4M de Tris e 2 mM de EDTA, com pH final de 8,15, reduzida com DTT (14M) e carboximetilada com ácido iodoacético, marcado como descrito por Marangoni et al., 1995. Após a redução e alquilação radioativa as amostras foram dessalificadas por meio de uma coluna de exclusão molecular em G25, usando o ácido acético 1M como eluente. A dessalificação ocorreu à temperatura ambiente com fluxo livre, sendo coletadas amostras de 1 ml por tubo, o monitoramento da corrida foi realizado a 280 nm.

A proteína reduzida e carboximetilada, RC Proteína, foi processada na região N-terminal da proteína com um seqüenciador automático de aminoácidos (Applied Biosystem modelo Procise f). A identificação dos PTH aminoácidos foi realizada com um PTH Analyzer 120 A da Applied Biosystem. A identificação dos resíduos de Cys foi realizada como PTH ^{14}Cys e confirmada por contagem radioativa.

4.4.4. Dicroísmo Circular

A alteração das estruturas secundárias foi monitorada pelo dicroísmo circular. O dicroísmo circular (CD) é particularmente útil para o estudo de moléculas quirais, macromoléculas, sejam elas ou não de origem biológica, tais como proteínas, carboidratos, etc., compostos esses, que possuem unidades opticamente ativas, ou seja, podem exibir sinal na espectroscopia de dicroísmo circular. Quando tais moléculas interagem com a luz circularmente polarizada provocam uma alteração nessa luz incidente. A técnica de CD detecta exatamente a alteração através da medida da diferença da absorção da luz

circularmente polarizada à esquerda e à direita, após a luz passar pela amostra. O CD pode ser utilizado para detectar:

- Mudanças conformacionais de macromoléculas;
- Composição de misturas quirais;
- Interação dessas macromoléculas com outras moléculas menores, especialmente aquirais.

O aparelho utilizado foi o Jasco J 720 espectropolarímetro (Tókio – Japão), equipado com um IBM PC-AT PS/2 com monitor multiscan CNS-3436 e com uma impressora Hewlett Packard 7465. As amostras protéicas foram dissolvidas em fosfato de sódio 10 mM pH 7,4 com concentração de 0,1mg/mL a temperatura ambiente. Utilizou-se uma cela de 1 mm e monitoração realizada a 195-250 nm. Esta técnica foi realizada no Laboratório de Biologia Estrutural e Cristalogrfia – Prof. Dr. Ricardo Aparicio - Instituto de Química da Unicamp.

4.5. Atividade biológica

4.5.1. Atividade de agregação plaquetária.

A) Preparo da solução de plaquetas

Para este ensaio foram coletados sangue de voluntários sadios, que não tomaram medicamentos por 15 dias. O sangue foi colocado em tubos plásticos contendo citrato de sódio 3,8% (1:10 v/v), e centrifugado a 200 x *g* por 15 minutos a 25^{o}C para obtenção do plasma rico em plaquetas (PRP); o plasma pobre em plaquetas (PPP) foi obtido centrifugando-se o sangue remanescente a 800 x *g* por 15 min a 25^{o}C. Para preparar as plaquetas lavadas, o PRP foi centrifugado a 800 x *g* por 12 min na presença de 9µl de

iloprost (8 nM), um análago da prostaciclina. O sobrenadante foi desprezado e o precipitado de plaquetas foi ressuspenso em solução de Krebs livres de Ca^{2+} e centrifugado a 200 x *g*. O processo foi repetido por mais duas vezes (Radomski & Moncada, 1983). Após a lavagem, as plaquetas (50µl) foram incubadas em tudos tipo eppendorf, com 950 µl de oxalato de amônia 1% por 10 min. e a contagem de plaquetas foi feita em Câmara de Neubauer, ajustada para 3 x 10^8 plaquetas/ ml. Cloreto de cálcio (1 mM) foi adicionado à suspensão final das plaquetas. O sangue para obtenção das PL contém ACD-C (124 mmol/L Na_3-citrato, 130 mmol/L ácido cítrico e 110 mmol/L glicose) (1:10, v/v).

B) Medida da Agregação plaquetária.

A medida de agregação foi realizada, usando-se um agregômetro de dois canais (Payton Scientif Instruments, Inc, Buffalo, NY). O PRP representa 0% de agregação, determinando-se uma linha de base para o PRP o aparelho foi calibrado contra PPP, que apresenta 100% de agregação, determinando-se um pico máximo. Com isso foi possível determinar uma amplitude para o registro de agregação deste plasma. Para as PL (0%) a calibração foi realizada contra solução de Krebs (NaCl 118mM, $NaHCO_3$ 25mM, d glicose anidra 5,6mM e KCl 74g/L) (100%). Uma suspensão de PRP (400 µl) foi mantida sob agitação constante (900 rpm) no agregômetro em cuvetas à 37ºC. Os experimentos de agregação foram realizados em triplicatas utilizando-se concentrações crescentes das proteínas purificadas nativas e modificadas do veneno total de *C. d. ruruima* e *C.d. cumanenses*. Como controles positivos para a agregação foram utilizados ADP (PRP) e trombina (PL).

4.5.2 Experimento de Edema em pata de rato

De acordo com Antunes et al (1990), ratos machos Wistar (120-150g) foram anestesiados com halotano (inalação), o edema de pata foi induzido por uma única injeção subplantar de PLA_2 nativa ou tratadas com ρ-bromofenacil e cumarina (5-30 μg/pata). O volume da pata foi medido 5 minutos após a injeção da amostra e nos intervalos de tempo de 15, 30, 60, 120 e 240 minutos, utilizando um hidropletismômetro (modelo 7150, Ugo Basile, Itália). Todas as amostras foram dissolvidas em solução salina estéril 0,9% e o volume injetado na pata foi sempre de 0,1 mL. Os resultados foram expressos em volume de pata (mL) calculado pela subtração do volume basal pelo volume final. A área sobre a curva (AUC) foi também calculada (regra trapezoidal) e os resultados expressos como volume total do edema (mL/pata).

4.5.3 Ensaio Antibacteriano

Foram empregadas a bactéria Gram negativa *Xanthomonas axonopodis pv. Passiflorae* (Xap) (IBSBF1445) e a bactéria Gram positiva *Clavibacter michiganensis* subsp. *michiganesis* (Cmm) (IBSBF979). Ambas as bactérias sobrevivem em aerobiose, meio nutriente ágar (extrato de carne 3%, peptona 5%, NaCl 5% e ágar 16%) em estufa a 28ºC.

As bactérias ($3x10^3$UFCs) foram incubadas com a PLA_2 de *Crotalus durissus ruruima* na concentração de 1mg/mL durante 30 minutos à temperatura ambiente, após esse período foram semeados 100 L da suspensão em meio de cultura nutriente ágar. A fração da PLA_2 também foi semeada em meio nutriente ágar para confirmar sua pureza. As placas foram incubadas por 48 horas em estufa a 28ºC.

Nas placas controle em meio ágar nutriente foram semeadas as bactérias na mesma concentração de $3x10^3$ UFCs, determinada por espectrofotometria, na absorbância de 0,063 para a Xap e de 0,080 para a Cmm, em comprimento de onda de 600λ.

4.5.4 Atividade miotóxica

Foi avaliada a liberação da creatina quinase no plasma de camundongos em células musculares danificadas. Três grupos de animais (18-22 g) foram divididos e foi injetado no músculo gastrocnêmio direito 50 mL de 0,5 mg / mL sPLA2, em amostras não tratadas ou tratadas com cumarina sintética ou ρ-BPB (n = 4), enquanto o grupo controle recebeu PBS. Camundongos foram sangrados na cauda 3 h após a injeção e sangue foi coletado em tubos com heparina, uma alíquota foi utilizada para a determinação de creatina quinase. A atividade da creatina quinase sobre plasma foi deterimanda usando o kit 47-UV (Sigma Chemical Co). A atividade foi expressa em unidades por litro a 30 ° C.

4.6. Análise estatística

Os resultados foram expressos como média +/- erro padrão da média. A significância das diferenças observadas foi determinada pelo teste não pareado *t*-Student, com valor $P< 0,05$ considerado significante.

5. RESULTADOS

5.1 Cromatografia em exclusão molecular dos venenos de *Crotalus durissus ruruima* e *Crotalus durissus cumanenses* em HPLC (Superdex 75)

Os venenos totais de *Crotalus durissus ruruima* (**Figura 4**) e de *Crotalus durissus cumanenses* (**Figura 5**) foram fracionados por cromatografia de exclusão molecular a um fluxo de 0,2ml/min e as amostras foram coletadas em tubos de 400µl. O monitoramento das corridas cromatográficas foi realizado a 280 nm. e resultaram na eluição de 5 frações principais para o veneno de *Crotalus durissus ruruima* nomeadas e identificadas como crotacetina, convulxina (I), giroxina (II), crotoxina (III) e crotamina (IV) e 3 frações principais que o veneno de *Crotalus durissus cumanenses* nomeadas e identificadas como giroxina (II), crotoxina (III) e crotamina (IV). A atividade enzimática foi monitorada a intervalos de 75- 92 minutos aproximadamente.

A análise cromatográfica dos venenos de *Crotalus durissus ruruima* e *Crotalus durissus cumaneneses* revelou diferenças significativas do ponto de vista quantitativo e qualitativo. Comparando os perfis cromatográficos de *Crotalus durissus ruruima* e *Crotalus durissus cumanenses* notou-se a ausência das frações crotacetina e convulxina para *C.d.cumanenses* e a presença majoritária de crotoxina para os dois venenos.

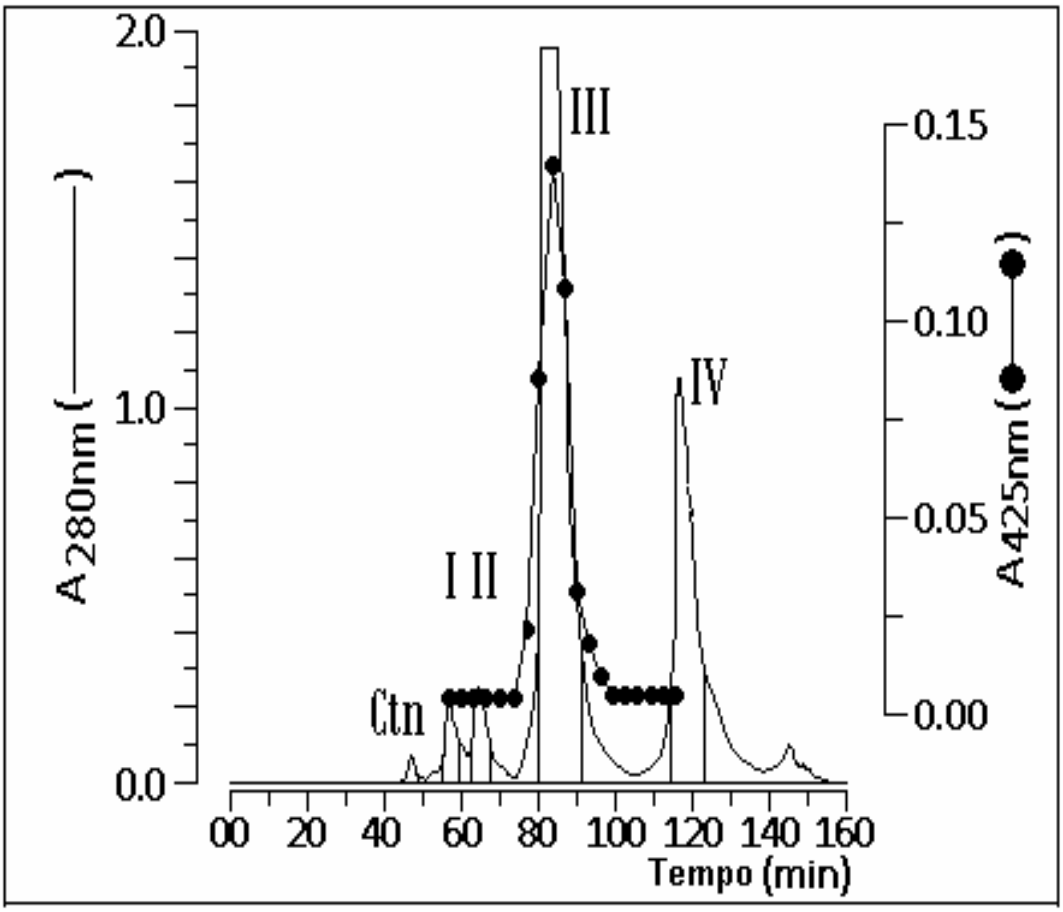

Figura 4. Perfil cromatográfico da purificação do veneno de *Crotalus durissus ruruima* obtido por cromatografia de exclusão molecular (Superdex 75) usando tampão bicarbonato de amônio. A primeira corrida cromatográfica isocrática com fluxo constante de 0,2ml/min foi monitorada a 280 nm. As frações em números romanos correspondem a: I (convulxina), II (giroxina), III (crotoxina) e IV (crotamina) e a fração Ctn é a crotacetina. A atividade PLA_2 foi encontrada no pico III e foi monitorada a 425nm.

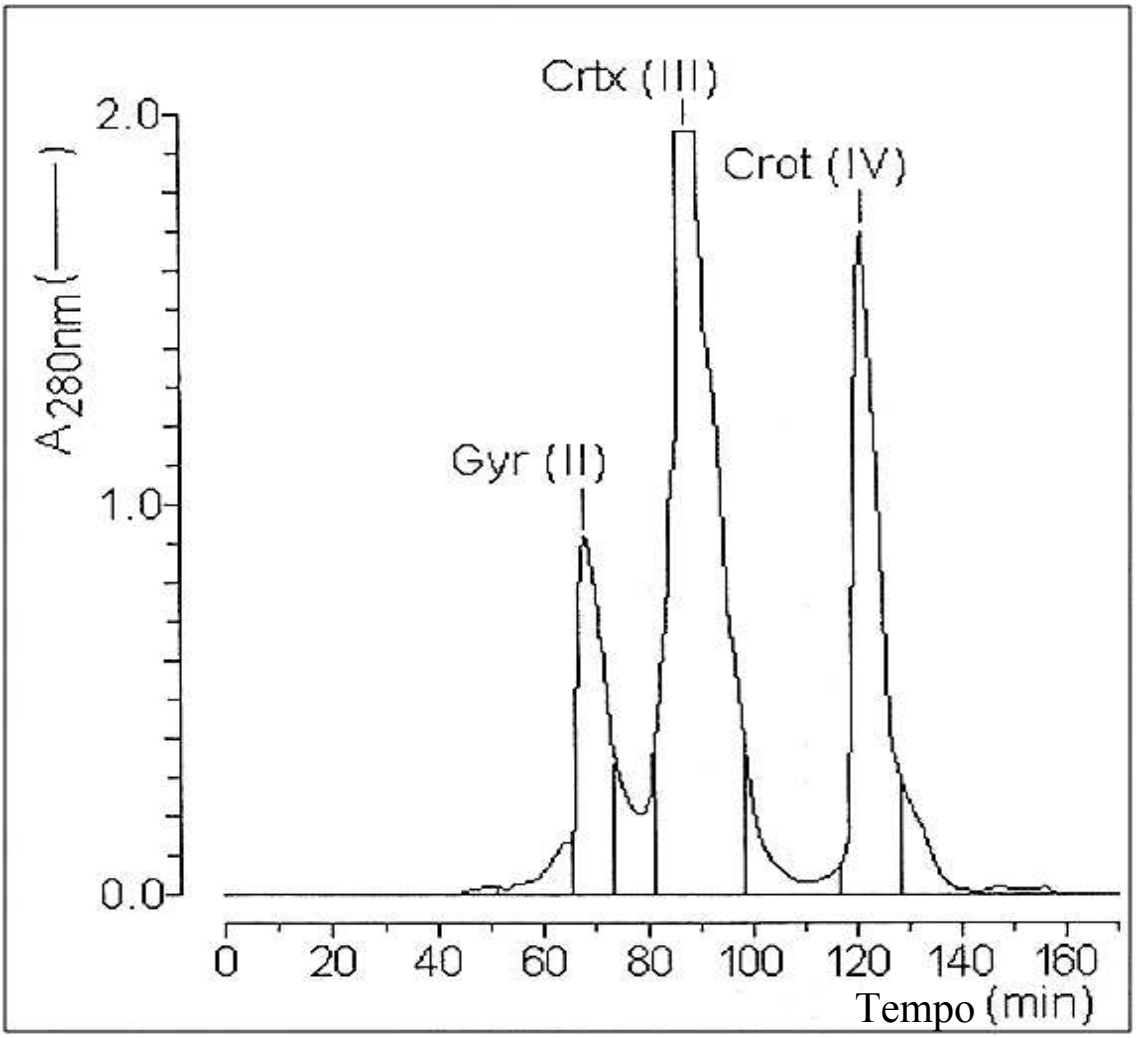

Figura 5. Perfil cromatográfico da purificação do veneno de *Crotalus durissus cumanenses* obtido por cromatografia de exclusão molecular (Superdex 75) usando tampão bicarbonato de amônio. A primeira corrida cromatográfica isocrática com fluxo constante de 0,2ml/min foi monitorada a 280 nm. As frações em números romanos correspondem a: II (giroxina), III (crotoxina) e IV (crotamina).

5.2 Purificação da crotoxina em HPLC de fase reversa do veneno de *Crotalus durissus ruruima* e *Crotalus durissus cumanenses*

As frações crotoxina do veneno de *Crotalus durissus ruruima* (**Figura 6**) e *Crotalus durissus cumanenses* (**Figura 7**) após o isolamento por exclusão molecular foram então submetidas a um segundo passo de purificação por coluna preparativa de fase reversa usando coluna μ-bondapack C-18 (0,78 x 30 cm).

As condições cromatográficas em que ambas crotoxinas foram fracionadas permitiram caracterizar as principais isoformas de PLA_2 e crotapotina. Na **figura 6**, observamos que o fracionamento por cromatografia de fase reversa da crotoxina de *Crotalus durissus ruruima* (C.d.ru) mostrou a presença de três isoformas principais de crotapotina (Crtp) e 2 isoformas de PLA_2 (PLA_2 iso 1 e iso 2), que foram eluídas aos 30,6 min e 34,3 min respectivamente da corrida cromatográfica.

O fracionamento da crotoxina do veneno de *Crotalus durissus cumanenses* (C.d.cu) em cromatografia de fase reversa (**figura 7**) mostrou a presença de uma forma majoritária de crotapotina e 2 isoformas de PLA_2, uma mais hidrofílica (PLA_2 iso 1) e uma mais hidrofóbica (PLA_2 iso 2), que foram eluídas aos 30,2 min e 36,0 min respectivamente.

A análise comparativa das duas crotoxinas em HPLC de fase reversa mostra que quantitativamente a crotoxina de *Crotalus durissus ruruima* apresenta uma concentração menor de PLA_2 e crotapotina em comparação com as de *Crotalus durissus cumanenses*.

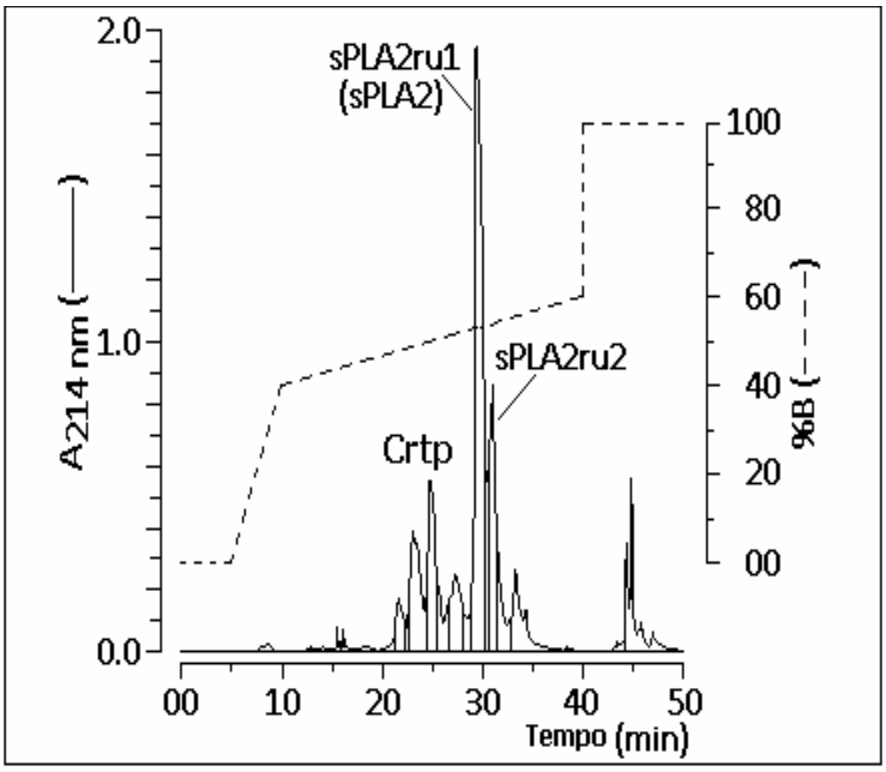

Figura 6. Perfil cromatográfico da fração crotoxina de *Crotalus durissus ruruima* obtida por coluna preparativa de fase reversa em HPLC resultando em 3 isoformas de crotapotina e 2 isoformas de PLA_2. A corrida cromatográfica foi monitorada a 214nm com fluxo constante de 2,0 mL/min usando gradiente não-linear de acetonitrila em presença de bicarbonato de amônio.

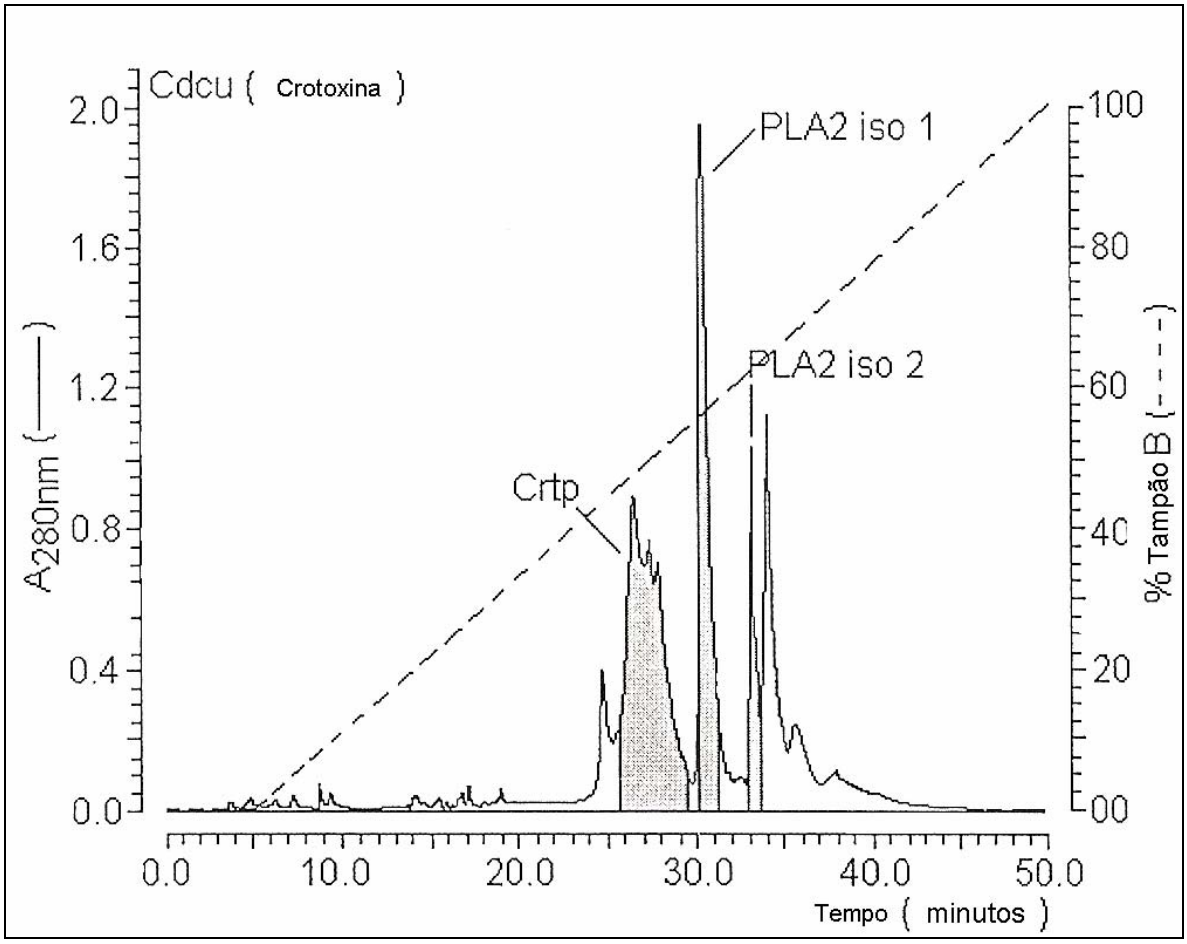

Figura 7. Perfil cromatográfico em HPLC de fase reversa da crotoxina purificada do veneno total de *Crotalus durissus cumanenses*. A corrida cromatográfica foi conduzida em fluxo constante de 2,0 mL/min com gradiente linear de acetonitrila e monitorada a 280 nm. Nessas condições, obteve-se a partir do veneno total de *Crotalus durissus cumanenses* uma fração crotapotina principal (Crtp) e duas frações PLA_2 nomeadas como PLA_2 iso 1 (isoforma principal) e PLA_2 iso 2 (isoforma menor).

5.3 Eletroforese em PAGE-SDS

O veneno total e sua fração PLA_2 foram submetidos à análise em PAGE-SDS para verificar o grau de pureza. Os marcadores de massa molecular utilizados foram: fosforilase b (94KDa), BSA (67 KDa), ovoalbumina (43 KDa), anidrase carbônica (30 KDa), inibidor de tripsina de soja (20,1 KDa) e α-lactoalbumina (14,4 KDa). Na **figura 8** são mostrados os perfis de massa molecular em PAGE-SDS a 12,5% dos venenos de *C.d. ruruima* e *C.d. cumanenses* e suas frações PLA_2. As PLA_2 mostraram neste estudo eletroforético a presença de uma única banda ao redor de 15 KDa sugerindo alto grau de homologia

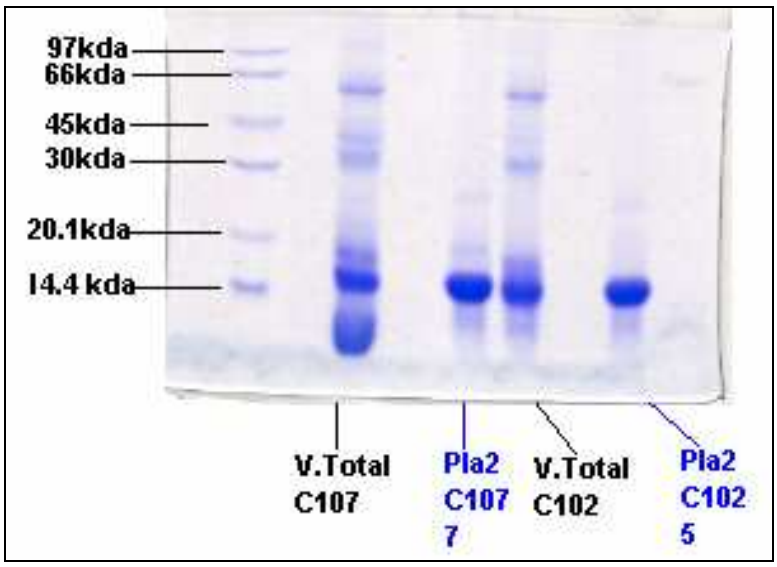

Figura 8. Perfis eletroforéticos dos venenos totais e das fosfolipases A_2 de *C.d ruruima* (C 102) e *C.d. cumanenses* (C 107) em PAGE-SDS 12,5% com voltagem constante de 70 V

5.4 Espectrometria de massa

As amostras de PLA_2 de *Crotalus durissus ruruima* (**Figura 9a**) e *Crotalus durissus cumanenses* (**Figura 9b**) mostraram a partir da análise espectométrica das massas moleculares de 14599,5 Da e 13989,14 Da respectivamente. A análise também revelou o grau de pureza da amostra que apresentou um único pico detectado.

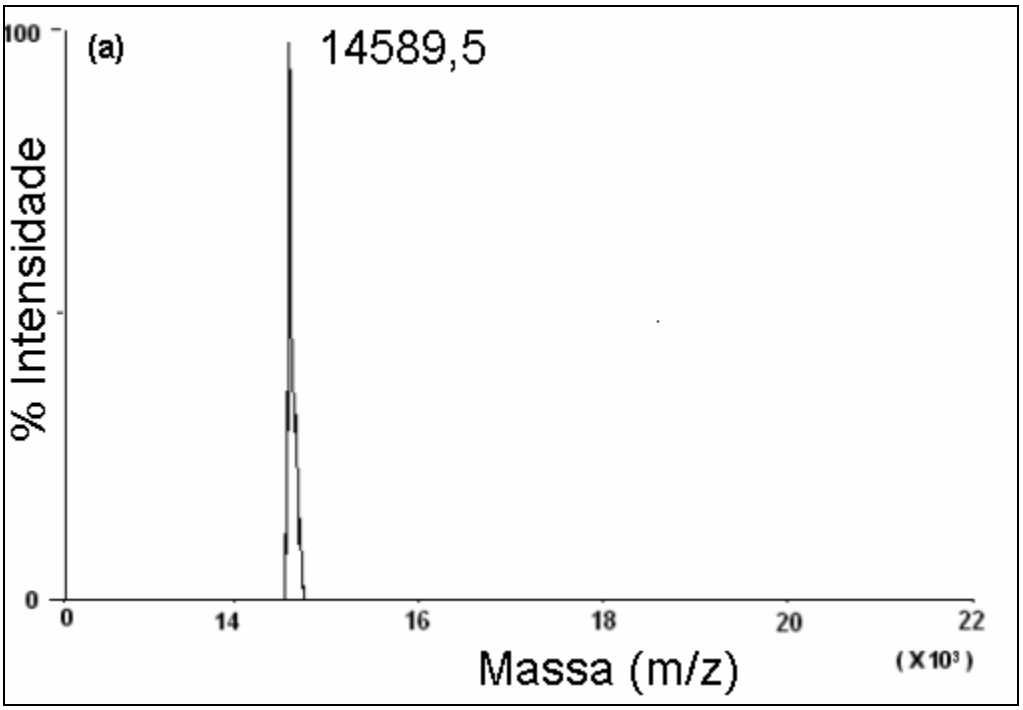

Figura 9. a) Determinação das massas moleculares das PLA_2 de *C.d.ruruima* no eixo das abscissas x 10^3. As massas no eixo x representam as massas moleculares x 1000.

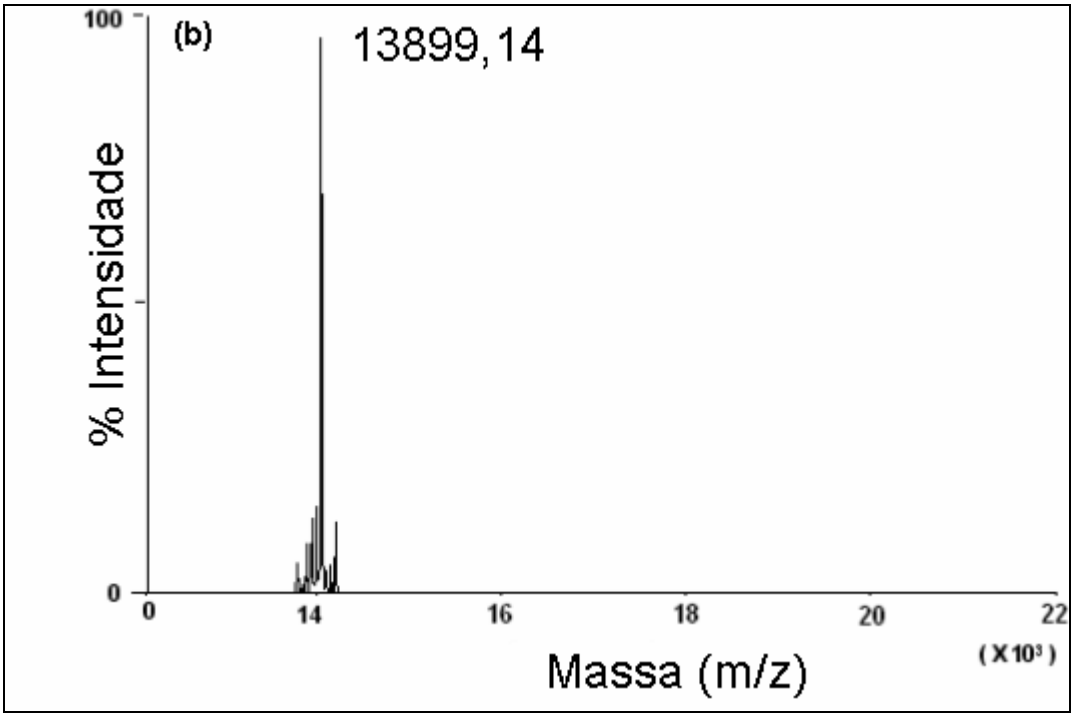

Figura 9. b) Determinação das massas moleculares das PLA_2 *C.d.cumanenses* no eixo das abscissas x 10^3. As massas no eixo x representam as massas moleculares x 1000.

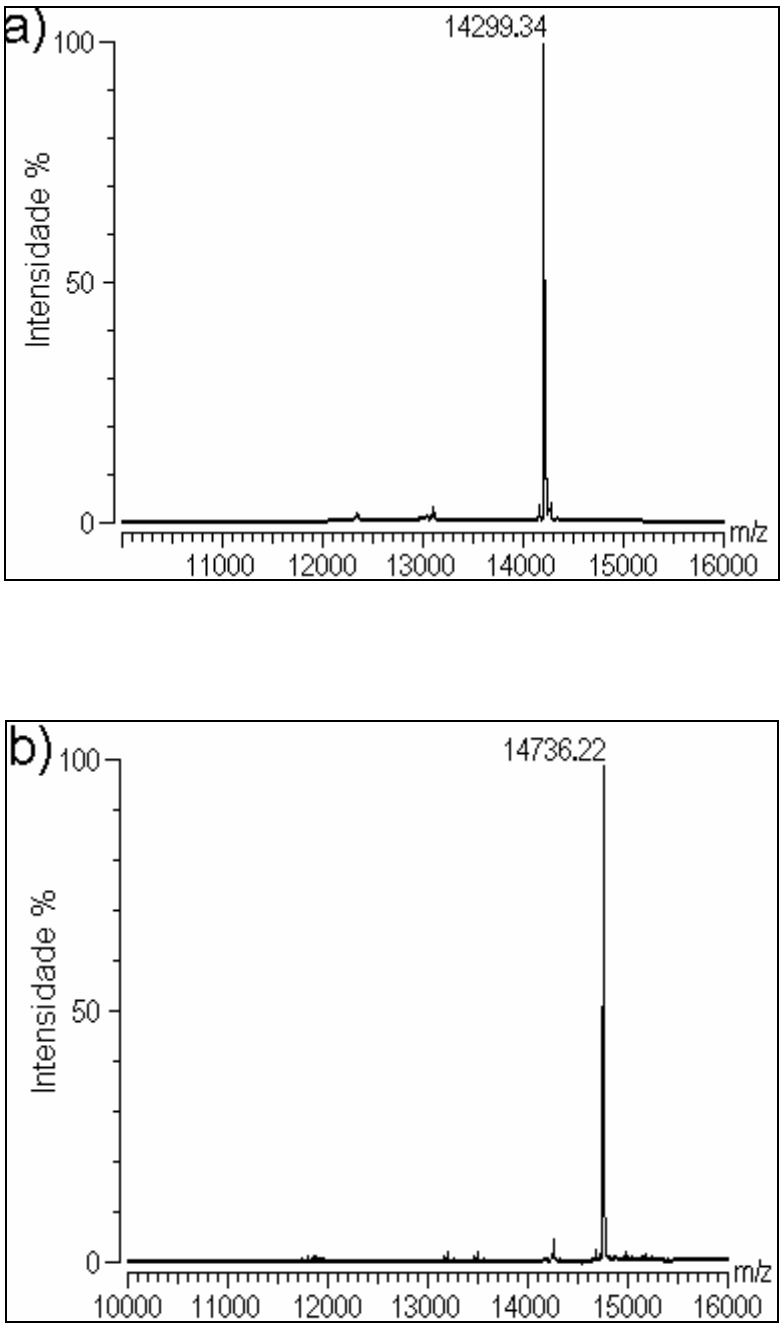

Figura 10. a) A principal isoforma de PLA_2 de *C.d ruruima* purificada foi analisada com espectometria de massa em MALDI-TOF, revelando uma massa molecular de 14.299,34 Da. b) A massa molecular de $sPLA_2$ tratada com cumarina sintética (EOCC) foi analisada com espectrometria de massa MALDI-TOF, revelando uma massa molecular de Da 14,736.22. Este resultado sugere que duas moléculas de EOCC foram adicionados a cada molécula de $sPLA_2$.

5.5 Atividade fosfolipásica nos venenos de *C.d. ruruima* e *C.d.cumanenses*

A medida da atividade fosfolipásica foi examinada usando o substrato cromogênico sintético e laminar 4-nitro-3-(octanoyloxy)-benzóico (NOBA) e o monitoramento da velocidade enzimática (Vo) para a PLA_2 nativa e tratada foi realizado no espectofotômetro com absorbância de 425 nm. Como podemos observar no gráfico da **figura 11a** constatamos redução da atividade fosfolipásica após prévia incubação da PLA_2 com cumarina sintética e ρ-bromofenacil brometo. Sob estas condições a $sPLA_2$ apresentou após 20 min Vo de 2,5 nmoles/min, entretanto a PLA_2 pré-incubada com cumarina sintética (EOCC) apresentou uma redução de Vo de 0,5 nmoles/min e a PLA_2 pré-incubada com ρ-bromofenacil apresentou uma redução de Vo de 0,4 nmoles/min.

As comparações do efeito inibitório de EOCC (cumarina sintética) sobre outras fontes de PLA_2. após 20 min foram apresentadas no gráfico da **figura 11b**, para a PLA_2 de plaqueta quando nativa o Vo foi de 2,3 nmoles/min e após prévia incubação com EOCC o Vo foi de 0,8 nmoles/min, portanto a redução variou em torno de 1,5 nmoles/min, para a PLA_2 bovina quando nativa o Vo foi de 4,2 nmoles/min e após prévia incubação com EOCC o Vo foi de 2,3 nmoles/min, portanto a redução variou em torno de 1,9 nmoles/min, para a PLA_2 de *Apis mellifera* quando nativa o Vo foi de 5,3 nmoles/min e após prévia incubação com EOCC o Vo foi de 0,6 nmoles/min, portanto a redução variou em torno de 4,7 nmoles/min e para PLA_2 de murinos GXII quando nativa o Vo foi de 4,2 nmoles/min e após prévia incubação com EOCC o Vo foi de 2,8 nmoles/min, portanto a redução variou em torno de 1,4 nmoles/min.

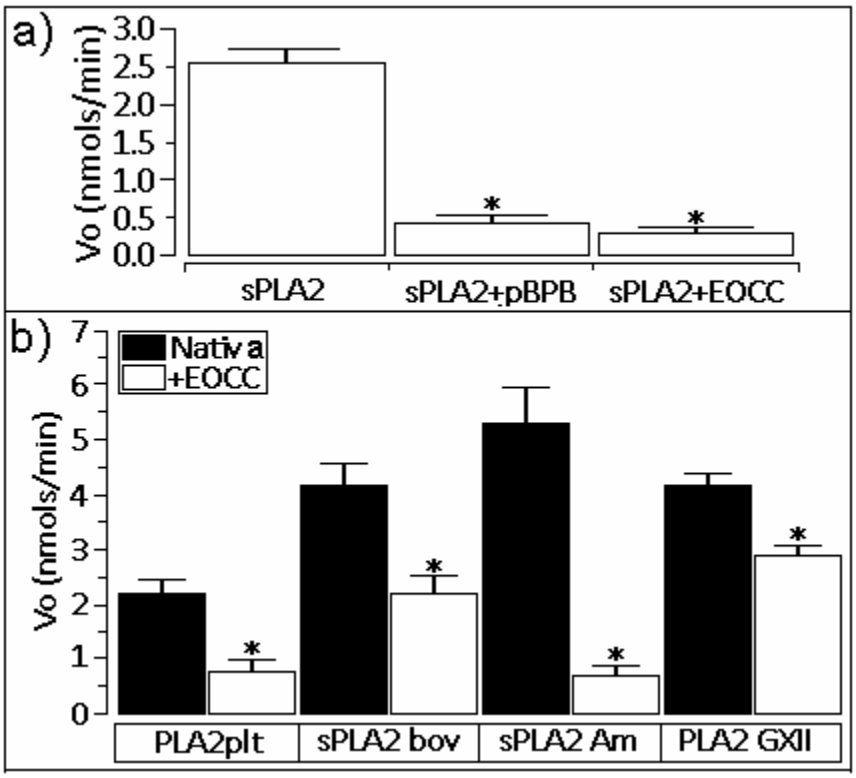

Figura 11. a) Comparação da velocidade enzimática (nmoles/min) das amostras de $sPLA_2$ nativa e $sPLA_2$ tratada de *Crotalus durissus ruruima* com cumarina sintética e ρ-bromofenacil ρ(BPB). Redução da atividade fosfolipásica após a prévia incubação. Leitura monitorada a 425nm de absorbância, (n = 12). *p < 0.05. b) Comparação do efeito inibitório de EOCC (cumarina sintética) contra outras fontes de PLA_2. Em todos os ensaios, 10 nmol EOCC foi previamente incubado com a solução sPLA2 (1 mg / mL) por 30 min antes do ensaio enzimático. Cada ponto representa a média ± EPM (n = 12). * P <0,05. Os efeitos da EOCC em PLA_2 de plaquetas, PLA_2 bovina, PLA_2 de abelha e PLA_2 de murinos GXII foram avaliados e comparados com as PLA_2. nativas das respectivas fontes. Neste experimento observou-se que EOCC inibiu fortemente $sPLA_2$ de *Apis mellifera* e inibiu moderamente o efeito enzimático das PLA_2 de plaquetas e das PLA_2 proveniente de murinos GXII.

5.6 Análise da composição de aminoácidos de PLA_2 dos venenos de *Crotalus durissus ruruima* e *Crotalus durissus cumenenses*

A análise de aminoácidos (**Figura 12**) mostra que as PLA_2 de *C.d.ruruima* e *C.d.cumanenses* possuem alto conteúdo de aminoácido básico (Lys) e a presença de 7 resíduos de ½ cisteína para a PLA_2 de *C.d.ruruima* e 8 resíduos de ½ cisteína para a *C.d.cumanenses*.

Mw	Amino ácido	PLA2 ru	Mw/AA	PLA2 cum	Mw/AA
133	Asp	13	1729	11	1463
147	Glu	9	1323	10	1470
105	Ser	6	630	5	525
75	Gly	14	1050	16	1200
155	His	2	310	4	620
174	Arg	7	1218	10	1740
119	Thr	6	714	5	595
89	Ala	7	623	5	445
115	Pro	6	690	5	575
181	Tyr	9	1029	10	1810
117	Val	4	468	5	585
149	Met	1	149	1	149
242	Cys	7	1694	8	1936
131	Ile	5	655	5	655
131	Leu	8	1048	7	917
165	Phe	4	660	2	330
148	Lys	13	1924	12	1776
204	Trp	1	204	1	204
	Total	122		122	

Figura 12. Análise composição de aminoácidos (PTC derivatização)

5.7 Análise da região N-terminal das PLA_2 dos venenos de *Crotalus durissus ruruima* e *Crotalus durissus cumenenses*

A partir do alinhamento das seqüências N-terminal dos resíduos de aminoácidos de PLA_2 de *C.d ruruima* e *C.d.cumanenses* com seqüências de PLA_2 de diferentes espécies e subespécies de serpentes verificou-se alto grau de homogeneidade com as demais PLA_2 de serpentes do gênero *Crotalus* (**Figura 13 a** e **b**). Nota-se que a sequência N-terminal dos resíduos de aminoácidos para *Crotalus durissus ruruima* e *Crotalus durissus cumanenses* diferem nas posições 1, 14 e 15 e são semelhantes nas demais posições.

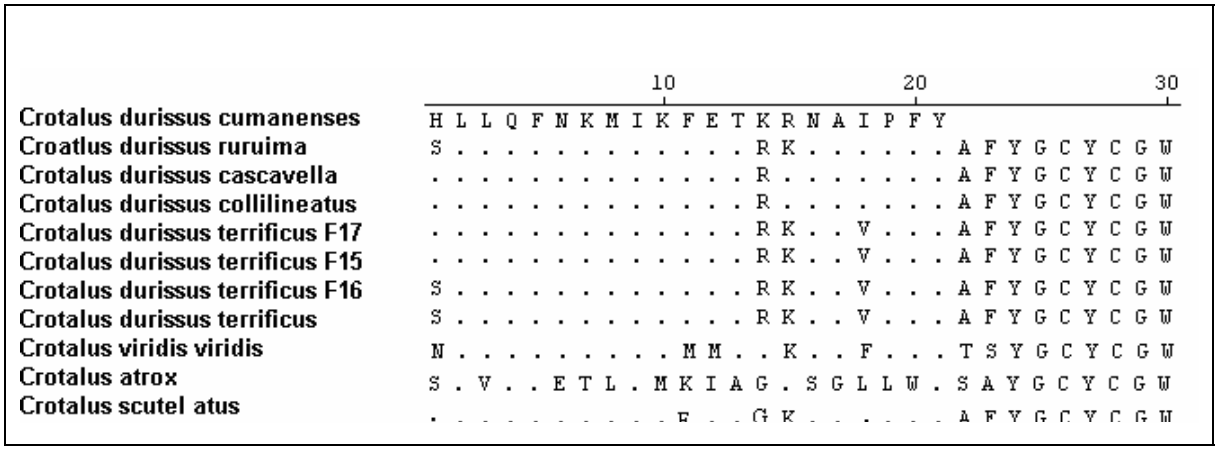

Figura 13. a) Comparação da seqüência N-terminal dos resíduos de aminoácidos de PLA_2 de *C.d.ruruima* e *C.d. cumanenses* com PLA_2 de outras subespécies crotálicas. Os pontos indicam similariedade.

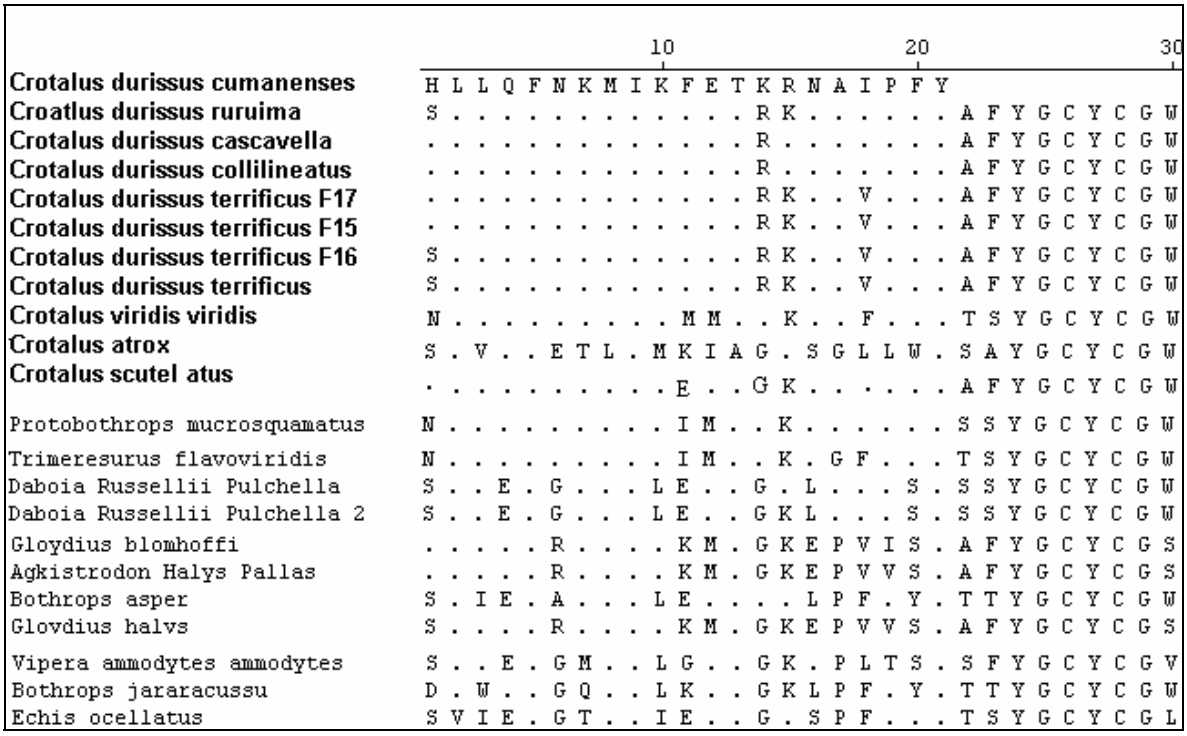

Figura 13. b) Comparação da seqüência N-terminal dos resíduos de aminoácidos de PLA_2 de *C.d.ruruima* e *C.d. cumanenses* com PLA_2 de outros gêneros de serpentes. Diferenças visíveis nas posições de 1 a 23.

5.8 Dicroísmo Circular

Observa-se na PLA_2 do veneno de *Crotalus durissus cumanenses* a presença de grande concentração de α-hélice, seguida pela presença de folhas beta e uma significativa presença de "random coils". Porém a incorporação de p-bromofenacil e cumarina à estrutura fosfolipásica ocasionou uma modificação estrutural. (**Figura 14**)

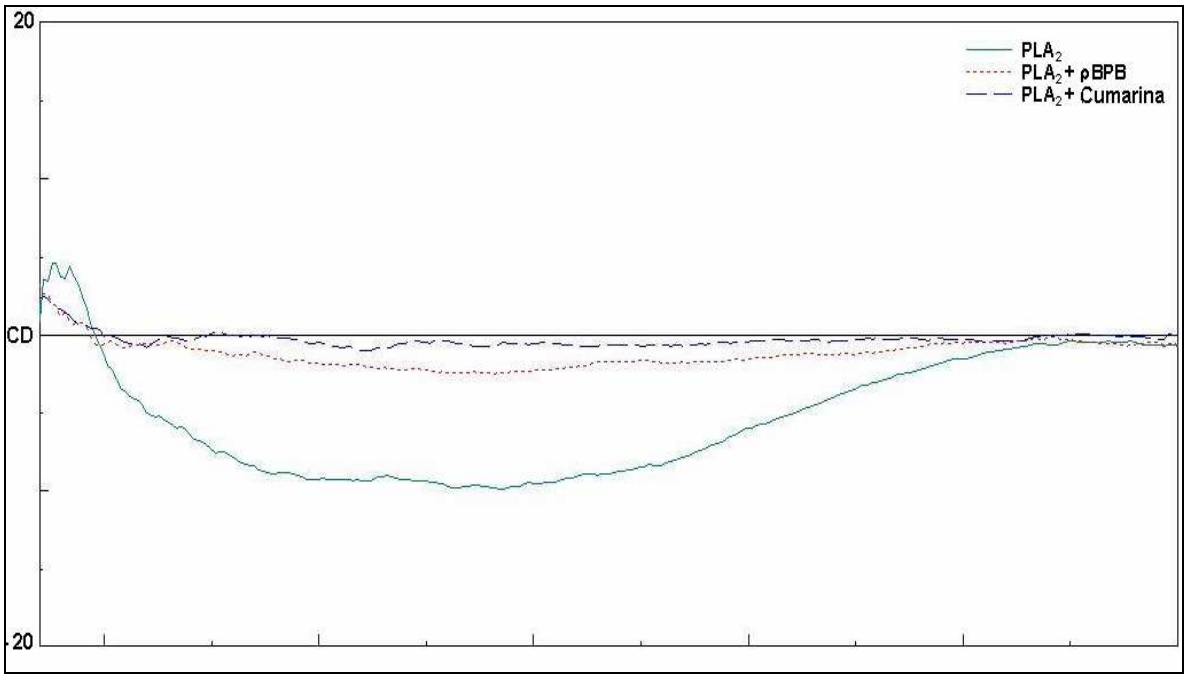

Figura 14. Perfil espectral - Dicroísmo Circular de PLA_2 nativa e PLA_2 tratada previamente com p-bromofenacil e cumarina monitorados em intervalo de 3 nm entre os 180 e 250 nm.

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5.9 Agregação plaquetária

No plasma rico em plaquetas (PRP), a PLA_2 de *Crotalus durissus ruruima* e *Crotalus durissus cumanenses* induziram uma forte e evidente agregação plaquetária para doses de 15 μg; enquanto as amostras de PLA_2 previamente incubadas com cumarina e ρ-BPB evidenciaram a redução no percentual de agregação plaquetária (**Figura 15 a e b**).

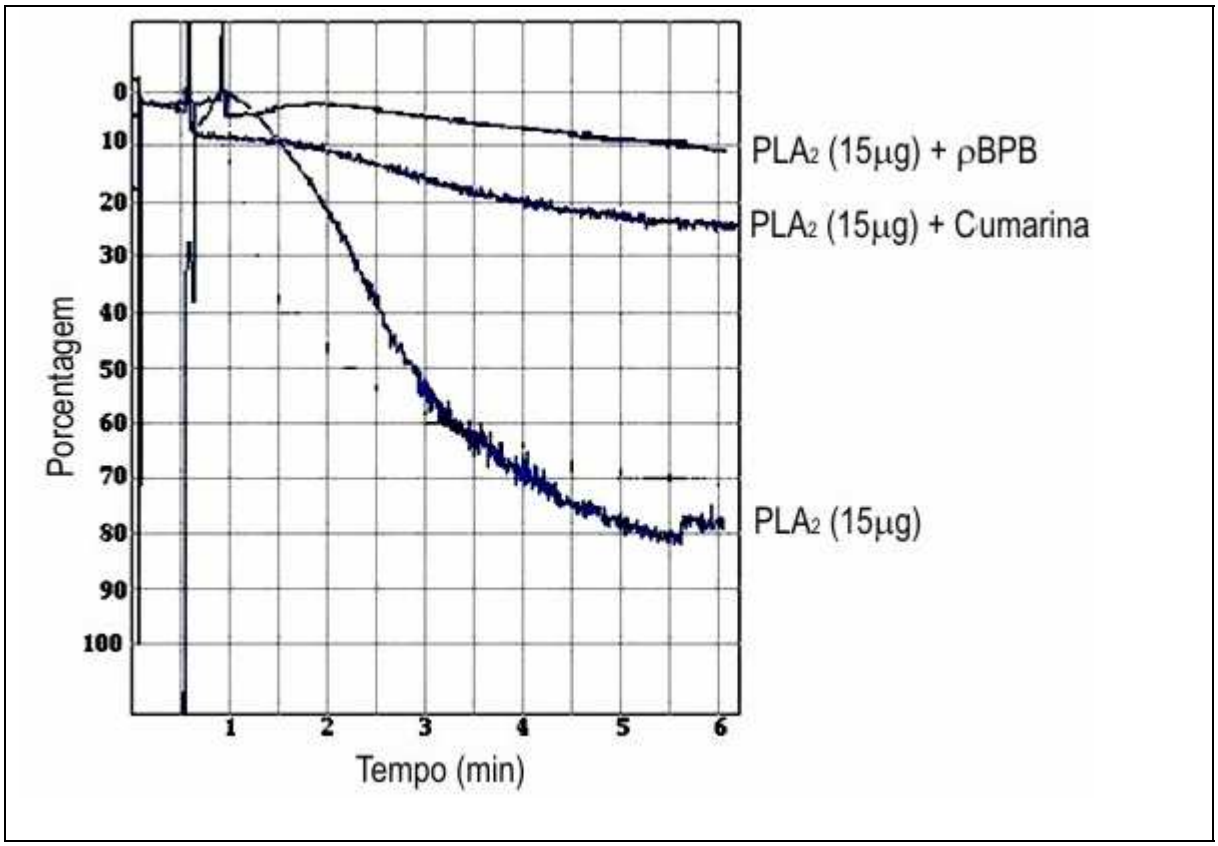

Figura 15. a) Percentual de agregação plaquetária para amostras de PLA_2 nativa (79%) e PLA_2 previamente incubadas com cumarina (22%) e ρ-BPB (8%) de *Crotalus durissus ruruima* monitorada por 6 minutos.

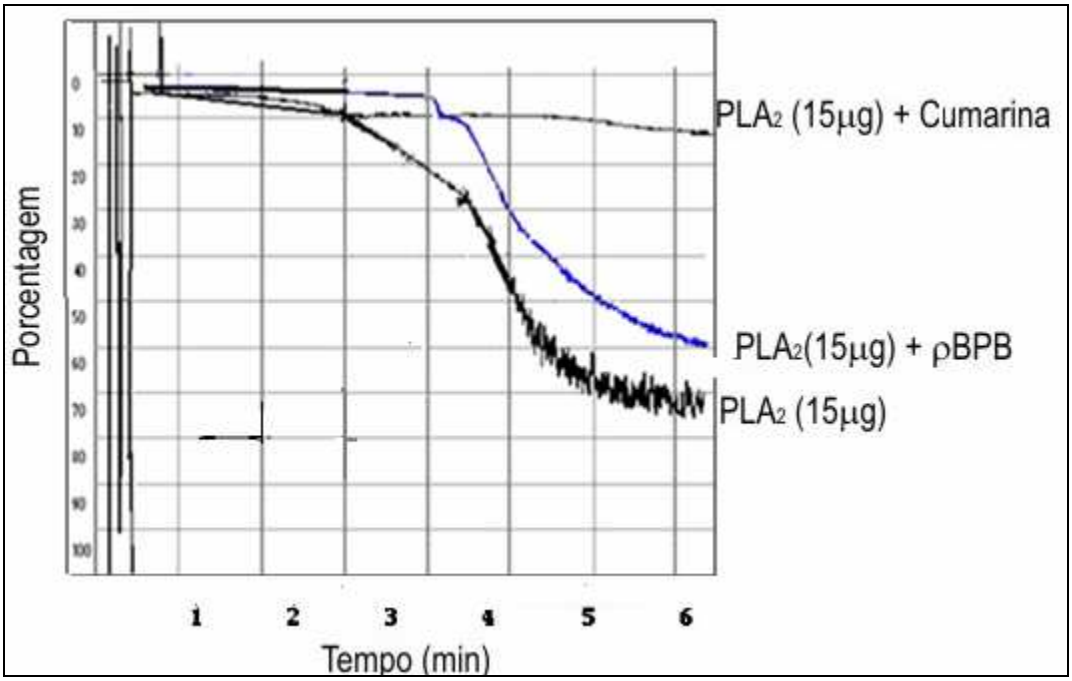

Figura 15. b) Percentual de agregação plaquetária para amostras de PLA_2 nativa (72%) e PLA_2 previamente incubadas com cumarina (8%) e ρ-BPB (54%) de *Crotalus durissus cumanenses* monitorada por 6 minutos.

Sob condições experimentais similares, a PLA_2 induziu a agregação plaquetária dose-dependente a diferentes concentrações (1 µg, 3 µg, 5 µg, 10 µg, 15 µg e 30 µg) (**Figura 16**). A mínima dose que PLA_2 induziu a agregação plaquetária foi estimada em 3 µg.

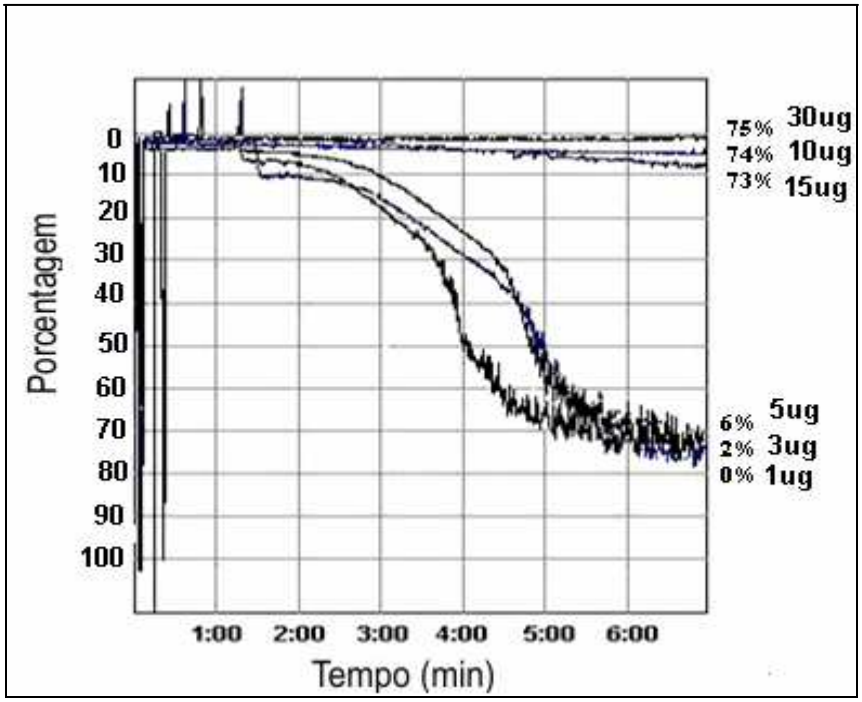

Figura 16. Curva de dose-resposta da fração PLA_2 purificada da fração Crotoxina do veneno total de *C. d. cumanenses* na agregação plaquetária usando sistema de plasma rico em plaquetas (PRP)

4.10 Edema em pata de rato

Ratos machos Wistar machos (120-150g) foram anestesiados com halotano (inalação) e em seguida o efeito edematogênico foi induzido por injeção subplantar de 100µl da amostra na concentração de 5µg/pata. O volume da pata foi mensurado imediatamente após a aplicação a intervalos regulares (15, 30, 60, 90 e 120 minutos) utilizando hidropletismômetro (modelo 7150, Ugo Basile, Italy).

A PLA_2 do veneno de *Crotalus durissus ruruima* e de *Crotalus durissus cumanenses* causou grande edema, e na mesma condição experimental a PLA_2 incubada com cumarina e ρ-BPB exibiu valores edematogênicos menores quando comparados com a PLA_2 nativa (**Figura 17 a e b**).

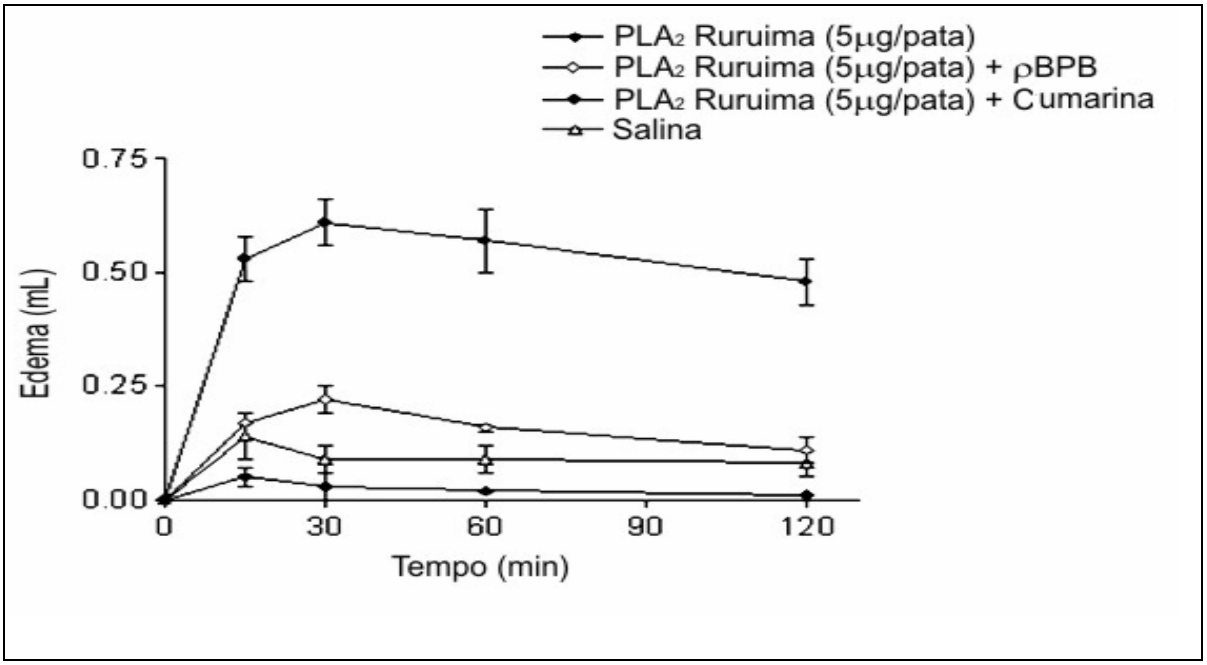

Figura 17. a) Edema em pata de rato – *Crotalus durissus ruruima*

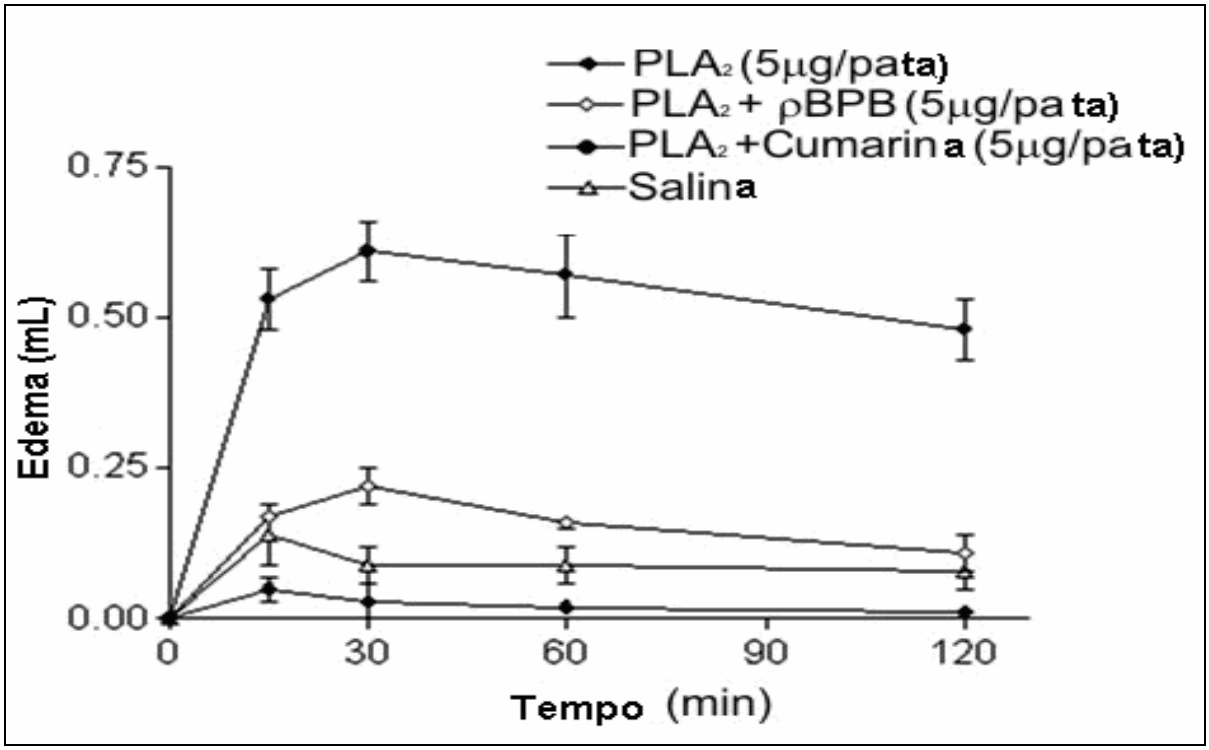

Figura 17. b) Edema em pata de rato – *Crotalus durissus cumanenses*

Sob condições experimentais similares, a PLA_2 de *Crotalus durissus cumanenses* induziu edema de pata em diferentes concentrações (1 µg, 3 µg, 10 µg e 30 µg), sendo o valor máximo de efeito edematogênico verificado no intervalo de tempo de 30 min. (**Figura 18**).

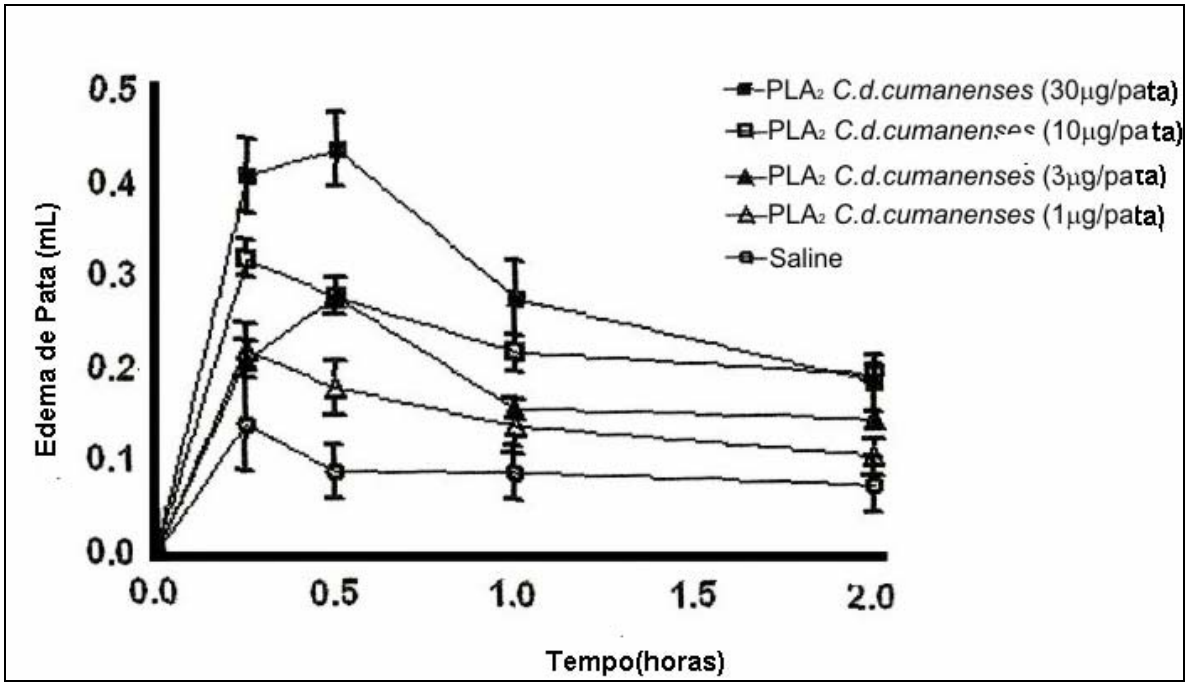

Figura 18. Curva dose-resposta para o efeito edematogênico da fração PLA_2 do veneno total de *C. d. cumanenses*.

A área sobre a curva foi calculada a fim de determinar a significância dos resultados. Como podemos observar na Figura 19, neste experimento de edema em pata de rato, a PLA_2 de *Crotalus durissus cumanenses* causou uma atividade edematogênica menor para dose 1µg/pata. (**Figura 19**).

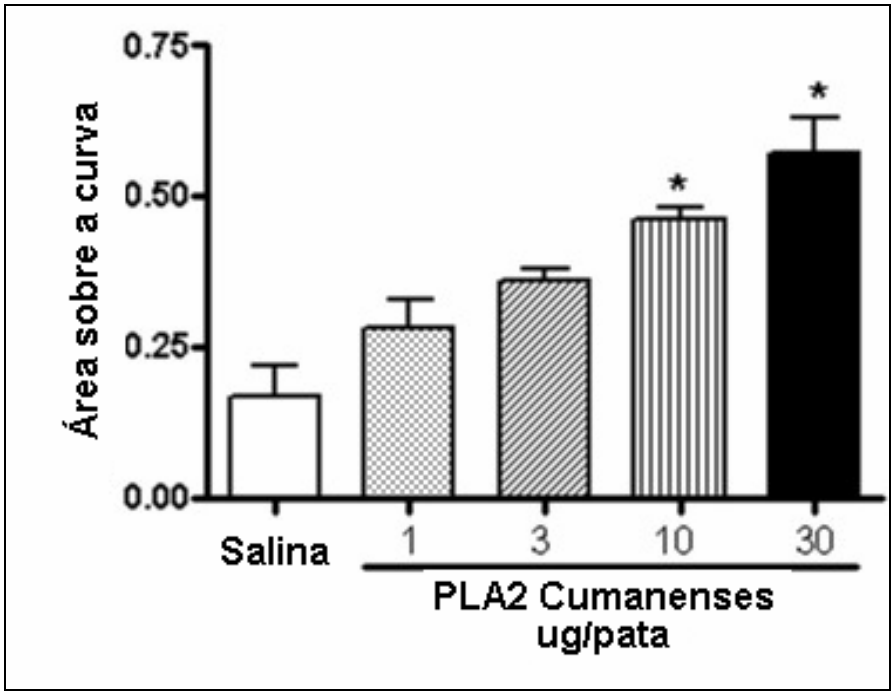

Figura 19. Gráfico da área sobre a curva do experimento de edema em pata de rato baseado na curva dose-resposta para a atividade edematogênica. Ocorreu aumento da área sobre a curva de acordo com o aumento da dosagem inoculada na pata de rato. ($n=5$, $p<0,05$)

5.11 Ensaio antibacteriano

As bactérias *Xanthomonas axonopodis pv. passiflorae* (Gram-negativas) e *Clavilobacter michiganensis michiganensis* (Gram-positivas) foram colhidas em placas de ágar e suspenso em água destilada esterilizada (A650 nm = 0.3/cc; $1x10^3$ UFC / ml). Alíquotas da suspensão bacteriana foram diluídas até 10-5 UFC/mL e incubadas com $sPLA_2$ nativas ou $sPLA_2$ + EOCC (125 µg/ mL) por 1 h a 37 C. Após a incubação, a sobrevivência foi avaliada em nutriente (Difco) placas (n = 5), como descrito por Oliveira et al. (2002). (**Figura 20**).

O ensaio da atividade antibacteriana de sPLA2 contra *Xanthomonas axonopodis pv passiflorae* e *Clavibacter michiganensis michiganensis* corroborou o efeito de EOCC sobre a atividade enzimática da sPLA2 de *Crotalus durissus ruruima*. Este efeito foi particularmente evidente para a atividade da sPLA2 contra *Clavibacter michiganensis michiganensis*. Uma vez que a atividade antibacteriana de alguns componentes do veneno de serpente, como sPLA2 ou LAAO, é parcialmente ou completamente dependente de sua atividade enzimática, é possível que a inibição ou a diminuição na sua atividade enzimática inibe sua atividade antibacteriana (Braga et AL, 2008).

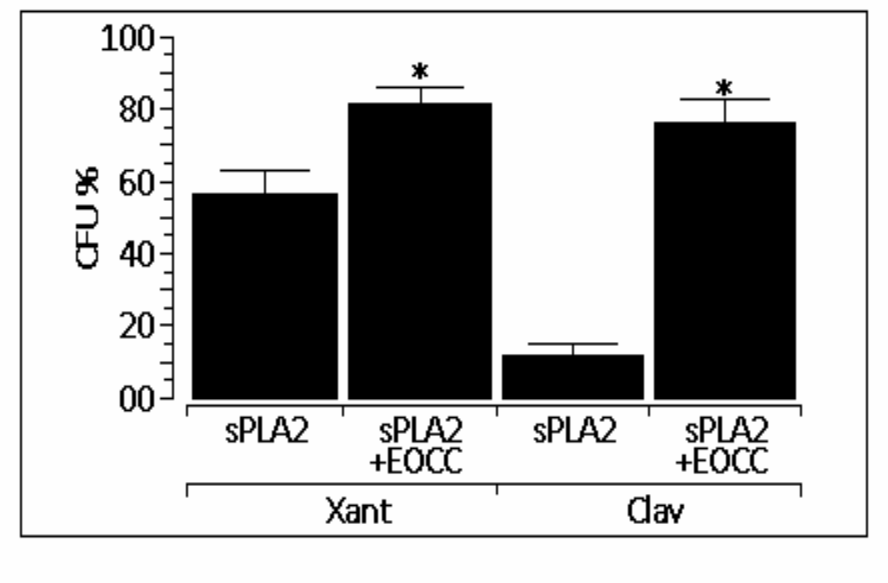

Figura 20. Comparação da atividade antibacteriana de PLA_2 nativa de *Crotalus durissus ruruima* e PLA_2 de *C.d ruruima* tratada com EOCC (cumarina sintética). As suspensões bacterianas que foram Gram negativas (Xant) e Gram positivas (Clav), ($p<0,05$)

5.12 Atividade Miotóxica

A dosagem para creatina quinase (CK) verificada após injeção de $sPLA_2$ nativa foi 2195,45 ±42,34 (n = 4, p<0,005), enquanto $sPLA_2$ tratada com EOCC apresentou apenas aumento dos níveis de CK em torno de 421,34 ±24,56 (n = 4, p<0,05), já o ρ-BPB inibiu fortemente a mionecrose, como mostrado pelo aumento no nível de CK de apenas 383,87 ± 28,23 (n = 4, P <0,05). Foi utilizado como controle negativo o PBS cujos níveis de CK ficaram em torno de 81,84 ±37,70 (n = 4, p<0,05).

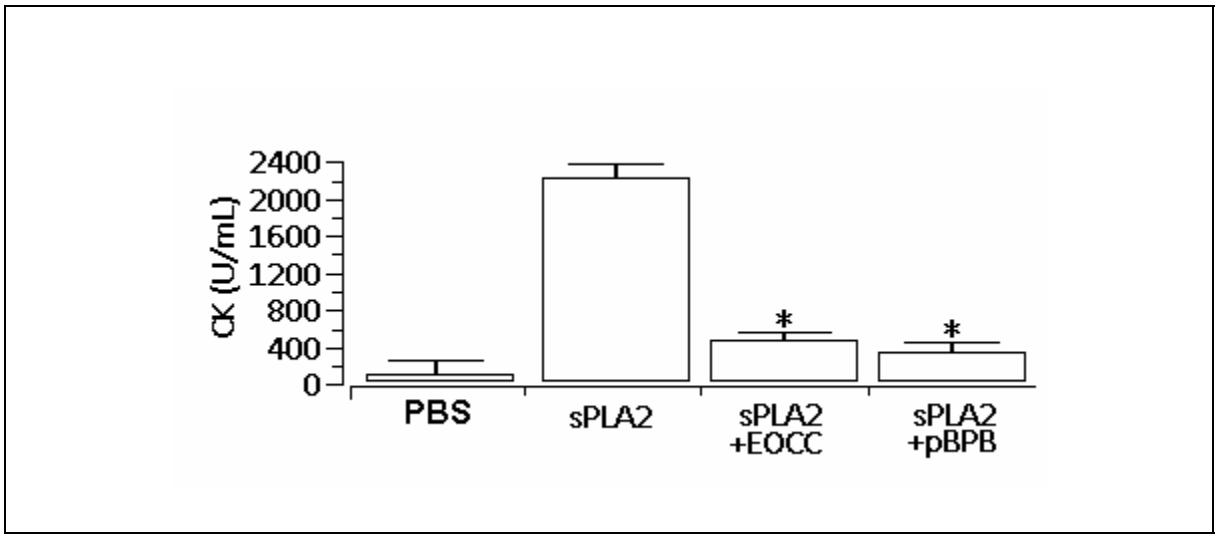

Figura 21. Medida da atividade da creatina quinase no soro de animais tratados com $sPLA_2$ nativa de *Crotalus durissus ruruima* e PLA_2 de *C.d ruruima* tratada com EOCC e *p*-BPB. Os resultados dos níveis de creatina quinase foram expressos em unidades de atividade enzimática por mililitro (U mL^{-1}). PBS foi utilizado como controle negativo. (p<0,05).

6. DISCUSSÃO

As crotoxinas crotálicas são consideradas proteínas heterodiméricas (Breithaupt, 1976), sendo composta por uma subunidade básica com atividade catalítica (PLA_2) e uma subunidade ácida sem atividade catalítica denominada crotapotina. A crotapotina potencializa a toxicidade e inibe a atividade enzimática das fosfolípases A_2 crotálicas (Rubsamen et al, 1971).

As PLA_2 presentes nos venenos de serpentes exibem ampla variedade de efeitos farmacológicos. No estudo da relação estrutura-função, é importante analisar seus efeitos farmacológicos *in vivo*, ao invés de *in vitro*. Os estudos *in vitro* às vezes evidenciam efeitos não específicos devido à atividade enzimática inerente, levando a conclusões errôneas. Só em alguns casos os efeitos farmacológicos podem ser analisados em sistemas *in vitro* como, por exemplo, em cultura celular (Lomonte, et al*.,* 2003).

As PLA_2s provenientes de veneno de serpente tem sido largamente empregadas como ferramentas farmacológicas para investigar seu papel em diversos processos fisiopatológicos. Segundo proposto por Gutierrez e Ownby (2003), as PLA_2 são os componentes miotóxicos mais importantes nos venenos de serpentes, induzindo eventos de degeneração muscular. As PLA_2s miotóxicas ligam-se aos aceptores da membrana plasmática, os quais poderiam se tratar de lipídeos ou proteínas, podendo diferir de sua afinidade pelas PLA_2.

A Fosfolipase A_2 é uma enzima importante no metabolismo celular e sua ação envolve a hidrólise de fosfolipídios específicos dentro de uma complexa rede de vias de sinalização, ligando-agonistas, oxidantes e citocinas pró-inflamatórias para a liberação do

ácido araquidônico e síntese de eicosanóides (Sun et al. 2004). Eicosanóides incluem as prostaglandinas, tromboxanos, prostaciclinas e leucotrienos (Granstrom, 1984), que atuam como mediadores inflamatórios. Além disso, no metabolismo oxidativo do ácido araquidônico e na interrupção da cadeia respiratória mitocondrial, que são mediadas por fosfolipase A_2 (PLA_2) e hidrólise da cardiolipina, podem contribuir para a geração de espécies reativas de oxigênio (ROS) e estresse oxidativo (Adibhatla Hatcher et al., 2006). Os efeitos farmacológicos da $sPLA_2$ do veneno das serpentes pode agir por meio da mobilização do ácido araquidônico ou por interação com um receptor específico, em ambos os casos, $sPLA_2$ provocaria a ativação de vias de sinalização da rede e liberação de ácido araquidônico e promover a síntese de eicosanóides (Valentin e Lambeau , 2000; Fuentes et al., 2002; Oliveira et al., 2008, Toyama et al., 2009).

Variados efeitos farmacológicos desencadeados pelas PLA_2 podem ser explicados também pela presença de receptores alvos específicos, que estão localizados na superfície das células ou do tecido alvo. Estes receptores alvos seriam reconhecidos por sítios farmacológicos localizados na superfície das PLA_2 que de forma geral são independentes, mas algumas vezes estes se sobrepõem com o catalítico. (Kini & Evans, 1989). De acordo com esta hipótese haveria uma necessidade de complementaridade entre o(s) receptor (es) farmacológico(s) e possíveis receptores das células alvos em termos de equilíbrio de cargas, interação hidrofóbica e interações de Van der Walls (Kini, 2003).

Relações estrutura-função de vários homólogos de PLA_2 miotóxica de veneno de serpente têm sido amplamente investigadas (Soares & Giglio, 2003). Visando a elucidação dos determinantes estruturais da toxicidade, várias metodologias têm sido exploradas, a saber: modificação química dos resíduos de aminoácidos específicos, mutagênese sítio-

dirigida, cristalografia, espectrofotometria e técnicas fluorimétricas, bem como ressonância magnética nuclear e os estudos sobre a ligação dos inibidores e substratos naturais ou artificiais. A tradicional modificação química específica de aminoácidos e os conseqüentes efeitos sobre as atividades enzimáticas e farmacológicas da PLA_2 miotóxica tem sido uma das principais estratégias utilizadas para explorar as relações estrutura-função.

A PLA_2 de veneno de serpente tem um sítio ativo localizado perto da His-48 e a enzima pode ser inativada por modificação química do resíduo com ρBPB. Alquilação de His-48 com ρBPB tem sido amplamente utilizado para avaliar o papel da atividade enzimática na ação farmacológica da PLA_2 do veneno de serpente. O conhecimento da mudança conformacional induzida pelo ρBPB pode ser útil para compreender os resultados experimentais (Zhao et al, 1980).

Foi demonstrado segundo a literatura que a modificação na PLA_2 de bovinos por ρBPB levou a mudanças conformacionais significativas em algumas partes da molécula da enzima. Conseguimos constatar que este inibidor induziu alterações na conformação de PLA_2 do veneno de serpentes.

Benzopiranos e seus principais metabólitos (cromenos) estão entre os mais importantes metabólitos secundários e são importantes para a produção de flavonóides, cumarinas e outros compostos polifenólicos encontrados na natureza. Novos derivados benzopiranos como dihidroquinolina, benzotiopiran e dihydronapthalene têm mostrado efeito anti-inflamatório promissor e estes compostos parecem preferencialmente e seletivamente inibir a ciclooxigenase-2 a ciclooxigenase-1 (Watzl, 2008). Entre os benzopiranos mais comuns estão os compostos derivados de flavonóides, que foram caracterizados como inibidores de várias enzimas, incluindo oxidase, xantina redutase, fosfodiesterase, Ca (+2)-ATPase, lipoxigenase, ciclooxigenase, etc (Rathee et al., 2009) .

As atividades destes compostos envolvem antioxidantes de alguns resíduos de aminoácidos hidrofóbicos e formação da ligação de hidrogênio ou reações químicas com alguns aminoácidos (Rathee et al., 2009).

Neste estudo, observou-se que EOCC diminuiu fortemente a atividade enzimática da $sPLA_2$, semelhante ao efeito induzido pelo *p*-BPB (p- bromofenacil brometo). Além disso, a espectrometria de massa sugeriu que EOCC formou um complexo estável com $sPLA_2$ em uma relação estequiométrica de 2 moléculas de EOCC para 1 molécula de $sPLA_2$. Assim, o modo de ação de EOCC sobre sPLA2 é semelhante ao de umbelliferone (Toyama et al., 2009). A mudança no tempo de retenção de sPLA2 antes e após incubação com EOCC também indica uma forte interação entre essas moléculas. O mecanismo de inibição da cumarina envolve o ataque nucleofílico de alguns resíduos de aminoácidos, bem como a presença de grande quantidade de quimiotripsina no bolsão hidrofóbico. Esses recursos têm sido caracterizados como necessários para a adequada interação deste composto com proteínas (Ghani et al. 2001, e Hyun et al., 2004), o bloqueio da estrutura benzopirano cumarina, assim como mudanças estruturais significativas da estrutura de proteínas observadas após a reação química entre certos resíduos de aminoácidos e 7-hidroxicumarina (7-HOC). É possível que EOCC use um mecanismo de ação semelhante. No caso de EOCC, observou-se que duas moléculas reagem com o inibidor da bolsa hidrofóbica desta $sPLA_2$, semelhante ao que tem sido observado por outras $sPLA_2$ (Toyama et al., 2009).

A perda da integridade da bolsa hidrofóbica induziu uma perda irreversível da atividade enzimática, conseqüentemente, todas as atividades biológicas que envolvem a atividade catalítica da $sPLA_2$ foram praticamente abolidas. Uma vez que a estabilidade da alfa hélice na cavidade hidrofóbica deve ter sido afetada pela estabilidade das estruturas,

tais como o cálcio obrigatório no loop ou folhas beta, é possível que os efeitos farmacológicos induzidos pela interação de $sPLA_2$ com o receptor devem ser igualmente afetados, como observado por Valentin e Lambeau (2000), Oliveira et al. (2008) e Toyama et al. (2009). Esta hipótese foi fortemente apoiada pelos resultados de Iglesias et al. (2005) e Toyama et al. (2009) para sPLA2 modificado por compostos polifenólicos, assim como outros (Harper & Power, 1985 e Liu et al., 2008).

Aqui mostramos que a estrutura da PLA_2 covalentemente modificada pela ρBPB e por cumarina quando comparada com a estrutura nativa apresentou tanto para agregação plaquetária quanto para atividade fosfolipásica e edema de pata uma redução na indução destes efeitos biológicos.

7. CONCLUSÕES

- A purificação total do veneno de *Crotalus durissus ruruima* e *Crotalus durissus cumanenses* objetivando a obtenção de PLA2 é baseado em 2 passos cromatográficos. A utilização de apenas um método de purificação não é capaz de isolar o complexo crotoxínico.
- A comparação da purificação do veneno de *C.d.ruruima* e *C.d cumanenses* revelou que ambos apresentaram isoformas de crotapotina e PLA2, porém em diferentes quantidades.
- A análise da espectrometria de massa da crotoxina demonstra através da presença de mais de uma banda a natureza instável do complexo crotoxínico.
- A comparação das seqüências n-terminal das PLA_2s de *Crotalus durissus ruruima* a aproximaram de *C.d terrificus* enquanto que a PLA_2 de *Crotalus durissus cumanenses* a aproximaram de *C.d cascavella* e *C.d collilineatus*.
- A análise dos resultados de dicroísmo circular para a PLA_2 de *C.d cumanenses* apresentou alta concentração de estruturas rígidas, folhas β e α-hélices, sugerindo que a conservação destas estruturas mantém sua atividade fosfolipásica.
- A modificação química induzida nas PLA_2s nativas confirmaram que as regiões tanto N-terminal como C-terminal destas isoformas crotálicas em estudo é crucial para sua toxicidade letal.

- EOCC apresentou uma oportunidade para melhorar a aplicação terapêutica de 2H-1-Benzopiranos (2H-cromenos) como nova droga anti-trombótica ou anti-inflamatória específica para combater a ação fosfolipásica de diferentes fontes.

8. APÊNDICES

8.1 Publicações

Autora

- Fonseca, F.V; Baldissera Jr, L; Camargo, E.A; Antunes, E; Diz-Filho, E.B.S; Corrêa, A.G; Beriam, L.O.S and Toyama, M.H. Effect of the synthetic coumarin, ethyl 2-oxo-2H-chromene-3-carboxylate, on activity of Crotalus durissus ruruima sPLA2 as well as on edema and platelet aggregation induced by this factor. Toxicon 2010 Mar 15. [Epub ahead of print]

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Toxicon

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Journal Manager

Toxicon

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Dear Dr Fonseca

MS 09-510

I am pleased to tell you that your paper has been accepted for publicationn in Toxicon. It has been sent to the publishers and you will receive proofs in due course.

Yours sincerely

Alan Harvey

Effect of the synthetic coumarin, ethyl 2-oxo-2H-chromene-3-carboxylate, on activity of *Crotalus durissus ruruima* sPLA2 as well as on edema and platelet aggregation induced by this factor

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Abstract

We show that ethyl 2-oxo-2H-chromene-3-carboxylate (EOCC), a synthetic coumarin, irreversibly inhibits phospholipase A_2 (sPLA2) from *Crotalus durissus ruruima* venom (sPLA2r) with an IC_{50} of 3.1 ± 0.06 nmol. EOCC strongly decreased the Vmax and Km, and it virtually abolished the enzyme activity of sPLA2r as well as sPLA2s from other sources. The edema induced by sPLA2r+EOCC was less than that induced by sPLA2r treated with *p*-bromophenacyl bromide, which was more efficient at neutralizing the platelet aggregation activity of native sPLA2r. Native sPLA2r induced platelet aggregation of 91.54 ± 9.3%, and sPLA2r+EOCC induced a platelet aggregation of 18.56 ± 6.5%. EOCC treatment also decreased the myotoxic effect of sPLA2r. Mass spectrometry showed that EOCC formed a stable complex with sPLA2r, which increased the mass of native sPLA2r from 14,299.34 Da to 14,736.22 Da. Moreover, the formation of this complex appeared to be involved in the loss of sPLA2r activity. Our results strongly suggest that EOCC can be used as a pharmacological agent against the sPLA2 in *Crotalus durissus sp*. venom as well as other sPLA2s.

Keywords: Crotalus durissus ruruima, edema, myonecrosis, platelet, sPLA2, synthetic coumarin

[1]2H-1-benzopyrans (2H-chromenes) are important intermediates in the synthesis of many natural products and medicinal agents (Ashwood et al., 1986; Kim et al., 2002) including flavonoids, coumarin, and derivatives of these compounds. 2-H chromenes have been used experimentally as anti-inflammatory and anti-thrombotic therapeutics. Related to their anti-inflammatory activity, synthetic coumarins are known to inhibit arachidonic acid metabolism. *Crotalus durissus sp.* venom has several pharmacological effects that appear dependent, at least in part, on the enzymatic activity of secretory phospholipase A_2 (sPLA2). The aim of this work was to evaluate the effect of ethyl 2-oxo-2H-chromene-3-carboxylate (EOCC), a synthetic coumarin derivative, on the enzymatic activity of sPLA2 from *Crotalus durissus ruruima* venom (sPLA2r) as well as on sPLA2r-induced edema, myotoxicity, and platelet aggregation.

Secretory PLA2r was fractionated in two steps as described by Diz Filho et al. (2009). Whole venom (45 mg) was first fractionated by size exclusion HPLC (Superdex 75, 1 × 60 cm, GE Healthcare), and the crotoxin fraction was purified by monitoring phospholipase A_2 (PLA2) activity (Fig. 1a). Crotoxin was then subjected to reverse-phase HPLC, which led to the identification of one main sPLA2 isoform (Fig. 1b). The molecular mass of purified sPLA2r was 14,299.34 Da, measured as described by Toyama et al. (2005).

Purified sPLA2r was chemically modified with EOCC using the procedure described by Iglesias et al. (2005). EOCC (100 nmol in 10 µL dimethyl sulfoxide) was incubated with sPLA2r (100 nmol in 1000 µL water) for 60 min at 37°C. The products

[1] *Abbreviations:* CK, creatine kinase; EOCC, ethyl 2-oxo-2H-chromene-3-carboxylate; 7-HOC, 7-hydroxycoumarin; pBPB, *p*-bromophenacyl bromide; PLA2, phospholipase A_2; sPLA2, secretory phosholipase A_2; sPLA2r, secretory phospholipase A_2 from *Crotalus durissus ruruima*.

were fractionated by analytical reverse-phase HPLC (C5 large pore column, Supelco). The resulting sPLA2r had a molecular mass of 14,736.22 Da. This, taken with the 14,299.34 Da mass of native sPLA2r, suggests that two molecules of EOCC (218.21 Da) were complexed to sPLA2r.

Amino acid analysis of native or treated sPLA2r samples was performed using the PICO-TAG system (Waters). Samples were hydrolyzed with 6N HCl in the presence of 1% of phenol over 24 h and derivatized with PITC. PTC-amino acids were then analyzed by reverse-phase HPLC. The global amino acid analysis revealed no significant differences between native sPLA2r and EOCC-treated sPLA2r (sPLA2+EOCC).

PLA2 activity was measured using a chromogenic substrate (4-nitro-3-octanoyloxy-benzoic acid, BIOMOL, USA) as described by Lima et al. (2008). The enzymatic activity, expressed as the initial velocity of the reaction (Vo), was calculated based on the increase in absorbance at 425 nm after 20 min. Absorbance was measured using a Spectramax 340 multiwell plate reader (Molecular Devices, Sunnyvale, CA, USA). Native sPLA2r had a Vo of 2.51 ± 0.34 nmol/min (n = 12). The chemical modification of sPLA2r with pBPB was done as described by de Casto et al. (2000). The Vo decreased to 0.48 ± 0.13 nmol/min ($n = 12$, $p < 0.05$) in the presence of the sPLA2 inhibitor, *p*-bromophenacyl bromide (pBPB). Similar to pBPB, EOCC decreased the Vo of sPLA2r to 0.38 ± 0.08 nmol/min ($n = 12$, $p < 0.05$). These results strongly suggest that EOCC and pBPB have comparable inhibitory potency against sPLA2r. To compare the half maximal inhibitory concentration (IC_{50}) of EOCC and pBPB, we used a substrate concentration of 10 mM and incubated sPLA2r with increasing amounts of each inhibitor (0 to 14 nmol in 10 μL) for 60 min prior to enzymatic evaluation. EOCC inhibited enzymatic activity in a dose-dependent manner, with maximal inhibition occurring in the presence of 8 nmol and no significant further

inhibitory effect being seen with doses of 10 to 14 nmol (Fig. 2a). The inhibitory effect of EOCC was significantly higher than that observed for pBPB at lower doses (2 to 8 nmol), but both showed similar inhibitory effects above 10 nmol. Next, we evaluated the effect of the substrate concentration on sPLA2r activity following the protocol described by Toyama et al. (2003). At different substrate concentrations, sPLA2r exhibited moderate allosteric behaviour. The addition of EOCC or pBPB to the enzyme strongly and irreversibly decreased the Vmax and Km (Fig. 2b). The enzymatic assay was performed as already described and in all cases, 1 mg/mL sPLA2 solution was incubated with 10 nmol EOCC for 30 min before the enzymatic assay. A comparison EOCC-induced inhibition of sPLA2r and sPLA2 from other sources produced the following results (listed without and with EOCC): 7.83 ± 0.56 and 0.98 ± 0.32 nmol/min for bovine sPLA2; 6.53 ± 0.28 and 2.32 ± 0.17 nmol/min for honey bee sPLA2; 11.43 ± 0.47 and 1.83 ± 0.53 nmol/min for *Naja mossambica mossambica* sPLA2 (Sigma-Aldrich, V1627); 8.78 ± 0.62 and 0.56 ± 0.12 nmol/min for PrTx-III sPLA2 from *Bothrops pirajai* venom; 10.4 ± 0.31 and 1.83 ± 0.35 nmol/min for sPLA2r (n = 12, Fig. 2c).

Next, we analyzed the effect of EOCC on sPLA2r-induced edema. Male Wistar rats (120 – 150 g) were anaesthetized with inhaled halothane, and hind paw edema was induced by a single subplantar injection of native or modified sPLA2 (10 μg dissolved in sterile 0.9% saline solution per paw). Paw volume was measured using a hydroplethysmometer (model 7150, Ugo Basile, Italy) immediately before the injection and at selected times thereafter (30, 60, 90, 120, 180 and 240 min). Results were expressed as the increase in the paw volume (in mL) from the basal volume. Native sPLA2r induced maximum edema at 30 min after injection (0.63 ± 0.03 mL, n = 4). Incubation of sPLA2r with EOCC virtually

abolished edema formation at this time (0.18 ± 0.05 mL, n = 4, $p < 0.05$). Similar results were seen with pBPB (0.24 ± 0.04 mL, n = 4, $p < 0.05$) (Fig. 3a).

Liberation of creatine kinase (CK) from damaged muscle cells was assessed by measuring CK enzyme activity in the plasma of mice. Four groups of four animals (18 – 22 g) were injected in the right gastrocnemius muscle with 50 μL of 0.5 mg/mL native sPLA2r, sPLA2r -treated EOCC, EOCC alone, or PBS (as a control). Three hours later, tail blood was collected into heparinized tubes for analysis of plasma CK activity (expressed as units per liter at 30°C) using a commercially available kit (47-UV, Sigma Aldrich). After injection of native sPLA2r, CK activity was 2195.45 ± 42.34 U/L (n = 4, $p < 0.05$), whereas EOCC-treated sPLA2r increased CK levels only to 421.34 ± 24.56 (n = 4, $p < 0.05$). Myonecrosis was also strongly inhibited by pBPB, as shown by a comparable increase in CK activity to 383.87 ± 28.23 U/L (n = 4, $p < 0.05$).

Finally, platelet aggregation was measured following the method described by Fonseca et al. (2006) and Oliveira et al. (2008). The washed platelet solution (400 μL) was kept at 37°C with constant stirring, and aggregation was recorded for 5 – 10 min with an aggregometer (Payton Scientific Instruments, Inc, Buffalo, NY). Aggregation experiments were performed using 10 μg sPLA2r or sPLA2r+EOCC in a final volume of 20 μL. Native sPLA2r induced irreversible platelet aggregation (91.54 ± 9.3%, n = 4, $p < 0.05$). On the other hand, sPLA2r+EOCC and sPLAr+pBPB induced platelet aggregation of only 18.56 ± 6.5% (n = 4, $p < 0.05$) and 6.4% ± 3.4 (n = 4, $p < 0.05$), respectively (Fig. 3c).

Our data show that, similar to pBPB, EOCC strongly decreases the enzyme activity of sPLA2r. Moreover, mass spectrometry suggests that EOCC forms a stable complex with sPLA2r. The stoichiometry of two molecules of EOCC per molecule of sPLA2r is similar to that of umbelliferone (Toyama et al., 2009). The shift in the retention time of sPLA2r

after incubation with EOCC also indicates a strong interaction. The inhibitory mechanism of coumarin involves nucleophilic attack of amino acid residues and binding to the large hydrophobic pocket of chymotrypsin. These features were shown to be necessary for the interaction of this compound with proteins (Ghani et al., 2001; Hyun et al., 2004). Blockade of the coumarin benzopyran structure and significant structural changes in the protein have been shown to occur after treatment with 7-hydroxycoumarin (7-HOC). EOCC may work by a similar mechanism of action. Two molecules of EOCC react with the hydrophobic pocket of sPLA2r, similar to what has been observed for 7-HOC (Toyama et al., 2009). The loss of hydrophobic pocket integrity induces an irreversible loss of enzyme activity. Consequently, all biological effects that depend on the catalytic activity of sPLA2 are virtually abolished. Since the stability of the alpha helix in the hydrophobic cavity should be affected by the stability of other structures such as the calcium-binding loop or beta wing, it is possible that pharmacological effects induced by sPLA2 interaction with the receptor are similarly affected, as observed by Valentin and Lambeau (2000), Oliveira et al. (2008), and Toyama et al. (2009). This hypothesis is strongly supported by studies of sPLA2 modified with other polyphenolic compounds (Iglesias et al., 2005; Toyama et al., 2009) as well as by other studies (Harper and Power, 1985; Liu et al., 2008).

In conclusion, EOCC is a useful tool for advancing our knowledge of the structure and function of sPLA2 as well as the pharmacological effects of related plant-derived polyphenolic compounds such as coumarins, flavonoids, terpenoids, and alkaloids.

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Conflict of interest

The authors have no conflict of interest.

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Figure captions

Fig 1. (a) Chromatographic profile of *Crotalus durissus ruruima* whole-venom fractionation by size-exclusion chromatography (Superdex 75, 1 × 60 cm). Clarified, dried venom (45 mg) was dissolved in the mobile phase (0.2 M ammonium bicarbonate buffer, pH 8.0) and separated using a flow rate of 0.2 mL/min. Fractions were eluted by monitoring absorbance at 280 nm. Enzyme activity was monitored in 10 – 20 µl samples from each fraction corresponding to crotacetin, convulxin, giroxin, crotoxin, and crotamine. PLA2 activity was detected in the crotoxin fraction and monitored spectrophotometrically at 425 nm. (b) Fractionation of crotoxin from *Crotalus durissus ruruima* by reverse-phase HPLC (C18 µ-Bondapack) using a non-linear gradient of acetonitrile (66% in 0.1% of TFA) and monitoring at A_{214nm}. The purity of the resulting fractions, termed sPLA2ru1 and sPLA2ru2, was evaluated by tricine SDS-PAGE and mass spectrometry on a MALDI-TOF instrument. The main sPLA2 fraction used in this work was designated sPLA2r.

Fig 2. (a) The effect of increasing concentrations of EOCC or pBPB (0 – 14 nmol/L) on the activity of sPLA2r (10 nmol/mL). Each compound was incubated with sPLA2r for 60 min, and sPLA2r was then added to the reaction well. sPLA2 activity was measured using 4-nitro-3-octanoyloxy-benzoic acid as a substrate. Enzyme activity, expressed as the initial velocity of the reaction (Vo), was calculated based on the increase in absorbance at 425 nm after 20 min. (b) The effect of varying substrate concentrations (0 – 16 mM) on the enzymatic activity of sPLA2r, which exhibited discrete allosteric behaviour. Using the same conditions, we also evaluated the effect of 10 nmol pBPB or EOCC on sPLA2r kinetics. (c) Comparison of the inhibitory effect of EOCC against sPLA2 from other sources. In all assays, 10 nmol EOCC was incubated with the sPLA2 solution (1 mg/mL)

for 30 min before the enzymatic assay. Each point represents mean ± SEM (n = 12). *p < 0.05.

Fig 3. (a) Effect of EOCC on sPLA2r-induced edema in the rat paw. Paw volume was measured prior to injection of the drugs and at selected times thereafter using a hydroplethysmometer. All drugs were dissolved in sterile 0.9% saline solution. Results were expressed as the increase in paw volume (mL) calculated by subtracting the basal volume. Maximal edema was observed at 30 min (approximately 0.6 mL for native aPLA2r), with edema being decreased by pBPB or EOCC co-incubation. Each point represents the mean ± SEM (n = 4). *p < 0.05. (b) Comparison of platelet aggregation induction in platelet-rich plasma (PRP). Aggregation experiments were performed with 10 μg sPLA2r or sPLA2r+EOCC in a final volume of 20 μL, and aggregation was recorded for 5 – 10 min with an aggregometer. Native sPLA2r induced 91.54 ± 9.3% platelet aggregation, while co-incubation of sPLA2r with pBPB or EOCC reduced the aggregation to 6.4 ± 3.4% and 18.56 ± 6.5%, respectively.

Figure 1:

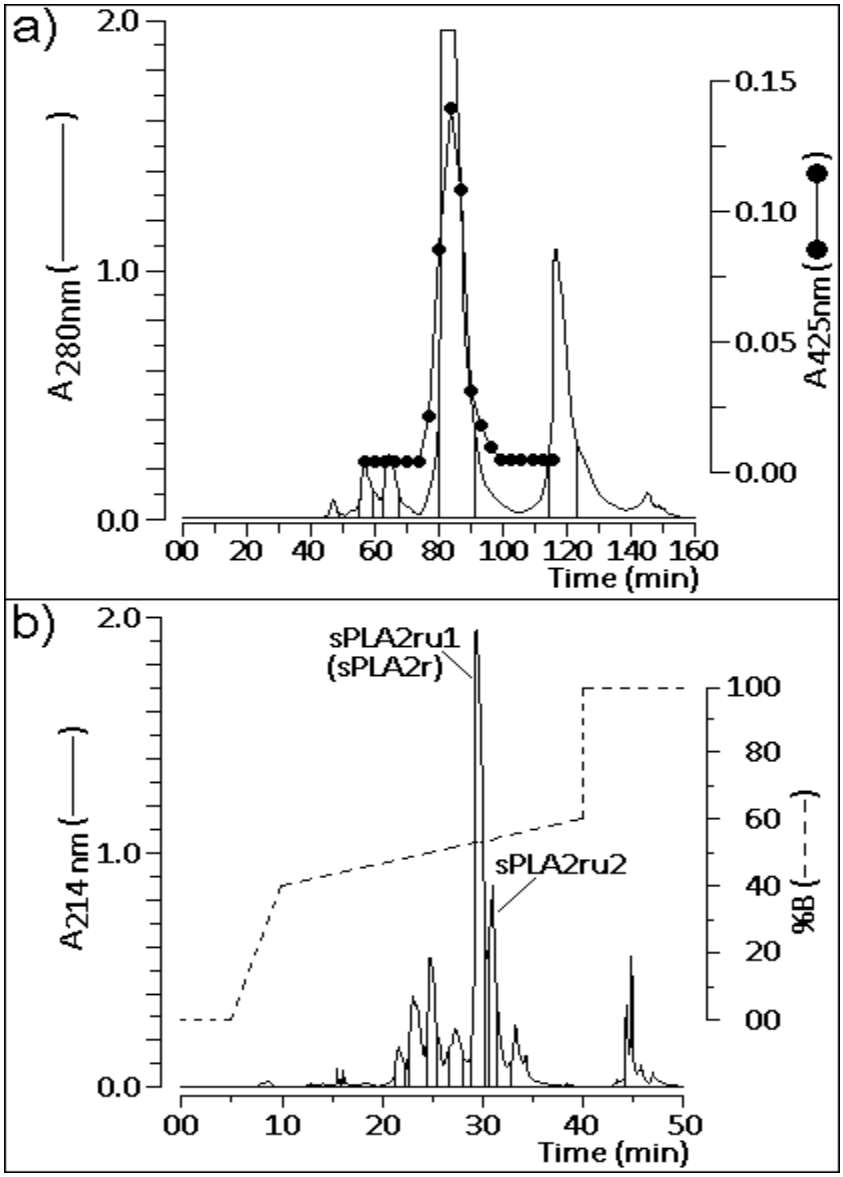

a)
2.0
1.0
0.0
A280nm (——)
0.15
0.10
0.05
0.00
A425nm (●—●)
00 20 40 60 80 100 120 140 160
Time (min)
b)
2.0
1.0
0.0
A214 nm (——)
sPLA2ru1
(sPLA2r)
sPLA2ru2
100
80
60
40
20
00
%B (- - - -)
00 10 20 30 40 50
Time (min)

Figure 2:

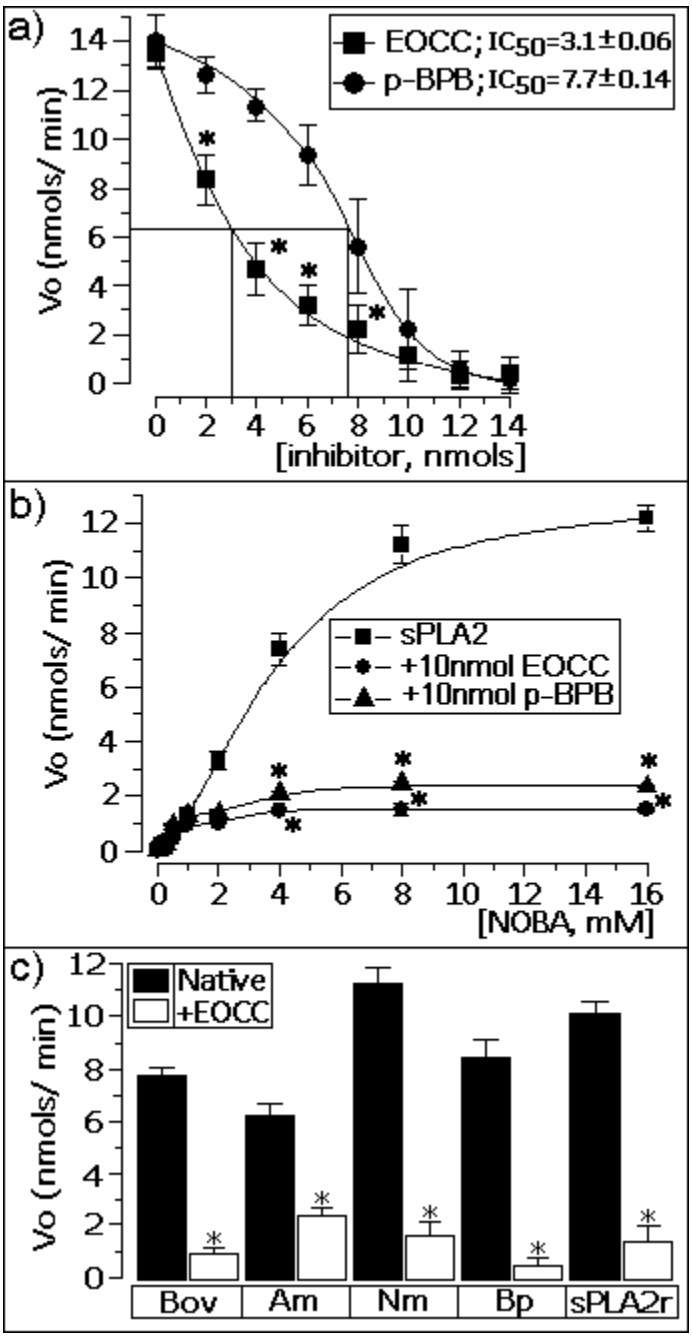
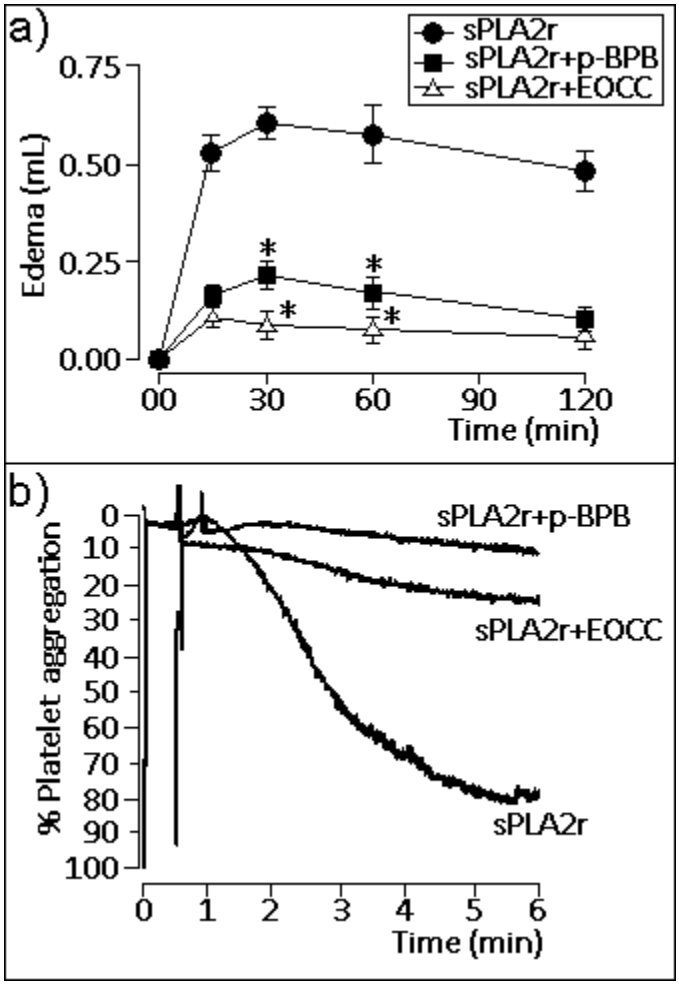

a)
EOCC; IC50=3.1±0.06
p-BPB; IC50=7.7±0.14
Vo (nmols/ min)
[inhibitor, nmols]
b)
sPLA2
+10nmol EOCC
+10nmol p-BPB
Vo (nmols/ min)
[NOBA, mM]
c)
Native
+EOCC
Vo (nmols/ min)
Bov
Am
Nm
Bp
sPLA2r

Figure 3:


a)
sPLA2r
sPLA2r+p-BPB
sPLA2r+EOCC
Edema (mL)
0.75
0.50
0.25
0.00
00
30
60
90
120
Time (min)
*
b)
% Platelet aggregation
0
10
20
30
40
50
60
70
80
90
100
sPLA2r+p-BPB
sPLA2r+EOCC
sPLA2r
0
1
2
3
4
5
6
Time (min)

Co-autora

- Simone CB Oliveira, Fabiana V Fonseca, Edson Antunes, Enilton A Camargo, Rafael P Morganti. Ricardo Aparício, Daniela O Toyama, Luís OS Beriam, Eudismar V Nunes, Benildo S Cavada, Celso S Nagano, Alexandre H Sampaio, Kyria S Nascimento and Marcos H Toyama. Modulation of the pharmacological of enzymatically-active PLA_2, an isolectin isolated from the *Bryothamnion triquetrum* red alga. *BMC Biochemistry* (Online), v. 9, p. 16, 2008.

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Modulation of the pharmacological effects of enzymatically-active PLA_2 by BTL-2, an isolectin isolated from the *Bryothamnion triquetrum* red alga

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Abstract

Background: An interaction between lectins from marine algae and PLA_2 from rattlesnake was suggested some years ago. We, herein, studied the effects elicited by a small isolectin (BTL-2), isolated from *Bryothamnion triquetrum*, on the pharmacological and biological activities of a PLA_2 isolated from rattlesnake venom (*Crotalus durissus cascavella*), to better understand the enzymatic and pharmacological mechanisms of the PLA_2 and its complex.

Results: This PLA_2 consisted of 122 amino acids (approximate molecular mass of 14 kDa), its pI was estimated to be 8.3, and its amino acid sequence shared a high degree of similarity with that of other neurotoxic and enzymatically-active PLA_2s. BTL-2 had a molecular mass estimated in approximately 9 kDa and was characterized as a basic protein. In addition, BTL-2 did not exhibit any enzymatic activity.

The PLA_2 and BTL-2 formed a stable heterodimer with a molecular mass of approximately 24–26 kDa, estimated by molecular exclusion HPLC. In the presence of BTL-2, we observed a significant increase in PLA_2 activity, 23% higher than that of PLA_2 alone. BTL-2 demonstrated an inhibition of 98% in the growth of the Gram-positive bacterial strain, *Clavibacter michiganensis michiganensis* (Cmm), but only 9.8% inhibition of the Gram-negative bacterial strain, *Xanthomonas axonopodis* pv *passiflorae* (Xap). PLA_2 decreased bacterial growth by 27.3% and 98.5% for Xap and Cmm,

respectively, while incubating these two proteins with PLA_2-BTL-2 inhibited their growths by 36.2% for Xap and 98.5% for Cmm.

PLA_2 significantly induced platelet aggregation in washed platelets, whereas BTL-2 did not induce significant platelet aggregation in any assay. However, BTL-2 significantly inhibited platelet aggregation induced by PLA_2. In addition, PLA_2 exhibited strong oedematogenic activity, which was decreased in the presence of BTL-2. BTL-2 alone did not induce oedema and did not decrease or abolish the oedema induced by the 48/80 compound.

Conclusion: The unexpected results observed for the PLA_2-BTL-2 complex strongly suggest that the pharmacological activity of this PLA_2 is not solely dependent on the presence of enzymatic activity, and that other pharmacological regions may also be involved. In addition, we describe for the first time an interaction between two different molecules, which form a stable complex with significant changes in their original biological action. This opens new possibilities for understanding the function and action of crude venom, an extremely complex mixture of different molecules.

Background

Lectins are carbohydrate-binding proteins of non-immune origin found in a wide variety of living organisms that decipher glycocodes in the structure of glycans attached to soluble and integral cell membrane glycoconjugates [1]. Mechanisms for sugar recognition in microorganisms, plants, and animals are independently involved in several protein frameworks [2]. Protein-carbohydrate interactions play biological roles in many cellular processes, such as cell communication, host defence, fertilization, development, parasitic infection and tumour metastasis. Although, in many instances, their exact biological roles remain unknown, many lectins have been extensively studied as biochemical tools in biotechnology and biomedical research. Marine algal lectins, however, have been described at a low pace, since the first report on their haemagglutinating activity 40 years ago [3]. Marine organisms are recognized as rich sources of diverse and biologically-active molecules, many lectins from red and green marine algae have been isolated and characterised to date from more than 40 species. Amongst activities of potential therapeutic and diagnostic interest are the inhibition of fungal growth [4], induction and inhibition of human lymphocyte transformation [5], identification of methacillin-resistant *Streptococcus aureus* [6], induction of mitogenic activity [7], antibiotic activity against marine vibrios [8], endothelium-dependent relaxation of the rat aorta [9], inhibition of platelet aggregation [10], and as an anti-HIV protein [11].

In each family of lectins, the structure of their carbohydrate recognition domains (CRDs) is essentially conserved. Similar domains have been found in some phospholipase A_2 (PLA_2) receptors named M and N-type and play an important role in the pharmacological activity of PLA_2 [12]. Phospholipases A_2 (PLA_2) (EC 3.1.1.4) from snake venom are very small proteins that catalyse the hydrolysis of glycerophospholipids at the sn-2 position in a Ca^{+2}-dependent reaction, releasing lysophospholipids and fatty acids [13]. Snake PLA_2s have many pharmacological effects, such as: neurotoxicity, myotoxicity, haemolytic activity, haemorrhagic and oedematogenic activities [13,14]. PLA_2s that have lysine at the position 49 (Lys 49 or K49) [15] structurally keep the same motifs of PLA_2s that have aspartate at the position 49 (Asp 49 or D49, catalytically active), but their enzymatic activity is lost. Despite this, these proteins display many biological activities, regardless of arachidonic acid release [16-18].

In 2000, Hori et al [19] developed the haemagglutinating activity assay, combining algal lectins (hypnin A) and PLA_2s from the snakes, *Naja naja* and *Crotalus adamanteus*, and found an interaction between proteins, demonstrated by the inhibition of haemagglutination at a relatively low concentration [19]; it was suggested that this effect probably was dependent on a protein-recognition site present in the lectin surface where the PLA_2 would bind. Thus, some algae lectin-like proteins may play other important biological functions, such as providing an alternative source of a novel class of PLA_2 inhibitors. In this article, we investigate the effects induced by a new isolectin from the red marine alga, *Bryothamnion triquetrum*, on some of the biological activities of PLA_2 and the interaction with catalytically-active PLA_2 from snake venom.

Results

After the first purification step, on a preparative DEAE column, the main fraction, named *Bryothamnion triquetrum* lectin (BTL), was obtained and then dialyzed and lyophilised. The chromatography of BTL showed the presence of two main fractions, further named BTL-1 and BTL-2, which is the most abundant isoform isolated (Fig. 1a). After Reverse Phase HPLC, lectins were obtained with a high degree of purity (Fig. 1b). SDS-PAGE of BTL-2 revealed the presence of one main protein band with an estimated molecular mass of 9.0 kDa (Fig. 1c). The N-ter-

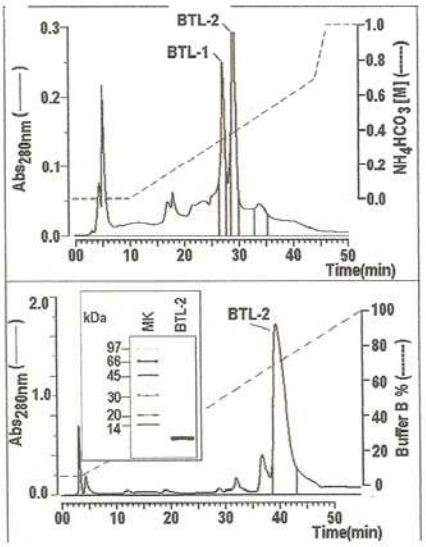

Figure 1

1a) HPLC profile of BTL-2 using an ion exchange column, protein Pack SP 5PW (Waters). Approximately 5 mg of the sample, obtained from the DEAE column, were dissolved in 0.05 M ammonium bicarbonate and applied onto the chromatographic column. The chromatography showed the presence of two main fractions containing haemagglutinating activity, named BTL-1 and BTL-2. The run was monitored at 280 nm and the elution was conducted using a discontinuous linear gradient of ammonium bicarbonate at a concentration of 1.0 M, pH 8.0, constant flow rate of 1 mL/min. 1b) The BTL-2 fraction was subjected to a final chromatography in a Reverse phase HPLC fractionation using a μ-Bondapack C18 column. The elution of samples was carried out using a linear gradient concentration of an aqueous solution of 66% acetonitrile and was monitored at 280 nm at a constant flow rate of 1 mL/min. Samples were previously dissolved in buffer A (0.1% TFA) that was used to equilibrate the column. 1c) Following the method [43], a discontinuous electrophoresis was performed with a final acrylamide concentration of 12% in the running gel. All the samples and the molecular markers were treated with SDS and DTT 1% and the run was conducted at 40 mA. The samples were stained with Coomassie brilliant blue R-250. The SDS-PAGE profile of BTL-2 showed high molecular homogeneity in a single band with estimated molecular mass of approximately 9.0 kDa.

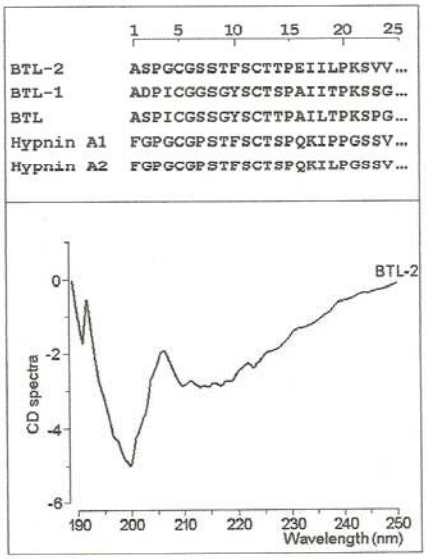

Figure 2

2a) BTL-1 and BTL-2 were previously reduced and carboxymethylated and later sequenced in an automatic gas-liquid sequencer Procise f (Applied Biosytem). 2b) The CD spectra were obtained using a J715 spectropolarimeter (Jarco Cop., Japan). BTL-2 was dissolved in water and the solution was adjusted to a final concentration of 10 mM of protein.

minal sequences of both lectins were deduced and showed a high homology with BTL [20] and hypnins [19] (Fig. 2a). In addition, BTL-1 and BTL-2 had a pI of around 8.6. Circular dichroism spectra of BTL-2 were obtained in the wavelength region of 190 – 250 nm, revealing mostly random coil structures and a low content of α-helices or β-sheets (Fig. 2b).

PLA_2 was purified to high molecular homogeneity by reverse phase HPLC (Figure 3a), which resulted in only one electrophoretic band (Figure 3b). The estimated pI value of this purified protein was 8.3 (Figure 3c). Molecular exclusion chromatography indicated a molecular mass of approximately 10.0 kDa for the isolated BTL-2; 15.0 kDa for the isolated PLA_2, while the PLA_2 and BTL-2, when incubated for 30 minutes, had a single peak with a molecular mass of approximately 24.0 – 26.0 kDa (Figure 3d).

The PLA_2 was characterized as an enzymatically-active D49 PLA_2 with moderate enzymatic activity in comparison with PLA_2 from *Naja naja*. In the presence of BTL-2, we observed a significant increase of 23% in the enzymatic activity of PLA_2, when compared with the PLA_2 alone (Figure 4a).

BTL-2 demonstrated an inhibition of 98% in the growth of the Gram-positive bacterial strain, *Clavibacter michiganensis subsp. michiganensis* (Cmm), but only a 9.8% inhibition of the Gram-negative bacterial strain, *Xanthomonas axonopodis* pv *passiflorae* (Xap). PLA_2 decreased Xap bacterial growth by 28.3% and Cmm growth by 98.5%, whilst incubation of these two proteins PLA_2-BTL-2 inhibited

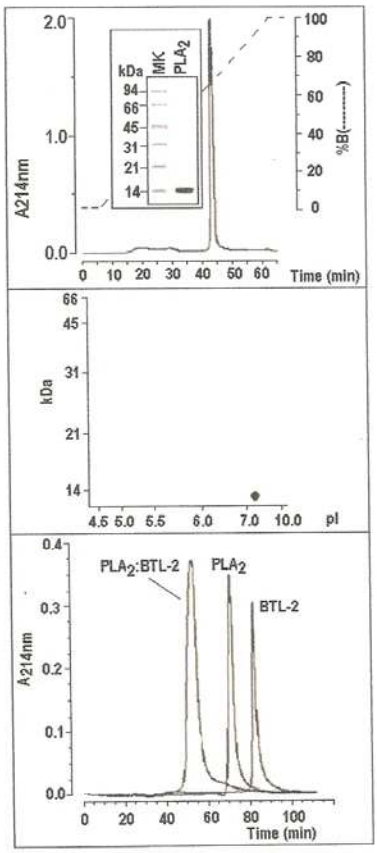

Figure 3

3a) The venom from the *Crotalus durissus cascavella* rattlesnake was fractionated by HPLC molecular exclusion chromatography (Superdex 75 column 1 × 60 Cmm) and the active fraction was subjected to Reverse Phase HPLC (μ-Bondapack C18 column) to obtain a high purity protein. 3b) Following the Laemmli method [43], a discontinuous electrophoresis was performed with a final acrylamide concentration of 12% in the running gel. All the samples and the molecular markers were treated with SDS and 1 M DTT and the run was conducted at 40 mA. The samples were stained with Coomassie brilliant blue R-250. The SDS-PAGE profile of PLA_2 showed high molecular homogeneity in a single band with an estimated molecular mass of approximately 14.0 kDa. 3c) Two dimensional electrophoresis showing one protein spot with molecular mass of around 14 kDa and pI of approximately 8.3. 3d) One milligram of sample was dissolved in 0.5 mL of 0.05 M phosphate buffer, pH 7.5, and applied to a Protein Pack TSK gel 3000 column (0.8 Cmm 30 Cmm) equilibrated with this same buffer. This procedure was performed with one milligram of each protein, incubated together and with all molecular mass markers to estimate the molecular weight of the samples. It is possible to observe a peak with an estimated molecular mass of 10 kDa from BTL-2, another with 15 kDa from PLA_2 and another of approximately 24–26 kDa that corresponds to both incubated proteins.

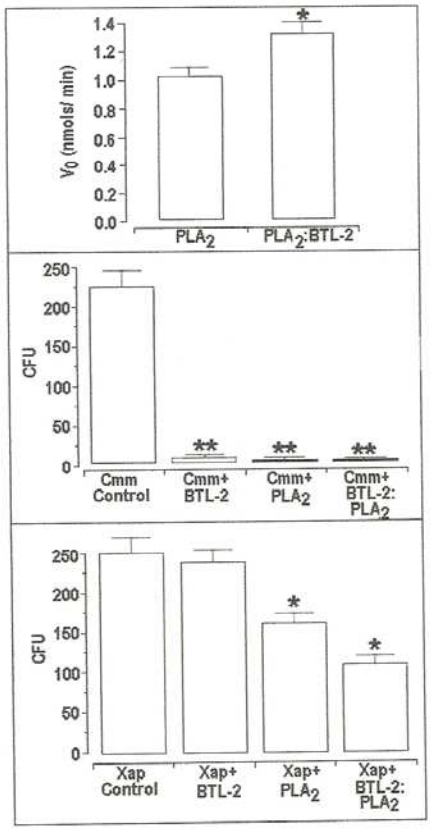

Figure 4

4a) The enzymatic activity assay [45] was analyzed using an Elisa plate containing 200 μL of buffer (10 mM Tris-HCl, 10 mM $CaCl_2$, 100 mM NaCl, pH 8.0), 20 μL of substrate (3 mM, 4-nitro-3-octanoyloxy-bencoic acid), 20 μL of water and 20 μL of the sample at a concentration 1 μg/μL or 2 μg/μL when both proteins were incubated together. After 20 minutes from the beginning of the reaction, the absorbance was measured at 425 nm using a SpectraMax 340 multiwell plate reader (Molecular Devices). It is possible to observe that the enzymatic activity of the PLA_2 was increased in the presence of BTL-2. 4b) and 4c) *Clavibacter michiganensis subsp. michiganensis* and *Xanthomonas axonopodis pv.passiflorae* were harvested from fresh agar plates and suspended in distilled and sterilized water (A600 nm = absorbance). BTL-2 demonstrated an inhibition of 98% of the growth of the Gram-positive bacterial strain, *Clavibacter michiganensis subsp. michiganensis* (Cmm), but only a 9.8% inhibition of the Gram-negative bacterial strain, *Xanthomonas axonopodis pv passiflorae* (Xap). PLA_2 decreased Xap bacterial growth by 28.3% and Cmm growth by 98.5%, whilst incubation of these two proteins PLA_2-BTL-2 inhibited their growths by 36.2% for Xap and 98.5% for Cmm.

their growths by 36.2% for Xap and 98.5% for Cmm (Figures 4b and 4c).

The PLA_2 isolated from *Crotalus durissus cascavella* showed a strong platelet aggregation activity in washed platelets (Figures 5 and 6). The aggregation induced by PLA_2 was very effective (Figure 5b), while BTL-2, at same concentration, did not produce any effect (Figure 5a). When PLA_2 was incubated with BTL-2, we observed a significant decrease in the effect of PLA_2 on platelet aggregation in the concentrations of 1 μg and 3 μg, respectivaly, 72.7% and 48.8%, when compared to PLA_2 alone (Figure 5c). The averaged platelet aggregation assay data is depicted in Figure 6.

BTL-2, injected in the paw of rats, did not induce an evident inflammation and its oedematogenic effect was similar to that of the saline control (Figure 7a). PLA_2 from *C. durissus cascavella* venom induced a strong oedema response at 15 minutes following injection. The maximum inflammatory response was observed at 30 min following injection of PLA_2. Under the same experimental conditions, PLA_2 incubated with BTL-2, exhibited a lower oedematogenic effect, compared to non-incubated PLA_2 (Figure 7a). In addition, BTL-2 did not inhibit the oedema induced by compound 48/80 (C48/80), a potent oedematogenic agent that induces the release of inflammatory mediators (Figure 7b).

PLA_2 from *Crotalus durissus cascavella* shared a high degree of amino acid similarity with other rattlesnake venom PLA_2s, such as the PLA_2s isolated from *Crotalus durissus terrificus* isoforms 15, 16 and 17 (Cdt F15, F16 and F17), CROTOX B and MOJAVE B (Figure 8). Furthermore, the amino acid sequence of PLA_2 was highly conserved at the N-terminal region, calcium-binding region, active site, α-helical and β-wing. Based on its three dimensional structure, *Crotalus durissus cascavella* PLA_2 exhibited a high number of random coil structures, mainly in the calcium binding and the C-terminal loops (Figure 9a and 9b). The 3D model also suggests the presence of an extensive loop that connects helix 1 to helix 2 and other significantly long random coil structures on the C-terminal region (Figure 9a) in this PLA_2.

Discussion and Conclusion

The interaction between a marine alga lectin and a PLA_2 from a rattlesnake was first detected by Hori et al 2000 [19]. Herein, we report the marine alga extract purification of BTL-2, one of the lectin isoforms isolated from the red marine alga *Bryothamnion triquetrum*. Lectins from *B. triquetrum*, with low molecular weight, share similar structural features with other low molecular weight lectins isolated from red algae, such as *Hypnea japonica* [19]. These structural similarities found suggest the importance of this

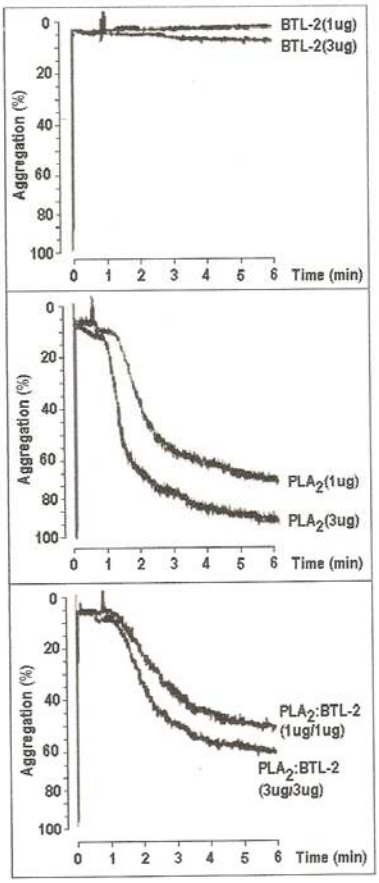

Figure 5
The washed platelets assay was performed with venous blood collected from healthy volunteers. The blood was centrifuged in 3.8% trisodium-citrate for 10 minutes at 200 × g and the residue was centrifuged again for 20 minutes at 1500 × g. Aggregations were performed in 1 μg and 3 μg concentrations in 20 μL of BTL-2, PLA_2 and the two proteins (v/v). 5a) BTL-2 induced low aggregation, however the PLA_2 induced high aggregation at these concentrations, and both proteins induced a lower aggregation when compared with the PLA_2 alone.

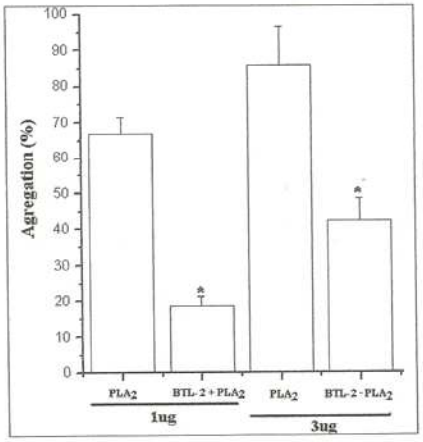

Figure 6
6a) For the concentrations 1 μg and 3 μg, the PLA_2 induced high aggregations. When PLA_2 was incubated for 30 minutes with BTL-2 at the same concentrations of 1 μg/1 μg and 3 μg/3 μg (v/v) in 20 μL volume it was possible to observe that the PLA_2 induced a lower aggregation when compared to the PLA_2 alone ($n = 5$; $p < 0.05$).

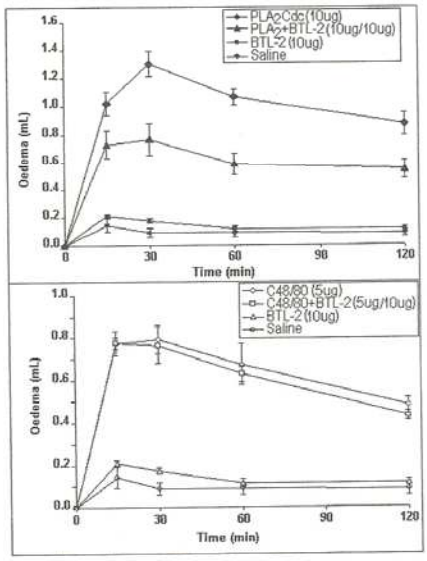

Figure 7
Male Wistar rats (120 g–150 g) were anaesthetised with halothane (inhaled). The paw oedema was induced by a single subplantar injection of 100 μL of sample 100 μg/paw). Paw volume was measured immediately before the injection and the intervals thereafter (15, 30, 60 and 120 minutes) using a hydroplethysmometer (model 7150, Ugo Basile, Italy). 7a) BTL-2 induced similar oedema similhat of the saline control. PLA_2 showed the highest oedema at 30 minutes before the injection. The presence of BTL-2 decreased the oedema induced by the enzyme. 7b) The compound 48/80 (C48/80) was used, incubated with BTL-2, to show that the lectin was not acting on the tissues to decrease the action of PLA_2, but was probably binding to the enzyme. Thus, the lectin did not decrease the action of C48/80 act.

protein for the development of these organisms and define a low molecular weight class of lectin.

Hori et al (2000) [19] suggested that the interaction between marine alga lectin and PLA_2 was correlated to the presence of a specific interaction domain. Experiments conducted by these authors showed that the haemagglutination activity of hypnins was strongly inhibited by the addition of PLA_2, which strongly suggests that the interaction between hypnins and PLA_2 involves specific molecular recognition. Accordingly [19], hypnins have two distinct binding sites; a carbohydrate-recognition site(s) (probably containing a C-type CRD motif) and a protein-recognition site(s) (phospholipase A_2-binding site). Interestingly, the interaction between PLA_2 with hypnins did not affect the hemolytic activity induced by PLA_2.

The N-terminal amino acid sequence of BTL-2 has a high homology with the lectins of *Hypnea japonica*, which probably possess the C-type CDR motif [19], as such BTL-2 probably possesses these same sites in its structure. The CD spectra analysis revealed that both BTL-2 and BTL-1 have a predominance of random coils, stabilized by disulfide bridges, conferring some interesting properties to these proteins such as resistance to high temperatures, very similar to hypnins which possess thermostability [19].

The catalysis of enzymatically-active PLA_2 can be affected by factors such as substrate, and PLA_2 activity is strongly enhanced by aggregated substrates that include micelles or monolayers [21]. Another important factor is substrate concentration, when the Critical Micelle Concentration (CMC) is reached, this enhances the enzymatic activity by several orders of magnitude [22,23]. Conformational

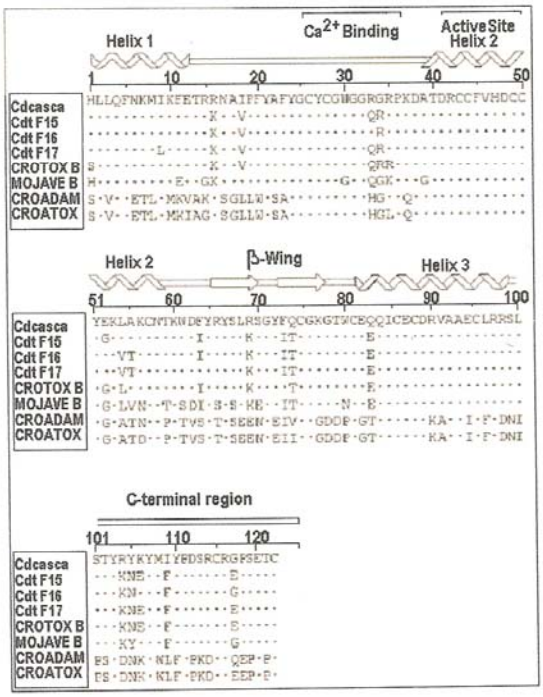

Figure 8
Amino acid sequence of PLA_2 isolated from *Crotalus durissus cascavella* and its estimated secondary structure. The calcium binding loop, active site and C-terminal region are present in the sequence. Note the highly conserved calcium binding loop and active site, and an extensive portion of basic amino acid residues, such as Arg and Lys, in the C-terminal. This PLA_2 showed high homology with other PLA_2s, such as the isoforms from *Crotalus durissus terrificus* (Cdt F15, F16 and F17). Crotoxin B (cloned basic subunit of crotoxin) and mojave b (PLA_2 subunit of *Crotalus scutulatus scutulatus*).

changes in the structure of PLA_2 are also an important factors that increase enzymatic activity [24]; these changes are located in the N-terminal residue and in the surface binding loop in which the calcium binding loop is located. The main conformation change, located in the N-terminal helix, has a small shift towards the calcium ion of PLA_2. The role and structural relevance of the N-terminal region have also been reported [25-27]. In addition, the suppression or the chemical modification of the N-terminal region results in a strong reduction in the enzymatic activity of catalytically-active PLA_2.

It has been reported that highly-purified heparin sodium salt is able to increase the enzymatic activity of catalytically-isolated PLA_2 from *Crotalus durissus terrificus* venom [28,29]. The potentialization of the enzymatic activity of heparin has been observed in the treatment of some vascular disorders in clinical trials. On the other hand, the inhibitory effects of the neurotoxic or other pharmacological activities of PLA_2 are well known. The mechanism of action of PLA_2 towards heparin involves inducible conformation changes to the active site, the N-terminal region, and strong binding with the cationic site located in the C-terminal region [30-32], which leads to the modifications

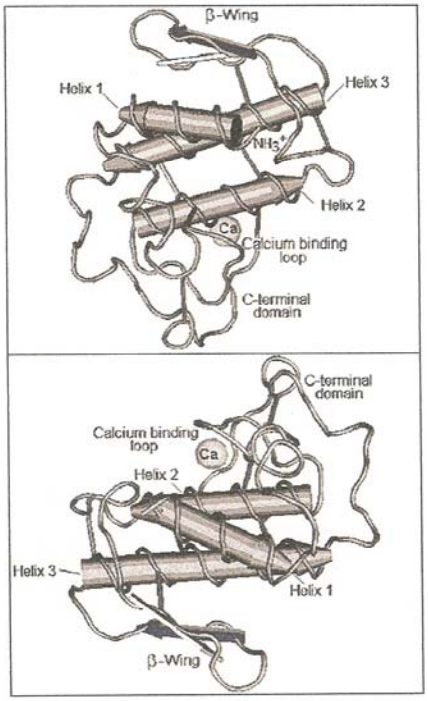

Figure 9
The three dimensional model of PLA_2 from *Crotalus durissus cascavella* venom was determined by comparative homology modelling using BLASTP in Protein Data Bank. The model shows the β-wing, α-helix, calcium binding loop, and C-terminal loop.

in the biological effects induced by the catalytically-active PLA_2.

Our results showed that this novel basic hypnin-like lectin, isolated from *Bryothamnion triquetrum*, seems to form a heterodimeric structure with PLA_2 from the *Crotalus durissus cascavella* venom and that its molecular binding strongly increases the enzymatic activity of PLA_2. Furthermore, our data suggest that the complex that is established between PLA_2, from *Crotalus durissus cascavella*, and lectin, from *Bryothamnion triquetrum*, probably involves a hydrophobic contact and indirectly affects the catalytic site of PLA_2. Thus, our results show that some pharmacological activities, induced by PLA_2 isolated from *Crotalus durissus cascavella*, did not involve its solely enzymatic activity, indicating that the specific actions induced by this PLA_2 are due to the presence of pharmacological sites on the protein surface, which are distinct from catalytic sites, allowing PLA_2 to bind specifically to soluble proteins or membrane-bound proteins that participate in the mechanism of action and this is not an isolated case, as observed for other PLA_2 from other sources [33,34]. The antibacterial activity, however, is very dependent on the ability of this PLA_2 to destroy the bacterial membrane. These facts are supported by the results of the bacterial growth rate of Xap, which was strongly inhibited by a complex formed by PLA_2 and BTL-2.

Conversely, the platelet aggregation assay showed that the presence of the lectin significantly decreased the PLA_2 aggregation effect, showing that the pharmacological activity of the enzyme was affected by BTL-2. This same effect occurred in the oedema assay, where in the presence of the lectin, the pharmacological activity of the PLA_2 was decreased, inducing a lower oedema. The hydrolysis of arachidonic acid (AA) by the PLA_2 generates free AA and lysophospholipids that are the precursors of eicosanoids and platelet-activating factor, respectively [35]. At first, it was thought that secretory PLA_2s served to provide substrates for the biosynthesis of proinflammatory lipid mediators; however, it was found that not all PLA_2 hydrolyze AA from intact mammalian cells, suggesting that the generation of lipid mediators is not a general function of this enzyme [36,37]. A great breakthrough was the identification of other biological effects of PLA_2 in cells involved in inflammatory and immune responses. As such, these results support the idea of the presence of another pharmacological site in the PLA_2, isolated from the *Crotalus durissus terrificus*, which is distinct from the enzymatic site. Something similar is observed in some other PLA_2 that interact with target cells via membrane peptidoglycans and with specific or promiscuous receptors [11] and, in some cases, lead to activation of some intracellular cytoplasmic PLA_2 and several proteins including PKA and PKC [37-39]. Some specific mutation studies carried out with PLA_2 showed that the binding of secretory PLA_2 to the receptor involves a specific recognition of the CRD region of this receptor [34,36,37], which is located near the catalytic site of PLA_2. Our results, however, suggest that the PLA_2 region involved in the binding with BTL-2 produces a structural change that results in the reduction of its pharmacological action, compared to untreated PLA_2. The C48/80 experiment supports this argument, since it shows that the lectin was not binding to the tissues to hinder the C48/80 or PLA_2 activity, but that it was acting in the PLA_2.

Class I and II secretory PLA_2s possess an antiparallel, poorly conserved β-wing. The structural conformation, though variable in relation to the rest of the enzyme, is internally consistent across species. The wing extends outwards into the solvent, and the 'wingtip' is poorly anchored. It has been suggested that biological properties such as neurotoxicity, myotoxicity or anticoagulation may be associated with the β-wing [40-42]. The presence of a similar β-wing motif in the 3D model of the PLA_2, isolated from *Crotalus durissus cascavella* venom, may explain its remaining pharmacological activity, which probably has two distinct pharmacological sites, one located in the random coil that links the N-terminal helix with the helix 2 and the β-wing.

The main conclusions of this study are that; 1) the pharmacological activity of PLA_2 from *Crotalus durissus cascavella* is completely dissociated from its enzymatic activity and also some of its biological activity. 2) The PLA_2 model revealed that the presence of an extensive hydrophobic random coiled region may be involved in the interaction with BLT-2, forming a random-coil based structure.

Methods

Source of algae

Specimens of the red marine alga *Bryothamnion triquetrum* were collected from the Atlantic Coast of Brazil (Pacheco Beach, Ceará) and kept in plastic bags at -20°C until processed.

Lectin purification

Algae were thawed, rinsed with distilled water, cleaned of epiphytes, and ground to a fine powder under liquid nitrogen. The powder was stirred for 4 hours with 0.02 M phosphate buffer (pH 7.0), with 0.15 M NaCl, filtered through nylon tissue, and then centrifuged at 7,000 × *g*, for 30 min, at 4°C. The supernatant was acidified and remained under refrigeration for 4 hours. The precipitated pigments were removed by centrifugation and the supernatant (crude extract) was adjusted to pH 7.0, followed by ammonium sulphate precipitation (0–60%). Precipitated proteins were recovered by centrifugation, dialyzed in distilled water and applied onto a DEAE-cellulose column, equilibrated and eluted with 0.02 M phosphate buffer (pH 7.6), followed by elution with 1.0 M NaCl solution in the same buffer, at a flow rate of 30 mL/h. The unabsorbed fractions with haemagglutinating activity were pooled and rechromatographed in the same column. Active fractions containing lectins were dialyzed and lyophilised (DEAE fraction).

After that, approximately 5 mg of freeze-dried DEAE-fraction was dissolved in 250 μL of ammonium bicarbonate buffer (0.05 M; pH 7.9) and homogenised until complete dissolution, followed by clarification by centrifugation (4,500 × *g* for 2 min, at 27°C). The supernatant was recovered and injected onto an ion exchange HPLC column (Protein Pack SP 5PW, 0.75 × 10 cm, Waters), previously equilibrated with the same buffer used for dissolving the fraction. The elution was conducted using a discontinuous linear gradient of ammonium bicarbonate at a concentration of 1.0 M, pH 8.0, at constant flow rate of 1 mL/min.

The chromatography was monitored at 280 nm and the peaks were collected and the two fractions containing haemagglutinanting activity were lyophilised and than subjected to the final chromatographic steps of the Reverse Phase (RP-HPLC). The elution of samples was carried out using a linear gradient concentration of an aqueous solution of 66% acetonitrile. Samples were previously dissolved in buffer A of RP-HPLC (0.1% TFA) that was used to equilibrate the column. The fractions were immediately concentrated by ultrafiltration (AMICON), followed by dialysis with 5.0 mM ammonium bicarbonate solution (pH 7.8), sterilized by microfiltration (0.22 μm) and stored at 4°C for further use.

Electrophoresis

The electrophoresis was carried out following the Laemmli method [43]. The degree of purity of fractions was assessed by discontinuous electrophoresis using a final acrylamide concentration of 12% in the running gels (1.0 M Tris-HCl, pH 8.8) and 5% in the stack gel (0.5 M Tris-HCl, pH 6.8). The electrophoretic run was carried out using a BIORAD electrophoretic system for SDS-PAGE. All samples and the molecular marker were treated with SDS and 1.0 M DTT and the run was conducted at 40 mA for both plates. After electrophoresis, samples were stained with Coomassie brilliant blue R-250.

Circular dichroism

The purified BTL-2 was dissolved in water and the solution was adjusted to a final concentration of 10 mM of protein. The sample was transferred to a 1 mm pathlength quartz cuvette. Circular dichroism spectra in the wavelength range of 185–300 nm were obtained using a J715 spectropolarimeter (Jasco Corp., Japan) using a bandwidth of 1 nm and a response time of 1s. Data collection was performed at room temperature with a scanning speed of 100 nm/min; a total of nine scans were accumulated for the sample.

PLA_2 purification

PLA_2 from the rattlesnake, *Crotalus durissussus casvella*, was purified by a combination of chromatographic procedures and its molecular homogeneity was determined by SDS-PAGE. The whole venom was fractionated by HPLC molecular exclusion chromatography using a Superdex 75 column (1 × 60 cm). The active PLA_2 fraction was then

subjected to Reverse Phase HPLC in a C18 μ-Bondapack column (0.78 cm × 30 cm) (Waters 991-PDA system). The column was eluted with a linear gradient (0–66.5%, v/v) of acetonitrile (solvent B) at a flow rate of 2 mL/min, and absorbance was monitored at 280 nm. The fractions were collected manually, lyophilized and stored at -20°C.

Amino acid sequence

Three milligrams of purified protein were dissolved in 200 μl of 6 M guanidine chloride (Merck, Darmstadt, Germany) containing 0.4 mM Tris-HCl and 2 mM EDTA (final pH 8.15). Nitrogen was flushed over the top of the protein solution for 15 min, which was then reduced with DTT (6 M, 200 μl) and carboxymethylated with ^{14}C-iodoacetic acid and cold iodoacetic acid. Nitrogen was again flushed over the surface of the solution and the reaction tube sealed. This solution was incubated in the dark at 37°C for 1 h and desalting was performed on a Sephadex G 25 column (0.7 × 12 cm) in 1 mM acetic acid buffer. The eluted reduced and carboxymethylated (RC) protein was lyophilised and stored at -20°C. The RC lectins were then purified and the N-terminal region sequenced.

For the RC-PLA_2, we determined the complete amino acid sequence of the protein by sequencing the N-terminal region and the peptides from enzyme-treated PLA_2. One sample of RC-protein was digested with *Staphylococcus aureus* protease V8 for 16 hours at 37°C, using an enzyme -substrate ratio of 1:30. The reaction was stopped by lyophilisation. A second sample of RC-PLA_2 was digested with Clostripain for 8 hours at 37°C and then lyophilised [44]; the products were separated by Reverse Phase HPLC using a Waters PDA 991 system with a C-18 μ-Bondapack column.

Peptide peaks were isolated using a linear gradient (0–100% of acetonitrile in 0.1% TFA (V/V)). A third sample (2 mg) was cleaved with a 15-fold molar excess of cyanogens bromide (CNBr) over methionine residues in 70% formic acid (4 ml) under nitrogen for 24 hours at room temperature. The reaction mixture was then diluted with 40 mL of water and lyophilised. Excess reagents were removed by gel filtration on a Sephadex G-25 column (1 × 20 cm) equilibrated with 10% acetic acid. The CNBr peptide fragments were separated by Reverse Phase HPLC, using an analytical μ-Bondapack C18 column (0.39 × 30 cm; Waters) with 0.1% TFA as solvent A and acetonitrile containing 30% of solvent A (solvent B). The elution profile was monitored at 214 nm. Analysis of the amino acid sequence of the RC-protein, as well as that of the enzymatically-digested fragments were performed with an Applied Biosystems model Procise f gas-liquid protein sequencer. The phenylthiohydantoin (PTH) derivatives of the amino acids were identified with an Applied Biosystems model 450 microgradient PTH-analyser.

PLA_2 activity

PLA_2 activity was measured [45]. The standard assay mixture contained 200 μL of buffer (10 mM Tris-HCl, 10 mM $CaCl_2$, 100 mM NaCl, pH 8.0), 20 μL of 2.98 mM substrate (4-nitro-3-octanoyloxy-benzoic acid; $4N_3OBA$), 20 μL of water, and 20 μL of PLA_2 (1 mg/mL) in a final volume of 260 μL. After the addition of PLA_2, the mixture was incubated for 20 min at 37°C. The increase in absorbance at 425 nm, due to product formation, was monitored during this period and the activity was expressed as nmols of product formed nmols/min/mg. The inhibitory effect of BTL-2 (1 mg/mL) on PLA_2 activity was investigated by incubating the two proteins at 37°C for 30 min prior to assaying the enzyme activity using the SpectraMax 340 multiwell plate reader (Molecular Devices).

HPLC molecular exclusion chromatography of BTL-2 andBTL-2-PLA_2

One milligram of PLA_2 from *C. d. cascavella* was dissolved in 0.5 mL of 0.05 M phosphate buffer, pH 7.5, and applied to a Protein-Pack TSK gel 3000 column (0.8 cm × 30 cm), previously equilibrated with the same buffer before use. BTL-2 and PLA_2 (1:1) were preincubated together for 30 minutes in phosphate buffer. Separately, molecular mass markers (bovine serum albumin, 66 kDa; egg album, 45 kDa; carbonic anhydrase, 29 kDa, and lysozyme 14 kDa) were used to estimate the molecular weight of PLA_2, BTL-2 and PLA_2:BTL-2. All molecular weight markers were dissolved in phosphate buffer at a concentration of 2 mg/mL. The proteins were eluted at a flow rate of 0.2 mL/min and the absorbance profile was monitored at 214 nm.

Platelet aggregation studies

Sample collection and aggregation studies

Venous blood was collected with informed consent from healthy volunteers who denied taking any medication in the previous 14 days. Blood was collected by a two-syringe technique using polypropylene syringes and 19-gauge needles, and immediately transferred into polypropylene tubes containing 1/10 of final volume of 3.8% trisodium citrate.

After removing the platelet-rich plasma (PRP), the remaining blood was prepared by centrifugation of citrated blood at 200 × *g* for 10 min and washed platelets (WP) were obtained from the residue by centrifugation of citrated blood at 1500 × *g* for 20 min. The platelets were left for 1 h at room temperature to recover their sensitivity to aggregating agents. Platelet counts were performed on a Coulter S Plus (Coulter Electronics, Hialeah, FL) or by phase-contrast microscopy. Platelet aggregation was carried out using 400 μL of the washed platelets solution in a cuvette and kept at 37°C with constant stirring.

The desired concentration of protein was added 3 minutes prior to the addition of platelet aggregation inducers (thrombin for washed platelets). Subsequently, the aggregation was recorded for 5–10 min by using an aggregometer (Payton Scientific Instruments, Inc, Buffalo, NY). Aggregation experiments were performed in two concentrations (1 μg and 3 μg) of BTL-2, PLA_2, and PLA_2 incubated with BTL-2 (v/v) in a final volume of 20 μL, which was previously incubated (30 min) at room temperature.

Paw oedema assay

Male Wistar rats (120–150 g) were anaesthetised with halothane (inhaled). The rat paw oedema was induced by a single subplantar injection of 100 μL of BTL-2 (10 μg/paw), PLA_2 (10 μg/paw), and PLA_2 incubated with BTL-2 (v/v). Paw volume was measured immediately before the injection of the samples and at selected time intervals thereafter (15, 30, 60, and 120 minutes) using a hydroplethysmometer (model 7150, Ugo Basile, Italy). The compound 48/80 (C48/80) was incubated with BTL-D2 to show that the lectin was not acting on the tissues to decrease the action of the PLA_2. All samples were dissolved in sterile saline solution (0.9%). Results were expressed as the increase in paw volume (mL) and calculated by subtracting the basal volume.

Antibacterial assay

Xanthomonas axonopodis pv.*passiflorae* (Xap) and *Clavibacter michiganensis subsp. michiganensis* (Cmm) were harvested from fresh agar plates and suspended in distilled and sterilized water (A600 nm = absorbance, corresponding to 3×10^8 CFU/mL). Aliquots of this suspension were diluted to 3×10^3 CFU/mL and incubated with 100 μL of BTL-2 (1 mg/mL) for 30 min at 37°C. We then determined the survival on Agar Nutrient (Difco) Plate (n = 5).

Prediction of the three-dimensional structure of PLA_2

Amino acid sequence similarity and alignment searches were carried out to compare the PLA_2 isolated from *Crotalus durissus cascavella* venom against the Protein Data Bank using BLASTP. The 3D structure of PLA_2 was determined by comparative homology modelling. Using computer graphics, the amino acid sequence of PLA_2 was fitted to the high-resolution X-ray structure of the homologous 1BJJ_A. This allowed the construction of a model, which was then subjected to energy minimization and molecular dynamic simulations.

Statistical analyses

The results were reported as the means ± SEM of *n* experiments. The significance of differences between means was assessed by analysis of variance, followed by a Dunnett's test when several experimental groups were compared with the control group. The confidence limit for significance was 5% ($p > 0.05$).

Authors' contributions

SCBO -carried out all the purifications, activity of the PLA_2, eletrophoresis, participated in all the assays, coordination of the study, helped to draft the manuscript and performed the statistical analysis. FVF; EA; EAC; RPM – carried out the pharmacological assays e.g. platelet aggregation studies and the paw oedema assay. RA – carried out the circular dichroism.

DOT; LOSB – carried out the antibacterial assays. EVN; BSC; CSN; AHS; KSN -collected the marine alga and performed the initial purification of the alga lectin. MHT – carried out the amino acid sequencing, prediction of the three-dimensional structure of PLA_2, coordination of the study, helped to draft the manuscript and performed the statistical analysis. All authors read and approved the final manuscript.

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Enzymatic and structural characterization of new PLA2 isoform isolated from white venom of *Crotalus durissus ruruima*

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ABSTRACT

This work reports the structural and enzymatic characterization of a new sPLA2 from the white venom of *Crotalus durissus ruruima*, nominated PLA2A. The homogeneity of the PLA2A fraction and its molecular mass were initially evaluated by SDS-PAGE and confirmed by MALDI-TOF spectrometry, indicating a molecular mass of 14,299.34 Da. Structural investigation, through circular dichroism spectroscopy, revealed that PLA2A has a high content of alpha helix and beta-turn structures, 45.7% and 35.6% respectively. Its amino acid sequence, determined by Edman degradation and "*de novo amino acid sequencing*", exhibited high identity to PLA2 Cdt F15 from *Crotalus durissus terrificus*. The enzymatic investigation, conducted using the synthetic substrate 4-nitro-3-(octanoyloxy)-benzoic acid, determined its V_{max} (7.56 nmoles/min) and K_M (2.76 mM).
Moreover, PLA2A showed an allosteric behavior and its enzymatic activity was dependent on Ca^{2+}. Intrinsic fluorescence measurements suggested that Ca^{2+} induced a significant increase of PLA2A fluorescence, whereas its replacement for Mg^{2+}, Mn^{2+}, Sn^{2+} and Cd^{2+} apparently induced no structural modifications. The optimal pH and temperature for the enzymatic activity of PLA2A were 8.4 and 40 °C, respectively, and the minimal concentration of p-BPB and crotapotin that significantly inhibited such activity was 0.75 mM and 0.4 μM, respectively. In addition, PLA2A showed a significant antibacterial effect that was not strictly dependent on the enzymatic activity of such sPLA2.

1. Introduction

Crotalus durissus venom is a complex mixture of substances, such as crotamine, gyroxin, crotoxin (a complex formed by phospholipase A2 and crotapotin), tissue Kallikrein-like activity, trombine-like enzyme, phosphodiesterase, 5′-nucleotidase, L-amino oxidase, convulsin, and peptides (Bercovici et al., 1987). Barrio (1954) analyzed samples of *Crotalus durissus terrificus* from several parts of Brazil and found three different types of electrophoretic profiles. His study allowed him to divide these snakes according to their origin into northern, central, and southern populations. He reported that the venom of specimens collected in southern Brazil differed from others on the presence of crotamine (Barrio, 1954). Gonçalves and Vieira (1950) also showed variation in *C. d. terrificus* venom composition related to geographical origin of specimens. In *C. durissus*, interspecific variability has been demonstrated for *C. d. terrificus*, *Crotalus durissus cascavella* and *Crotalus durissus collilineatus* rattlesnakes (Santoro et al., 1999). Interspecific variations have been also reported in pools of

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venoms from adult specimens of *C. d. terrificus* and *Crotalus durissus ruruima* (Dos Santos et al., 1993), a rattlesnake subspecies commonly found in cultivated lands in the northern Brazilian state of Roraima and in savannas in southern Venezuela. Venoms of *C. d. ruruima* may belong to one of two varieties, designated white or yellow venom, according to their appearance. By other side, some studies performed with these venoms indicated evidently different pharmacological properties, as reported by Dos Santos et al. (1993, 2005). A pool of white venoms of *C. d. ruruima* was shown to display lethal, coagulant, myotoxic, edematogenic and hemolytic activities very similar to *C. d. terrificus* venoms (Dos Santos et al., 1993). In addition, hemorrhage, necrosis, caseinolytic and crotamine activities were present in a pool of *C. d. ruruima* yellow venom (Dos Santos et al., 1993, 2005) from which Ponce-Soto et al. (2007) have recently isolated and characterized the biological activity of a PLA2.

Crotoxin accounts for approximately 40–50% of whole dried venom and is involved in the main symptoms caused by *C. durissus* bites. Crotoxin has neurotoxic, myotoxic, hemolytic, and platelet aggregating activities (Bon et al., 1989; Faure et al., 1994). This protein is primarily constituted of a reversible heterodimeric association of a basic and weakly neurotoxic compound (basic phospholipase A2 or PLA2) and an acidic compound, devoid of toxic effects (crotapotin or Crtp) (Faure and Bon, 1988). Crotapotin has been reported to possess *in vivo* anti-inflammatory activity as demonstrated by the inhibition of carrageenin-induced rat paw edema (Landucci et al., 1995, 2000). It was demonstrated that part of this immunosuppressive effect is due to an increase in the production of prostaglandin E2, by macrophages previously stimulated with crotapotin (Garcia et al., 2003). Furthermore, this compound also showed other activities such as a moderate antimicrobial activity and it was able to induce significant changes in isolated kidneys (Oliveira et al., 2003). Moreover the presence of different PLA2 isoforms in snake venoms may show significant changes in induced pharmacological activities in view of neurotoxicity, for example (Oliveira et al., 2003). Intraspecific variation in phospholipase A2 activity, as reported in *Bothrops asper*, *Bothrops neuwiedii*, *Crotalus ruber*, and *Daboia russelli*, may be also found for *C. d. ruruima* venom.

In the present study, a new PLA2 isoform from *C. d. ruruima* white venom was identified and its primary structure was determined by combination of Edman degradation and mass spectrometry. In addition, we compared its amino acid sequence to other sPLA2s and determined several of its kinetic properties. Furthermore, the antibacterial activity of this new PLA2 and Ca^{2+} effect on its activity was investigated.

2. Materials and methods

2.1. Venom and reagents

The venom of *C. d. ruruima* was gently donated by Regional Ophidism Nucleus (NUROF/Federal University of Ceará/Fortaleza-CE). The reagents and chemicals used in this study were ACS, HPLC or sequencing grade, purchased from Sigma–Aldrich, Merk, BioRad, GE Healthcare or Applied Biosystems.

2.2. Isolation of PLA2A

The PLA2 from *C. d. ruruima* white venom was purified by a combination of two chromatographic procedures. A molecular exclusion chromatographic step was initially performed, followed by a second step in a reverse phase (RP) HPLC. This procedure has been previously described by Oliveira et al. (2002). For the molecular exclusion chromatographic step, 10 mg of whole dried venom were dissolved in a 1 M ammonium bicarbonate (AMBIC) solution, pH 7.8 until complete homogenization, and then clarified by centrifugation at 4500 × *g* for 4 min. The resulting supernatant was applied onto a Superdex 75 column (1 cm × 60 cm Waters) previously equilibrated with 0.2 M AMBIC, pH 7.8. Elution of fractions of the venom was conducted at a flow rate of 0.2 ml/min and the elution profile was monitored at 280 nm (Waters LC650 system). All fractions were collected at the absorbance peak and then lyophilized and stored at −20 °C. The whole crotoxin was afterward purified by reverse phase HPLC. Approximately 5 mg of whole crotoxin were dissolved in 200 µl of HPLC buffer A (0.1%TFA), also used for the equilibration of the chromatographic column. After homogenization, the sample was centrifuged at 4500 × *g* for 5 min, for clarification. The resulting supernatant was recovered and applied on the reverse phase HPLC column (µ-Bondapack C18, 0.39 × 30 cm). Fractions were eluted using a linear concentration range 0–100% of buffer B (acetonitrile 66% in buffer A) at flow rate of 1 ml/min. The chromatographic run was monitored at 280 nm and each fraction was recovered and lyophilized. The degree of purity of the isolated PLA2 was evaluated by Tricine SDS-PAGE according to Schägger and von Jagow (1987) and MALDI-TOF mass spectrometry. PLA2 activity was monitored after each chromatographic step.

2.3. PLA2 activity

PLA2 activity assay was conducted following the protocol described by Lee et al. (1999) and adapted by Toyama et al. (2003) for 96-well plates. The standard assay mixture contained 200 µl of buffer (10 mM Tris–HCl, 10 mM $CaCl_2$, 100 mM NaCl, pH 7.8), 20 µl of substrate (4-nitro-3-octanoyloxy-benzoic acid (NOBA, BIOMOL, USA)), 20 µl of water and 20 µl of PLA2 sample, adding to a final volume of 260 µl. After the addition of PLA2 sample (20 µg), the mixture was incubated for up to 40 min at 37 °C, its absorbance at 425 nm being read at 10-min intervals. Enzyme activity was calculated based on the increase in absorbance after 20 min. The pH and temperature optima of PLA2 were determined by incubating the enzyme in buffers of different pHs (4.0–10.0), and at different temperatures in Tris–HCl, pH 8.0 in the presence of calcium. The effect of substrate concentration on enzyme activity was determined by measuring the increase in absorbance after 20 min of incubation in Tris–HCl buffer, pH 8.0, at 37 °C. The effect of temperature on the PLA2A was investigated following the protocol described by Toyama et al. (2003), over a temperature range from 20 to

60 °C and with temperature increments of 5 °C. Moreover, we examined the effect of p-bromophenacyl bromide (p-BPB) and crotapotin on the enzymatic activity. In order to do so, we performed a chemical modification of PLA2 with p-BPB according to the protocol described by Toyama et al. (2005). The effect of crotapotin was determined by mixing different molar concentrations of this compound, ranging from 0 to 0.8 μM, with 1.5 μM of PLA2. All assays were conducted in triplicate and absorbances were measured at 425 nm using a SpectraMax 340 multiwell plate reader (Molecular Devices, Sunnyvale, CA, USA). The effect of different ions, such as Mg^{2+}, Mn^{2+}, Sn^{2+} and Cd^{2+} on the PLA2 activity and the Ca^{2+} concentration for the optimal activity were determined by incubating the enzyme in Tris–HCl buffer pH 8.0 with each cation (10 mM) and diverse Ca^{2+} concentrations, respectively.

2.4. Spectroscopic analyses

2.4.1. Circular dichroism spectroscopy

The purified enzyme was dissolved in 10 mM sodium phosphate buffer pH 7.4 and the solution was adjusted to a final concentration of 8.7 μM of protein. After centrifugation at 4000 × *g* for 5 min for clarification, samples were transferred to a 1 mm path-length quartz cuvette. Circular dichroism spectra in the wavelength range 185–300 nm were acquired in house on a J720 spectropolarimeter (Jasco Corp., Japan) using a bandwidth of 1 nm and a response time of 1 s. Data collection was performed at room temperature. With a scanning speed of 100 nm/min, a total of nine scans were accumulated for each sample and all the spectra were corrected by subtraction of buffer blanks.

2.4.2. Intrinsic fluorescence

The relative intrinsic fluorescence intensity of native PLA2 was monitored with Shimadzu spectrofluorimeter. A reaction mixture of 2.0 ml in 1 cm path-length quartz cuvette contained 100 mM Tris–HCl buffer (pH 7.4), PLA2 (200 μg/ml) in presence or absence of 10 mM of Ca^{2+} or in presence of 10 mM of Mn^{2+}, Mg^{2+}, Sn^{2+} and Cd^{2+}. Fluorescence spectra were acquired over the wavelength range from 300 to 450 nm after excitation at 280 nm.

2.5. Primary structure

Five mg of the purified PLA2 were dissolved in 200 μl of 6 M guanidine chloride (Merck, Darmstadt, Germany) containing 0.4 mM Tris–HCl and 2 mM EDTA (final pH 8.15). Nitrogen was flushed over the top of the protein solution for 15 min, which was then reduced with DTT (6 M, 200 μl) and carboxymethylated with ^{14}C and cold iodoacetic acid. Nitrogen was again flushed over the surface of the solution and the reaction tube sealed. This solution was incubated in the dark at 37 °C for 1 h and desalting was done on a C5 reverse phase HPLC column with 30% acetonitrile in 0.1% TFA as elution buffer. The resulting fraction designated as RC-protein was then lyophilized. For the N-terminal amino acid determination, 1 nmol of this RC-protein was sequenced in an Applied Biosystems model Procise f gas–liquid protein sequencer. Phenylthiohydantoin (PTH) derivatives of the amino acids were identified with an Applied Biosystems model 450 microgradient PTH-analyzer. The N-terminal amino acid sequencing was done up to the 25th amino acid residue.

The remaining undisclosed amino acid sequence was determined by "de novo" sequencing of tryptic peptides. All mass spectra were acquired using a quadrupole-time of flight (Q-TOF) hybrid mass spectrometer Q-TOF Ultima (Micromass, Manchester, UK) equipped with a nano Z-spray source, operating in a positive ion mode. The ionization conditions were: capillary voltage of 2.3 kV, cone voltage of 30 V, RF1 lens voltage of 100 V, and collision energy of 10 eV. Source temperature was 70 °C and cone gas, N_2, at a flow of 80 l/h. Nebulizing gas was not used to obtain the sprays. Argon was used for cooling and ion fragmentation in the collision cell. External calibration with sodium iodide was made over a mass range from 50 to 3000 *m/z*. Alkylated tryptic peptides fractionated by RP-HPLC were lyophilized and resuspended in 20% acetonitrile in 0.1% TFA prior injection into the mass spectrometry source at a flow rate of 500 nl/min. Before performing a tandem mass spectrum, an ESI/MS mass spectrum (TOF MS mode) was acquired for each HPLC fraction over the mass range of 400–2000 *m/z*, in order to select the ion of interest; subsequently, these ions were fragmented in the collision cell (TOF MS/MS mode). Different collision energies were used, depending on the mass and charge state of the ions. The resulting product-ion spectra were acquired in the TOF analyzer and processed using the MassLynx-MaxEnt 3 algorithm. Single charge spectra were processed manually using the PepSeq application including the MassLynx. In addition we used the MASCOT software package for determination of final amino acid sequence.

We also digest this protein with highly purified V8 protease from *Staphylococcus aureus* (Promega) in phosphate buffer pH 4.5 that allowed the enzyme to cut the polypeptide sequence in D and E. Briefly, HPLC-purified RC-protein was digested with the V8 protease using a protein:enzyme ratio of 1:30. After 12 h the resulting peptides from the enzymatic cleavage were purified by reverse phase HPLC (C18 columns). The peptides were used to confirm PLA2A amino acid sequence through comparison of sequencing data of these peptides to data of peptides derived from trypsin digestion of PLA2A, acquired by means of mass spectrometry.

2.6. Antibacterial activity

Xanthomonas axonopodis pv. passiflorae (Gram-negative) bacteria were harvested from fresh agar plates and suspended in distilled sterilized water (A650 nm = 0.3/cc; 103 CFU/ml). Aliquots of the bacterial suspension were diluted to 10–5 CFU/ml and incubated with PLA2A (75 μg/ml) or saline buffer for 1 h at 37 °C. After incubation, survival was assayed on nutrient (Difco) plates ($n = 5$) as described by Oliveira et al. (2002). Electron microscopic assessments for observation of morphological alterations of *X. a. pv. passiflorae* were made for both the control samples and samples containing PLA2A-treated bacteria (A650 nm = 0.3/cc; 103 CFU/ml). Samples were fixed with 4% glutaraldehyde in buffer A (0.1 M cacodylate buffer, pH 7.4) for 1 h at 4 °C, embedded in 3% low-melting

temperature agarose (Sea Plaque, FMC Corp.), and stored for 12 h in fixative at 4 °C.

Agarose pellets were immersed in fixative for 2 h at 25 °C, washed three times for 10 min with buffer A, and fixed with 1% osmium tetroxide (Agar Scientific Ltd.) in buffer A, for 2 h at 25 °C. Sections were washed three times, dehydrated in increasing concentrations of ethanol and propylene oxide, and embedded in Epon resin (Agar Scientific). Polymerisation was performed at 60 °C for 48 h, and ultra-thin sections were prepared with a Sorvall MT2 ultra microtome. The sections were placed on 5% collodion-coated 100-mesh grids, and stained with 4% uranyl acetate (Agar Scientific) for 15 min, followed by staining with 2.6% lead citrate (Agar Scientific) for 15 min. Samples were observed under a Hitachi 1100 transmission electron microscope (Hitachi Scientific Instruments) operating at 100 kV.

2.7. Statistical analysis

The results are reported as the means ± SEM of n experiments, when appropriate. The significance of differences between means was evaluated by analysis of variance followed by Student's t-test when various experimental groups were compared with the control group. The confidence limit for significance was 5%.

3. Results

3.1. Structural characterization

The purification of the whole white variety venom from *C. d. ruruima* resulted in two main fractions, named as IV and V, and four minor fractions, named as I, II, III and VI. Fraction IV was positively identified as crotoxin by pharmacological and biochemical assays as shown in Fig. 1a. Positive characterization of each fraction was performed by means of specific assays for some protein groups. In the case of crotamine, convulxin and crotamine we used specific assays for each one as described by Toyama et al. (2000, 2001) and Passero et al. (2007). After purification, crotoxin was subjected to a new chromatographic procedure on a reverse phase column. In this condition, we purified two crotapotins and two new PLA2 isoforms (Fig. 1b) named as PLA2A and PLA2B. PLA2A accounted for approximately 26% of the whole crotoxin whereas PLA2B represented only 7%. Thus, we conducted most of the subsequent experiments with the PLA2A isoform. The investigation conducted with PLA2B was only enzymatic, especially in comparison with PLA2A. After the HPLC fractionation of crotoxin, both PLA2s (PLA2A and PLA2B) were subjected to Tricine SDS-PAGE that revealed the presence of only one electrophoretic band with molecular mass of approximately 14 kDa. Mass spectrometry analysis determined a molecular mass of 14,299.34 Da for PLA2A (Fig. 2a) and a molecular mass of 14,756.45 Da for PLA2B (data not shown). Examination of PLA2A by CD spectroscopy indicated a high content of alpha-helical structures (46.7%), beta-turns (35.6%), and random coils (17.6%). This result suggested that the predominant secondary structure of this PLA2A consisted of alpha-helices (Fig. 2b).

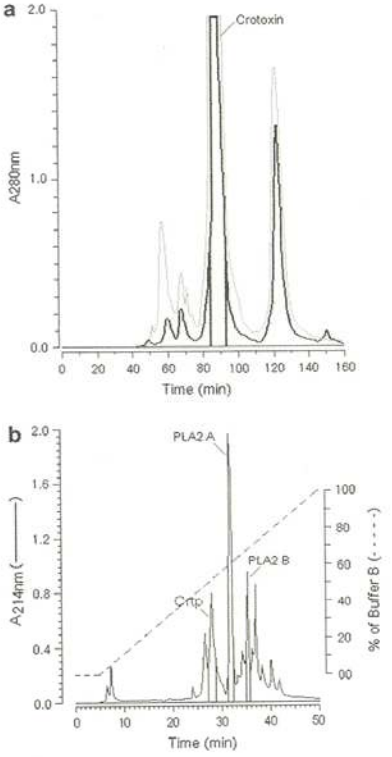


Fig. 1. a) Purification of whole venom of *Crotalus durissus ruruima* on the molecular exclusion chromatography. 10 mg of whole venom were dissolved in 400 µl of 1 M ammonium bicarbonate solution, homogenized and then centrifuged. The resulting supernatant was filtered and applied onto the chromatographic column (AP 1 × 60 cm – Waters, completed with Superdex 75 – GE Healthcare). This first chromatographic run was isocratic with constant flow rate of 0.2 ml/min and monitored at 280 nm. In this figure, the chromatograms of *Crotalus durissus ruruima* white venom and yellow venom are represented as black continuous line and light gray line, respectively. b) Chromatographic profile of reverse phase HPLC of crotoxin purified from the whole venom. Approximately 5 mg of this fraction were dissolved in 200 µl of buffer A. Crotoxin was fractionated in a linear gradient of concentration of buffer B. The chromatographic run was conducted at constant flow rate of 1.0 ml/min and monitored at 280 nm. In this condition, we purified from the whole venom of *Crotalus durissus ruruima* one main crotapotin fraction (Crtp) and two PLA2 fractions named as PLA2A (major isoform) and PLA2B (minor isoform).

PLA2A amino acid sequence was determined by N-terminal amino acid sequencing up to the 25th amino acid residue. "De novo" sequencing by mass spectrometry was performed in order to complete the remaining sequence determination. Alkylated PLA2A was digested with trypsin and the resulting peptides were fractionated

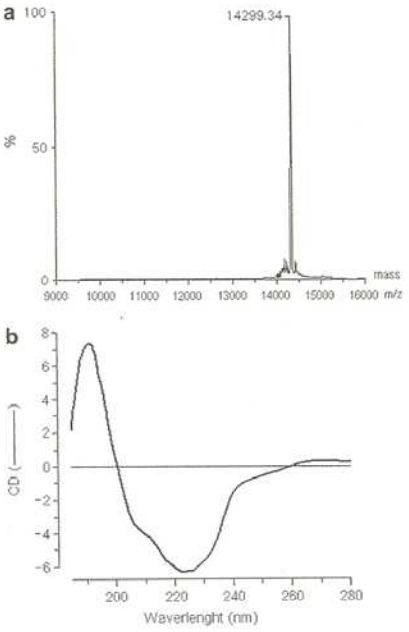
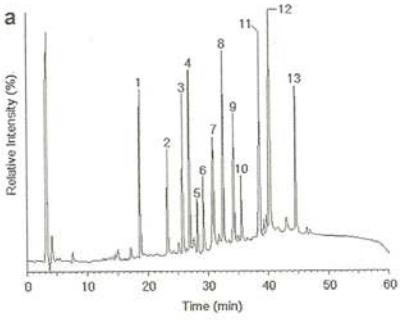


Fig. 2. The main purified PLA2 isoform, PLA2A, was subjected to Tricine SDS-PAGE electrophoresis in which only one electrophoretic band was observed (data not shown). a) Second aliquot of PLA2A was analyzed in the MALDI-TOFF mass spectrometry resulting in a molecular mass of 14,299.34 Da for PLA2A. b) CD spectra of native PLA2A. Data over the range 185–250 nm are shown. The CD spectra are expressed in theta machine units in millidegrees.



b

Fractions	Peptide sequence	[M+H]+
01	MIKFETRK	923.59
02	KNAIPFYAFYGCYCGWGGR	2171.97
03	GRPKDATDR	1014.52
04	CCFVHDCCYGK	1276.45
05	CNTK	464.21
06	WDIYPYSLK	1183.64
07	SGYITCGK	827.38
08	GTWCEEQICECDR	1570.59
09	HLLQFNK	983.76
10	VAAECLRR	916.49
11	SLSTYK	697.36
12	YGYMFYPDSR	1297.58
13	CRGPSETC	826.45

Fig. 3. a) RP-HPLC of the peptides obtained by tryptic digestion of the alkylated PLA2A eluted using a linear gradient of acetonitrile 66% in aqueous solution of TFA (0.1%). Each digest was designated as 1–13. All fractions were lyophilized and stored for subsequent amino acid sequencing in mass spectrometer. b) Summarized table of the $[M+H]^+$ ions of peptides resulted from trypsin digestion purified in the reverse phase HPLC (fractions 1–13). The molecular mass of the ions as determined by mass spectrometry, as wel as the related amino acid sequence, is presented.

by RP-HPLC (Fig. 3a). Peaks were numbered from 1 to 13 and manually collected, lyophilized and "de novo" sequenced by ESI-Q-TOF/MS/MS. Isoleucine and leucine residues were not discriminated in the reported sequence since they were indistinguishable in the low-energy CID spectra. Due to the external calibration applied to all spectra, it was also not possible to resolve the 0.036 Da difference between glutamine and lysine residues, except for the lysine that was deduced based on cleavage and missed cleavage of the protein by trypsin. The deduced sequence and measured masses of alkylated peptides of PLA2A are summarized in Fig. 3b. The sequence of each peptide was then independently compared to the NCBI non-redundant protein database using the search algorithm BLAST-P. Using the position matches of the "de novo" sequenced peptides to homologous proteins present in the database, it was possible to deduce their original position in PLA2A (Fig. 4a).

Primary structure of PLA2A revealed some amino acid replacements in comparison with Cdr-12 (*C. d. ruruima*) such as H(1) → S(1); R(33) → Q(33); F(45) → I(45); I(63) → F(63); E(83) → Q(83); K(104) → R(104); G(107) → E(107). In relation to Cdr-13 (*C. d. ruruima*), PLA2A showed other distinct replacements H(1) → S(1); L(3) → V(3); N(6) → E(6); R(5) → G(5); I(18) → V(18); F(45) → I(45); A(55) → V(55); I(63) → F(63); I(73) → F(73); K(104) → R(104) and G(107) → E(107) (Fig. 4a). The computational analysis of the primary structure of PLA2A showed a high amino acid sequence identity to PLA2 Cdt F15, isolated from *C. d. terrificus*, Cdr-12 and MOJAVE PLA2, from *Crotalus scutulatus scutulatus* (Fig. 4a). The amino acid sequence analysis revealed that PLA2A had a strong evolutionary relationship with Cdt F15 (Fig. 4b). The tandem mass spectra shown in Fig. 5, relative to the peptide eluted in fraction 4 (Fig. 3b), uncovered the sequence CCFVHDCCYGK, which allowed us to classify both enzymes as D49 PLA2. In addition, in Fig. 4a, the N-terminal amino acid sequencing of PLA2B up to the 30th amino acid residue is also presented.

The amino acid sequence analysis of PLA2A catalytic domain indicated high sequence identity with that of other sPLA2s, as shown in Fig. 4b. Thus, although not a good method for such purpose, we initially deduced this short

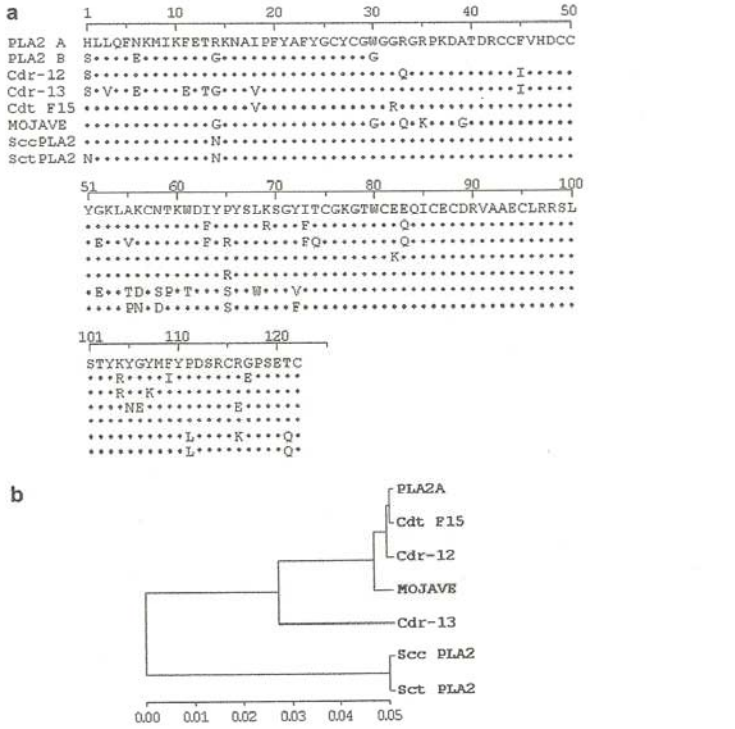


Fig. 4. a) Amino acid sequences of PLA2A (primary structure), PLA2B (N-terminal amino acid sequence at 30th residue). The primary structure of PLA2A was obtained by Edman degradation (N-terminal sequencing) and "de novo sequencing" by ESI-Q-TOF/MS/MS. The protocol used for this purpose followed the methodology described by Dal Bello et al. (2005). N-terminal sequence of PLA2A was determined up to the 25th residue and the remaining sequence was deduced by analysis of the results of $[M+H]^+$ ions of peptides. The PLA2B was submitted to the same treatment of PLA2A and its N-terminal was determined up to the 30th amino acid residue. Clustal X was employed for the comparison of PLA2A and PLA2B sequences with other PLA2 isoforms from the *Crotalus durissus ruruima* yellow venom (Cdr-12; and Cdr-13; Ponce-Soto et al., 2007), PLA2 isoform from *Crotalus durissus terrificus* (Cdt F15; Toyama et al., 2003), PLA2 from *Crotalus scutulatus scutulatus* (MOJAVE; MOJAVE toxin; John et al., 1994); PLA2 isoform from *Sistrurus catenatus catenatus* (SccPLA2; Gibbs et al., 1997) and PLA2 isoform from *Sistrurus catenatus tergeminus* (SctPLA2; Chen et al., 2004). b) Phylogeny relationship of PLA2A to other PLA2 isoforms statistically evaluated by Bootstrap method. PLA2B was excluded from this analysis.

sequence by comparison with other sPLA2s. Subsequently, we digested PLA2A with highly purified V8 protease from *S. aureus* (Promega) obtaining 13 different peptides, named fractions 01–13 that were purified by HPLC and sequenced. Fraction 10 as well as fraction 13 confirmed the presence of LAK in the catalytic domain. Other obtained peptides also confirmed the rest of PLA2A amino acid sequence, thus providing a verification of data from the first mass spectrometry sequencing.

3.2. Enzymatic studies

The PLA2 activity of PLA2A and PLA2B was examined using the synthetic substrate 4-nitro-3-(octanoyloxy)-benzoic acid (NOBA). PLA2A displayed a discrete sigmoidal behavior (Fig. 6a), especially at low substrate concentration (Fig. 6b). On the other hand, the PLA2B had an evident Michaelis–Menten behavior, as shown in Fig. 6a and b. V_{max} of the PLA2A and PLA2B were calculated to be 7.54 nmoles/min and 5.33 nmoles/min, respectively. These data also evoked that the K_M of PLA2A and PLA2B were 2.3 mM and 1.72 mM, respectively. Both PLA2A and PLA2B showed a maximum enzymatic activity at pH 8.0, the optimal temperature for the enzymatic activity was 40 °C (data not showed) and the PLA2B enzymatic activity was virtually abolished at temperatures higher than 60 °C, whereas PLA2A enzymatic activity was more resistant to higher temperatures. We also investigated the effect of different

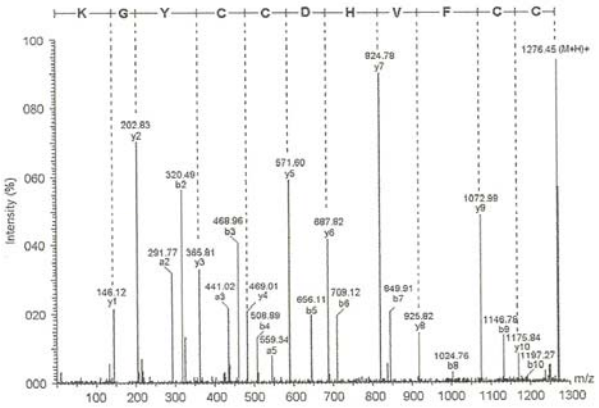


Fig. 5. ESI-Q-TOF/MS/MS spectrum of the 11-residue-long 1276.45 Da tryptic peptide eluted in fraction 4 of the RP-HPLC elution profile of PLA2A digest shown in Fig. 3a, containing the aspartic acid residue at position 48 in the amino acid sequence.

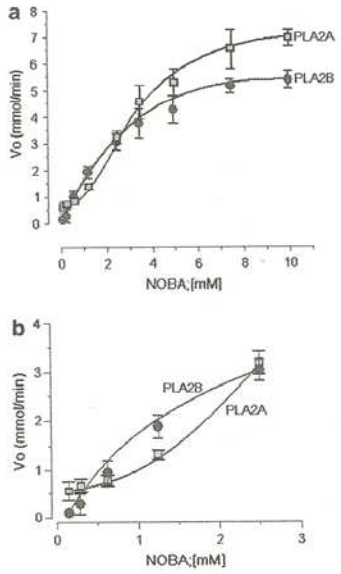


Fig. 6. a) Effect of substrate concentration on the enzymatic activity of PLA2A and PLA2B, the enzymatic activity was estimated by increase in absorbance of chromogenic product (p-anilide), the measurement of product was carried out using an ELISA system and wavelength at 425 nm, each point represent the mean of $n = 12$ experiment ($p < 0.05$). b) Detail of the curve shapes at low substrate concentrations.

p-bromophenacyl bromide (p-BPB), or crotapotin, concentrations on the enzymatic activity of PLA2A and PLA2B. The results shown in Fig. 7a suggested a significant difference between PLA2A and PLA2B in presence of different concentrations of p-BPB.

The minimal p-BPB concentration that virtually abolished the enzymatic effect of PLA2A was 0.75 mM. Yet the p-BPB concentration to completely inhibit PLA2B was higher than 3 mM (Fig. 7a). Fig. 7b shows the effect of different concentrations of crotapotin on the enzymatic activity of both PLA2A and PLA2B, indicating that the minimum concentration of such protein to inhibit the enzymatic activity of both PLA2A and PLA2B was 0.4 μM for 1 μM of each enzyme.

Both PLA2A and PLA2B showed a strict dependence on calcium ions. Complete replacement of Ca^{2+} for Mg^{2+}, Mn^{2+}, Sn^{2+} or Cd^{2+} strongly reduced the enzymatic activity of PLA2A to the same level as that in the absence of calcium (Fig. 8a). Fluorescence analyses suggested that calcium ions were effective to induce significant changes in the structure of PLA2A. However, the replacement for other divalent cations such as Mg^{2+}, Mn^{2+}, Cd^{2+} and Sn^{2+} did not induced the same modification of PLA2A structure (Fig. 8b).

3.3. Antibacterial activity

We evaluated the antibacterial properties of PLA2A on the *Xanthomonas axopodis pv passiflorae* that is commonly used as model. PLA2A displayed a notable antibacterial activity under the experimental conditions used, in which 75 μg of protein were capable of inhibiting about 96% of the bacterial growth rate (Fig. 9a). This antibacterial effect was strongly affected by EDTA (2 mM) or p-BPB (1 mM). Furthermore, incubation of PLA2A with heparin also strongly decreased this effect. Bacteria treated with PLA2A (75 μg/ml) exhibited massive and evident membrane

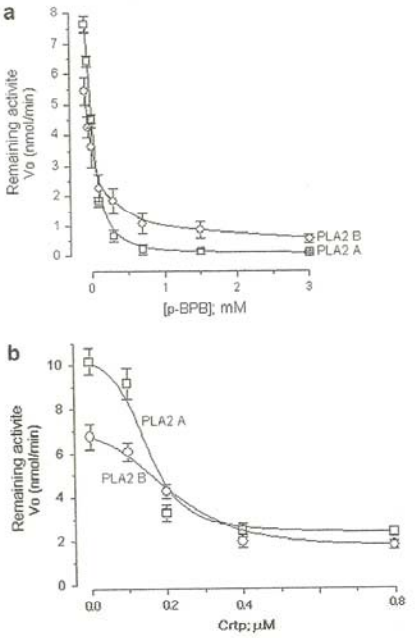


Fig. 7. a) Effect of different concentrations of p-bromophenacyl bromide (p-BPB) on the phospholipase A2 activity of PLA2A and PLA2B. b) Effect of different concentrations of crotapotin (Crtp) purified by reverse phase HPLC on the enzymatic activity of PLA2A and PLA2B. The measurement of chromogenic product (p-anilide) was carried out at 425 nm, each point representing the mean of $n = 12$ experiments ($p < 0.05$).

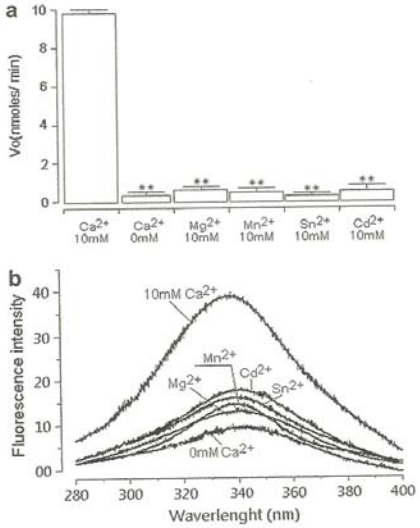


Fig. 8. a) Effect of different ions on the enzymatic activity of PLA2A, enzymatic activity was measured as described in Section 2. The measurement of chromogenic product (p-anilide) was carried out using an ELISA system and wavelength at 425 nm, each point representing the mean of $n = 12$ experiments ($p < 0.025$, in comparison with PLA2A with calcium 10 mM). b) Observed effect of different divalent ions on the fluorescence of native PLA2A.

perfusion followed by loss of their integrity. Membrane vesiculation and highly electron-dense structures in bacterial cells were observed probably due to the penetration of the fixative solution in cells (Fig. 9b).

4. Discussion

The presence of PLA2 isoforms in crotalinae snake venoms has been demonstrated by several authors and discussed by Valiente et al. (1992) and Fuly et al. (2002) reported in *Lachesis muta* venom the occurrence of a complex pattern of proteins that eluted at different ionic strengths from a DEAE-cellulose column, some of which displayed indirect hemolytic activity. Recently a comparison of the structures of 127 snake venom PLA2 enzymes indicated that the presence of mutational hot spots on the surface of such proteins (Kini and Chan, 1999). Natural substitutions occur about 2.6–3.5 times more in fully exposed residues than in buried residues. Surface residues are important in molecular recognition of target proteins. Thus, natural substitutions in such residues of an enzyme contribute directly towards modifying molecular surface and interactions, and consequently its specificity.

Several studies have shown that exons in PLA2 genes mutate faster than introns (Ogawa et al., 1992; Nakashima et al., 1995; Ohno et al., 2003). Such an accelerated evolution is proposed to result in the acquisition of diverse pharmacological properties (Ogawa et al., 1996). The surface substitutions and the accelerated evolution of exons play a significant role in the emergence of new isoenzymes by altering the target specificity. Through these mechanisms, nature appears to experiment with and modify the molecular surface to afford distinct and novel targeting to cells or tissues.

In this article we reported that the PLA2 fraction purified from the white venom of *C. d. ruruima* presented two distinct PLA2s (PLA2A and PLA2B), PLA2A being the main component.

The structural information and enzymatic studies on PLA2s from *C. d. ruruima* are not as detailed as those from *C. d. cascavella* or *C. d. terrificus* venoms. The structural characterization of PLA2A indicated that it was, at the primary structure level, very similar to Cdr-12 and Cdr-13, which were previously described by Ponce-Soto et al. (2007). These results suggest that *C. d. ruruima* venom has a large number of PLA2 isoforms with particular

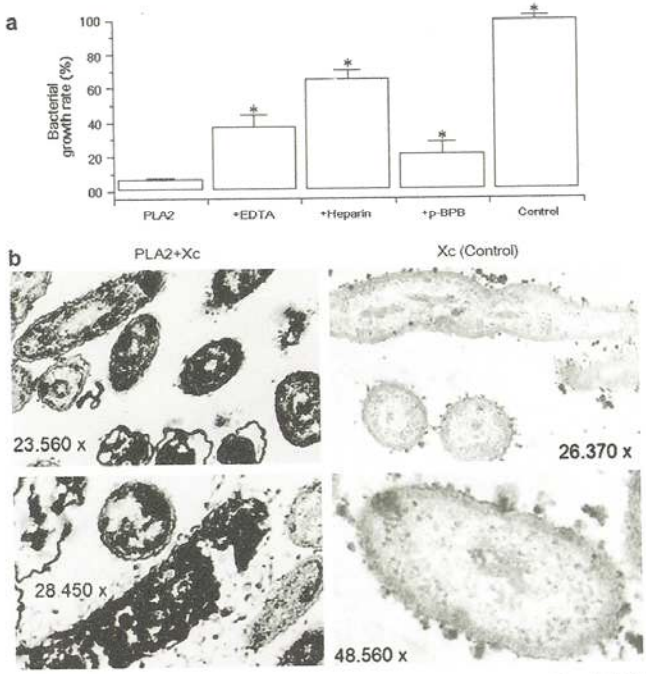


Fig. 9. a) Effect of EDTA, p-BPB and heparin on the antibacterial activity of PLA2A. b) Observed structural modifications induced by PLA2A on the *Xanthomonas axonopodis pv passiflorae*.

pharmacological characteristics as shown by Dos Santos et al. (1993, 2005).

The amino acid sequence of PLA2A compared with other isoforms of PLA2 from *C. d. ruruima* shows a histidine as the N-terminal of this protein. The N-terminal region of PLA2s displays important roles for catalysis as well as for pharmacological activities. The amino-terminal nitrogen lies at the centre of a hydrogen bond network that links it to the B-wing (residues 74–84) and Asp99 at the active site. Transaminating the N-terminal amino group of the bovine pancreatic enzyme induces structural change and reduces its catalytic ability on micellar, but not monomeric substrates (Scott and Sigler, 1994). It also helps to appropriately close the hydrophobic pocket of the PLA2. Thus mutations or chemical modifications in some of amino acid residues of such region strongly modified both enzymatic and pharmacological effect of these enzymes (Liu et al., 1995; Chang et al., 1994). The importance of the alpha helix for PLA2A is shown by CD spectra analysis.

The comparison of the amino acid sequence of PLA2A with that of PLA2B also revealed a slight modification. PLA2B N-terminal amino acids showed high amino acid similarity to Cdr-12 and Cdr-13. Probably such alteration affected the enzymatic activity of PLA2B, since PLA2A presented an allosteric behavior whereas PLA2B displayed a Michaelis–Menten behavior. The experimental results also showed significant differences between these two isoforms for optimal pH and temperature and for inhibition with crotapotin or p-BPB. Thus, it is possible to suggest that structural modifications found in the N-terminal region of PLA2A and PLA2B are responsible for each specifically observed enzymatic behavior, considering that the N-terminal octapeptide moiety of PLA2 possibly plays a role in maintaining the optimally arranged active site structure of the molecule. Comparison of data suggested that the N-terminal moieties of PLA2s from snakes of an elapid family and from mammalian pancreas were essential for catalysis of a micellar substrate, whereas those PLA2s from snakes of a viperid family, such as *T. flavoviridis*, were not (Oda et al., 1986).

The dependence on Ca^{2+} ions for full enzymatic activity of PLA2 was previously described by Oliveira et al. (2002) and Toyama et al. (2005). Accordingly, our experimental results showed that intrinsic fluorescence of PLA2A was

greatly enhanced upon the addition of Ca^{2+}. In class I and II enzymes, the Ca^{2+}-binding loop runs from positions 25–33, and contains four glycine residues which confer flexibility to the loop, allowing three carbonyl oxygen to coordinate the primary Ca^{2+} ion. In the substrate unbound form, the Ca^{2+} ion is also coordinated by the carboxy moiety of residue Asp 49 and two water molecules to result in a pentagonal bipyramidal arrangement (Chang et al., 1994). Since the I_{max} of tryptophan fluorescence in proteins varies over a 40-nm range, depending upon the polarity and rigidity of local environment (Ladokhin et al., 2000), the results obtained for PLA2A indicated that calcium, Ca^{2+}, ions induced a strong structural shift whereas its replacement of calcium for other divalent cations had no effect on the structure of PLA2A. In agreement to this fact, Chang et al. (2004) and Chu et al. (2005) proposed that the binding of Ca^{2+} induced a conformational change which activates the enzyme towards its toxic function, implying a role for the Ca^{2+}-binding loop in myotoxicity.

Since PLA2 enzymes require Ca^{2+} for their activity, chelators such as EDTA inhibit their enzymatic activity (Kini and Evans, 1989). The removal of Ca^{2+} ions sometimes affects normal functions of the PLA2 enzymatic system (Georgieva et al., 2000). Nonenzymatic mechanisms are not affected by EDTA or other metal ions, whereas the enzymatic contributions to the effect are compromised significantly. On the other hand, although alkylation does not strongly affect the conformation of PLA2 enzymes and its ability to bind to phospholipids, it could alter the ability of the enzyme to interact with specific proteins or ligands (Zhao et al., 1998). In our experiments, neither EDTA nor p-BPB abolished the antibacterial activity of PLA2A, only heparin significantly inhibited this activity (Fig. 9a). This result suggested that the enzymatic activity of PLA2A had a secondary role on the antibacterial activity, though the C-terminal region of PLA2A, or the positively charge amino acid located in this region, was probably determinant for this effect. Therefore the antibacterial activity of PLA2A is not strongly dependent on enzymatic activity of this enzyme.

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Conflict of interest

There is no conflict of interest.

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Quercetin as an inhibitor of snake venom secretory phospholipase A2

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Molecular docking

ABSTRACT

As polyphenolic compounds isolated from plants extracts, flavonoids have been applied to various pharmaceutical uses in recent decades due to their anti-inflammatory, cancer preventive, and cardiovascular protective activities. In this study, we evaluated the effects of the flavonoid quercetin on *Crotalus durissus terrificus* secretory phospholipase A2 (sPLA2), an important protein involved in the release of arachidonic acid from phospholipid membranes. The protein was chemically modified by treatment with quercetin, which resulted in modifications in the secondary structure as evidenced through circular dichroism. In addition, quercetin was able to inhibit the enzymatic activity and some pharmacological activities of sPLA2, including its antibacterial activity, its ability to induce platelet aggregation, and its myotoxicity by approximately 40%, but was not able to reduce the inflammatory and neurotoxic activities of sPLA2. These results suggest the existence of two pharmacological sites in the protein, one that is correlated with the enzymatic site and another that is distinct from it. We also performed molecular docking to better understand the possible interactions between quercetin and sPLA2. Our docking data showed the existence of hydrogen-bonded, polar interactions and hydrophobic interactions, suggesting that other flavonoids with similar structures could bind to sPLA2. Further research is warranted to investigate the potential use of flavonoids as sPLA2 inhibitors.

1. Introduction

Phospholipases A2 (PLA2, EC 3.1.1.4) are small proteins that catalyze the hydrolysis of glycerophospholipids at the sn-2 position in a Ca^{2+}-dependent reaction, releasing lysophospholipids and fatty acids [1–3]. These enzymes are the main component of snake venom and have been investigated not only because they have a wide range of biological effects, but also due to their similarity to mammalian phospholipases [4,5]. However, in contrast to their mammalian counterparts, several snake venom PLA2s are toxins that induce pharmacological effects [6] through arachidonic acid metabolism leading to the production of various lipid pro-inflammatory mediators such as prostaglandins, thromboxanes and leukotrienes [7]. Recent studies have shown that inhibition of cytosolic PLA2 (cPLA2) leads to a decrease in eicosanoid levels and, reduced inflammation [8].

Due to the role of PLA2s in the inflammatory process, there is pharmacological interest in PLA2 inhibitors, and among these, the flavonoids have been successfully studied. Flavonoids are widely produced in plants tissues making them suitable targets for pharmaceutical extraction and chemical synthesis [8,9]. The inhibitory effect of flavonoids on secretory PLA2 (sPLA2) was reported by Gil et al. [10], and Lindahl and Tagesson [11]. Their results showed that inhibition of sPLA2 from different sources following incubation with various flavonoids is dependent on the 5-hydroxyl group as well as the double bond and the double-bonded oxygen in the oxane ring, and that the hydroxyl groups at the 3′- and 4′-

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position are required for selective inhibition of PLA2 [11]. However, the exact mechanism by which flavonoids inhibit PLA2 remains unclear. Iglesias et al. [12] showed that morin modifies the secondary structure of sPLA2 from *Crotalus durissus cascavella* venom, but did not significantly affect its pharmacological activities.

Although there are studies of flavonoids as PLA2 inhibitors, the mode of binding of flavonoids to PLA2 as well as their inhibitory mechanism is still not clear. The aims of this article are to investigate the effect of quercetin, a widely spread flavonoid, on the structure and function of a sPLA2 isolated from *Crotalus durissus terrificus*, to increase our understanding of the mode action of polyphenolic compounds on the snake venoms, and to evaluate therapeutic application against the symptoms caused by snake bites. In the last decades the action of quercetin on the PLA2 has been studied. In 1993 Lindahl and Tagesson [13] showed that quercetin is a potent inhibitor of PLA2 (group II) from *Vipera russuli*. In addition, Lättig et al. [8] showed through computational studies chemical interactions between humans' sPLA2 and quercetin. Due to these characteristics, quercetin has been chosen as flavonoid model. Moreover we also propose, through high-resolution three-dimensional (3D) data (molecular docking), a structural model for understanding the molecular interactions between sPLA2 and quercetin, and how it can influence enzymatic and pharmacological activities of sPLA2.

2. Materials and methods

2.1. Venom, animals and reagents

C. durissus terrificus venom was purchased from Bio-Agents Serpentarium (Batatais, São Paulo, Brazil). Analytical HPLC- and sequencing-grade solutes and solvents were purchased from various suppliers (Bio Rad, Sigma Aldrich, Boehringer Mannheim, and Applied Biosystems). Female Swiss mice (18–20 g) used in the pharmacological assays were obtained from the Multidisciplinary Center of Biological Investigations (CEMIB-UNICAMP) and male chicks were obtained from Itu farm in Campinas City. All animal experiments were approved by the Ethics Committee of State University of Campinas (São Paulo, Brazil) under the number 1916-1.

2.2. Purification of sPLA2

Whole *C. durissus terrificus* venom was first fractioned as previously described by Toyama et al. [14]. Dried venom (45 mg) was dissolved in ammonium bicarbonate buffer (1.0 M; pH 8.0) and clarified by centrifugation (4500 × *g* for 1 min). The sPLA2 from *C. durissus terrificus* was eluted using a non-linear gradient of acetonitrile 66% in 0.1% of trifluoroacetic acid (TFA) by reverse-phase chromatography using a Supelco C5 column (0.10 cm × 25 cm) with flow rate of 1 mL/min with absorbance monitoring at 280 nm. The resulting PLA2 was termed sPLA2 and its purity was evaluated by SDS-PAGE.

2.3. Incubation of sPLA2 with quercetin and purification of modified sPLA2

The incubation of sPLA2 with quercetin (mol:mol) was according to the procedure described by Zhao et al. [15]. Quercetin was dissolved in dimethyl sulfoxide (DMSO), and its concentration never exceeded 1% during incubation. Quercetin (400 μL of a 0.1 mM solution) was added to 400 μL of a homogenized, purified sPLA2 solution (1 mg/mL). The mixture was incubated for 90 min at room temperature, and 200-μL samples of this mixture were loaded onto a preparative reverse-phase column to separate the treated enzyme (sPLA2:Q) from quercetin. After column equilibration with HPLC buffer A (aqueous 0.1% TFA), samples were eluted using a discontinuous gradient of HPLC buffer B (66.6% of acetonitrile in 0.1% TFA) at a constant flow rate of 1.0 mL/min. The chromatographic run was monitored at 280 nm.

2.4. Electrophoresis

Electrophoresis was carried out following the Laemmli method [16]. The degree of purity of fractions was assessed by discontinuous electrophoresis using a final acrylamide concentration of 12.5% in the resolving gels (1.0 M Tris–HCl, pH 8.8) and 5% in the stacking gel (0.5 M Tris–HCl, pH 6.8). Electrophoretic separation was carried out in a 250 Mighty Small (Hoefer Scientific Instruments) for SDS-PAGE. All samples and the molecular marker were treated with SDS and 1.0 M dithiothreitol (DTT), and the run was conducted at 60 mA for stacking gel and 90 mA for running gel. After electrophoresis, samples were stained with Coomassie brilliant blue R-250.

2.5. Circular dichroism spectroscopy

sPLA2 and sPLA2:Q (sPLA2 + quercetin) were dissolved in 10 mM sodium phosphate buffer (pH 7.4) and final protein concentrations were adjusted to 8.7 mM. After centrifugation at 4000 × *g* for 5 min, samples were transferred to a 1-mm path length quartz cuvette. Circular dichroism spectra in the wavelength range 185–300 nm were acquired in-house with a J720 spectropolarimeter (Jasco Corp., Japan) using a bandwidth of 1 nm and a response time of 1 s. Data collection was performed at room temperature, with a scanning speed of 100 nm/min. Nine scans were obtained for each sample, and all spectra were corrected by subtracting buffer blanks.

2.6. Intrinsic fluorescence.

The relative intrinsic fluorescence intensities of sPLA2 and sPLA2:Q were monitored with a Varian Cary Eclipse. The proteins were solubilized in water at room temperature. The measurements were performed in a 1.5-mL 1-cm path length quartz cuvette. Fluorescence was measured between 300 and 450 nm after excitation at 280 nm.

2.7. Mass spectrometry

The molecular mass of sPLA2 and sPLA2:Q were determined by matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry using a Voyager-DE PRO MALDI-TOF mass spectrometer (Applied Biosystems). One microliter of samples (sPLA2 and sPLA2:Q) in 0.1% TFA was mixed with 2 μL of the matrix α-cyano-4-hydroxycinnamic acid, 50% acetonitrile, and 0.1% TFA (v/v). The matrix was prepared with 30% acetonitrile and 0.1% TFA (v/v). Ion masses were determined with an acceleration voltage of 25 kV, the laser operated at 2890 μJ/com2, a 300-ns delay, and the linear analysis mode.

2.8. Measurement of sPLA2 activity

sPLA2 activity was measured following the protocol described by Lee et al. [17] and modified by Toyama et al. [14] in 96-well plates, using 4-nitro-3-octanoyloxy-benzoic acid (4N3OBA, BIOMOL, USA) as substrate. Enzyme activity, expressed as the initial velocity of the reaction (Vo), was calculated based on the increase in absorbance after 20 min. All assays were performed with absorbance at 425 nm using a SpectraMax 340 multiwell plate reader (Molecular Devices, Sunnyvale, CA). After the addition of native or treated sPLA2 (20 μg), the reaction mixture was incubated for up to 40 min at 37 °C and the absorbance read at 5-min intervals.

2.9. Antibacterial activity

The antibacterial activity was assayed as described by Santi-Gadelha et al. [18] and the structural modification was done following the method of Toyama et al. [19]. *Clavibacter michiganensis michiganensis* cells were harvested from fresh agar plates and suspended in sterile distilled water ($A_{600\,nm} = 3 \times 10^8$ CFU/mL). Aliquots of bacterial suspension were diluted to 10^3 colony-forming units/mL (CFU/mL) and incubated with sPLA2 or sPLA2:Q samples (75 μg/mL) for 60 min at 28 °C. Survival was assayed on nutrient agar (Difco) plates ($n = 5$). For both antibacterial assays, electron microscopy assessments of morphologic alterations were performed in the presence of saline (negative control), quercetin (100 μM, quercetin control), sPLA2, and sPLA2:Q.

2.10. Paw edema assay

Paw edema assays were performed with the aim of evaluate the inflammatory activity induced by sPLA2. For this assay female Swiss mice were used, since they show a lower aggressive behavior than male mice. Paw edema was induced by a single subplantar injection of 25 μL sPLA2 or sPLA2:Q (25 μg/paw). Paw volume was measured immediately before the injection and at selected time intervals thereafter (30, 60, 120, 180, and 360 min) using a hydroplethysmometer (model 7150, Ugo Basile, Italy). All samples were dissolved in sterile saline solution (0.9%). Results were expressed as the increase in paw volume (μL) and calculated by subtracting the basal volume from the volume following treatment.

2.11. Neurotoxic effect assay

Male chicks (4–8 days old) were killed with ether, and the biventer cervicis muscle was removed [20] and mounted under a resting tension of 1 g in a 4 mL organ bath containing aerated (95% O_2 + 5% CO_2) Krebs solution (118.7 mM NaCl, 4.7 mM KCl, 1.88 mM $CaCl_2$, 1.17 mM KH_2PO_4, 1.17 mM $MgSO_4$, 25.0 mM $NaHCO_2$ and 11.65 mM glucose, pH 7.5) at 37 °C. A bipolar platinum ring electrode was placed around the tendon, which ran the length of the nerve trunk supplying the muscle. Indirect stimulation was applied with a Grass S4 stimulator (0.1 Hz, 0.2 ms, 3–4 mV). Muscle contractions and contractures were recorded by connecting the preparation to a force displacement transducer (Narco Biosystems Inc.) coupled to a Gould RS 3400 recorder. Contractures to exogenous acetylcholine (ACh, 55 or 110 μM for 60 s) and KCl (5 mM for 120–130 s) were obtained in the absence of nerve stimulation prior to the addition of sPLA2 or sPLA2:Q (10 μg/mL) and at the end of the experiment. The preparations were allowed to stabilize for at least 20 min before the addition of Ach, KCl, or a single concentration (10 μg/mL) of the compounds.

2.12. Platelet aggregation studies

Platelet aggregation activities were assayed as described by Toyama et al. [19]. Briefly, venous blood was collected with informed consent from healthy volunteers who denied taking any medication in the previous 14 days. Collected blood was immediately transferred into polypropylene tubes containing one-tenth of final volume of acid citrate dextrose (ACD-C; citric acid 3%, trisodium citrate 4%, glucose 2%; 1:9 v/v). Platelet-rich plasma (PRP) was obtained by centrifuging whole blood at 200 × *g* for 15 min. PRP was washed in a wash buffer solution (NaCl 140 mM, KCl 5 mM, sodium citrate 12 mM, glucose 10 mM and saccharose 12 mM; pH 6; 5:7 v/v) and centrifuged at 800 × *g* for 12 min at 20 °C. The platelet pellet was gently resuspended in Krebs–Ringer solution and counts were performed on a Neubauer chamber. The final platelet suspension was adjusted to 1.2×10^8 platelets/mL. Platelet aggregation was carried out using 400 μL of the washed platelet solution in a cuvette and incubated at 37 °C with constant stirring. The desired concentration of protein was added 3 min prior to the addition of a platelet aggregation inducer (thrombin). Aggregation was subsequently recorded for 5–10 min with an aggregometer (Chrono-log Lumi-Aggregometer model 560-Ca, Havertown, PA, USA). Aggregation experiments were performed with a concentration of 15 μg/mL of sPLA2 and sPLA2:Q.

2.13. Myotoxic activity

The liberation of creatine kinase (CK) from damaged muscle cells was followed by use of the CK-NAc kit (Laborlab) to measure the enzyme activity in mice plasma. Five groups of animals (18–22 g) were injected in the right gastrocnemius muscle with 25 μL of 1.0 mg/mL of sPLA2, sPLA2:Q, or quercetin ($n = 4$) while the control group received an equal volume of 0.15 M NaCl. Blood was collected from the tail after 1 h into tubes containing heparin. The amount of CK was determined using 4 μL plasma, which was incubated for 3 min at 37 °C with 1.0 mL of the reagent according to the kit protocol. Activity was expressed in units per liter (U/L).

2.14. Molecular modeling (docking)

The structural optimization of the quercetin ligand was initially achieved using the quantum chemical AM1 method [21] implemented in the BioMedCache program (BioMedCache, 1989) with default values for the convergence criteria. Docking calculations were performed with the GOLD 4.0 program [22] to obtain the *in silico* affinity of quercetin to the Crotoxin B target, a basic sPLA2 from *C. durissus terrificus* venom. This sPLA2 structure was taken from the RCSB Protein Data Bank [PDB] under the PDB code 2QOG. The structure of the B chain was chosen for calculations, maintaining the Ca^{2+} ion and the water molecule number 188, located 3.16 Å from Ca^{2+}.

Docking calculations were performed to consider the flexibility of the quercetin ligand in such a way that torsions were considered active during the calculation. The active site was defined as all atoms within a 10-Å radius from His48, an important residue according to the literature [23,24].

2.15. Statistical analyses

Results were reported as means ± SEM of replicate experiments. The significance of differences between means was assessed by an analysis of variance, followed by a Dunnett's test when several experimental groups were compared to the control group. The confidence limit for significance was 5%.

3. Results

3.1. Purification of sPLA2 and Incubation of sPLA2 with quercetin

sPLA2 was isolated from *C. durissus terrificus* venom through reverse-phase chromatography (Fig. 1a) and its enzymatic activity was evaluated using 4-nitro-3-octanoyloxy-benzoic acid (4N3OBA) as a substrate. SDS-PAGE revealed the presence of one protein band with a molecular mass of 14 kDa (Fig. 1c), corresponding to sPLA2. After incubation with quercetin, sPLA2:Q (sPLA2 + quercetin) eluted at 23.7 min whereas sPLA2 had a retention time of 24.3 min (Fig. 1b). This difference indicates an interaction between sPLA2 and quercetin, which changed the hydrophobicity of sPLA2.

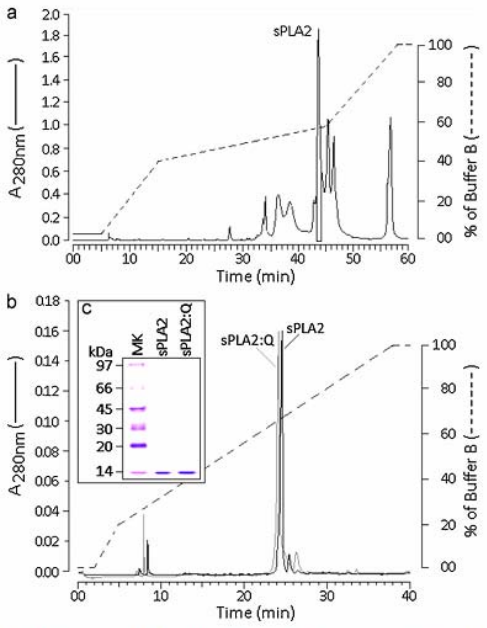

Fig. 1. Purification and chemical modification of secretory phospholipase A2 (sPLA2). (a) Fractionation of whole venom was performed by reverse-phase HPLC (C5 column 0.10 cm × 25 cm) using a non-linear concentration gradient of buffer to obtain a high purity protein. (b) Reverse phase HPLC profile of sPLA2 before and after modification with quercetin. (c) Tricine SDS-PAGE of sPLA2 from *Crotalus durissus terrificus*.

3.2. Circular dichroism spectroscopy

The effect of quercetin on the sPLA2 structure was evaluated by absorption spectra of sPLA2 and sPLA2:Q, circular dichroism, and fluorescence spectroscopy. As shown in Fig. 2a, few modifications were observed in the 270–280 nm wavelength region. However, some changes were observed on CD and fluorescence spectra after treatment with quercetin. CD spectra analysis showed modifications mainly in the region corresponding to the α-helices, suggesting that quercetin is able to induce changes in the secondary structure of this enzyme (Fig. 2b).

3.3. Intrinsic fluorescence

The presence of aromatic amino acids such as tryptophan and tyrosine in the protein chain allows the use of fluorescence spectra, which is sensitive for the investigation of protein conformation and ligand binding. Fig. 3a shows an increase in the intensity of fluorescence emission spectra after treatment with quercetin, suggesting that this flavonoid is able to change the structure of the protein at the tertiary structure level, however, fluorescence spectrum of quercetin has shown that this compound has a peak of fluorescence near the region of the tryptophan and possibly contributes to increase the fluorescence observed.

3.4. Mass spectrometry

Mass spectrometry by MALDI-TOF indicated that the exact molecular mass of the native protein is 14,425.56 Da while that of sPLA2:Q is 14,727.79 (Fig. 3b), an increase of 302.23 Da in sPLA2 treated with quercetin, suggesting that one molecule of quercetin is bound to the protein structure.

3.5. Measurement of sPLA2 activity and antibacterial activity

The effect of quercetin on sPLA2 enzymatic activity was evaluated. Both enzymes, sPLA2 and sPLA2:Q exhibit allosteric behavior (Fig. 4a), and quercetin strongly inhibits sPLA2 activity. The maximum velocity after 20 min for sPLA2 was 0.330 ± 0.04 vo/mol whereas for sPLA2:Q was 0.196 ± 0.02 vo/mol, showing a significant decrease of 40%. To analyze possible correlations between the enzymatic activity of sPLA2 and its antibacterial activity, bacterial viability was tested by CFU counting. The assay was performed using *C. michiganensis michiganensis* (Gram-positive). As shown in Fig. 4b, sPLA2 has a higher inhibitory potential on bacterial growth than sPLA2:Q, since sPLA2 decreased CFU levels to 9.8% (90.2% inhibition) compared to 63.5% (only 36.5% inhibition) for sPLA2:Q.

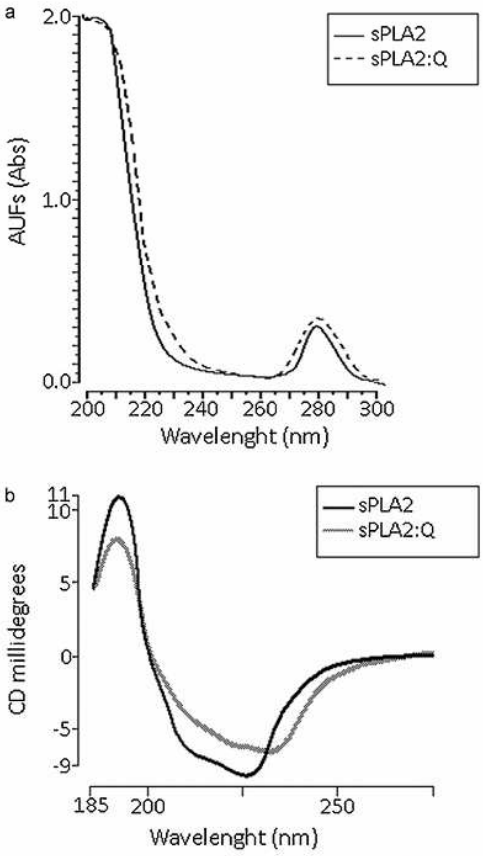

Fig. 2. UV/vis absorption and circular dichroism (CD) spectra. (a) Absorption spectrum of sPLA2 and sPLA2 after treatment with quercetin (sPLA2:Q) at wavelength intervals 200–300 nm. The proteins were analyzed at 280 nm. (b) CD spectra of native sPLA2 and sPLA2:Q. Data over the range 185–280 nm are shown. The CD spectra are expressed in theta machine units in millidegrees.

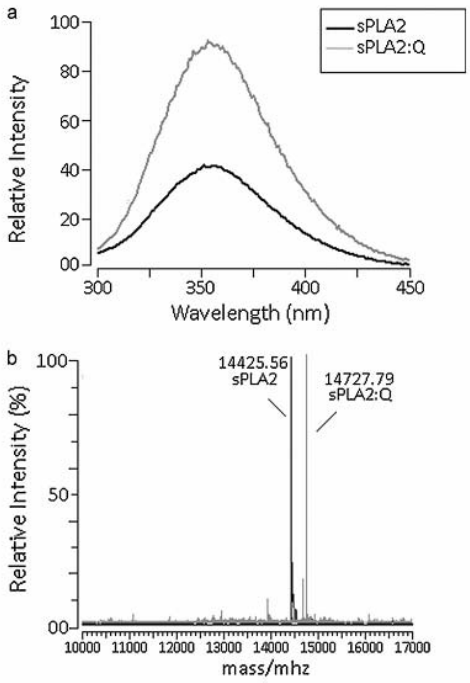

Fig. 3. Intrinsic fluorescence and mass spectrometry. (a) Intrinsic fluorescence of native sPLA2 and sPLA2:Q was measured with excitation at 280 nm and emission monitoring between 300 and 450 nm. (b) MALDI-TOF mass spectrometry analysis of native sPLA2 and sPLA2:Q shows a difference between the molecular masses corresponding to one molecule of bound quercetin.

3.6. Paw edema assay

Since flavonoids have shown a good capacity to inhibit sPLA2 and consequently decrease its pro-inflammatory activity [13,25], the effect of quercetin on sPLA2 was evaluated. Following subplantar injections on Swiss mice, sPLA2 had a huge potential to induce edema after 60 min (Fig. 5a). Under the same experimental conditions, sPLA2:Q did not show a decrease in edema effect, but the maximum edema was observed after 30 min.

3.7. Neurotoxic effect assay

When the neurotoxic activity was evaluated, both sPLA2 and sPLA2:Q induced neuromuscular blockage, but sPLA2 induced a faster effect at 80 min, whereas sPLA2:Q had a similar behavior at 100 min (Fig. 5b), suggesting that quercetin changed the velocity of native sPLA2 binding to the neurotoxic site of chick biventer muscle. However, this change is not able to abolish the neurotoxic effect of sPLA2.

3.8. Platelet aggregation studies

In order to evaluate the anti-coagulant potential induced by sPLA2 were performed platelet aggregation assays. The results have shown that sPLA2 from *C. durissus terrificus* has a moderate ability to cause aggregation of washed platelets. While sPLA2 from *C. durissus cascavella* induces about 85% aggregation at 3 μg/μL [26], sPLA2 from *C. durissus terrificus* induced 70% aggregation at a concentration of 20 μg/μL. Treatment with quercetin decrease this effect, with 32% aggregation induced by sPLA2:Q at the same concentration (Fig. 5c).

3.9. Myotoxic activity

The ability of sPLA2 to cause myonecrosis was also evaluated through measurement of released creatine kinase. Fig. 5d shows that sPLA2 induces an increase in plasma creatine kinase levels of 821.39 ± 107.8 U/L, indicating its ability to cause muscle damage. Treatment with quercetin significantly decreased the creatine kinase levels measured to 492.28 ± 71.5 U/L, a 40% decrease.

3.10. Molecular modeling (docking)

In order to analyze possible intermolecular interactions between sPLA2 and quercetin, we performed *in silico* studies using molecular modeling (docking). The best docking solution for the quercetin ligand is shown in Fig. 6a. The GOLD score for this result was 43.94, showing good affinity for the target. The presence of several important intermolecular interactions such as (i) hydrogen bonds with residues Cys45 and His48 at 2.69 and 3.00 Å, respectively; (ii) hydrogen bond with water molecule 188 at 3.16 Å; (iii) one polar contact with Ca^{2+} ion at 3.19 Å; and (iv) hydrophobic interactions with residue Phe5 can account for the stability of the quercetin–sPLA2 complex (Fig. 6a and b).

4. Discussion and conclusions

In this study, sPLA2 from *C. durissus terrificus* was modified by quercetin, a flavonoid known for its anti-inflammatory activ-

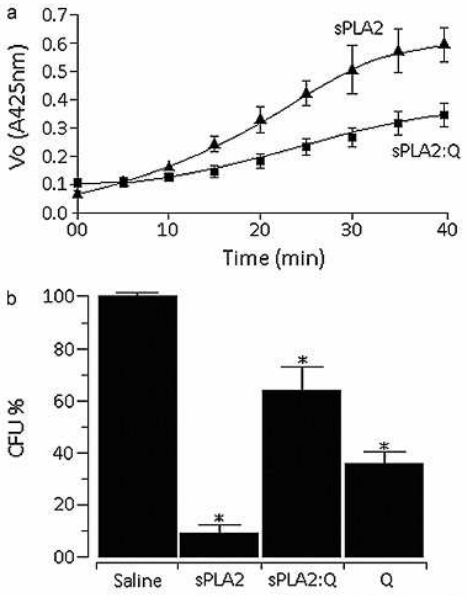

Fig. 4. Enzymatic and antibacterial assays. (a) Enzymatic activity was analyzed using 4N3OBA as substrate and monitored at wavelength 425 nm. sPLA2:Q shows a significant decrease compared to native sPLA2. (b) Effect of native sPLA2 and sPLA2:Q against Gram-positive bacteria. Quercetin decreases the ability of sPLA2 to inhibit Gram-positive bacteria. Error bars indicate SEM *$P < 0.05$ compared to saline control.

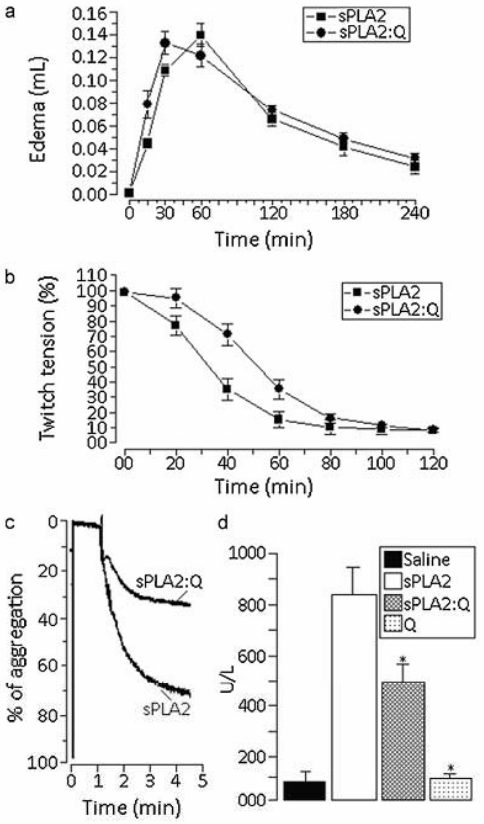

Fig. 5. Pharmacological assays. (a) Paw edema induced after the injection of sPLA2 and sPLA2:Q (25 μg/paw) into the right paw of Swiss mice. Measurements were done after 30, 60, 120, 180 and 240 min, with no differences observed after treatment with quercetin. (b) Neurotoxic effect of sPLA2 and sPLA2:Q on chick biventer cervicis muscle. Results were expressed as the percentage change in twitch tension. (c) Percent platelet aggregation caused by native sPLA2 and sPLA2:Q. The washed platelets assay was performed with venous blood collected from healthy volunteers and the concentration of both proteins was 15 μg/mL (d) Myonecrosis was assayed based on the creatine kinase levels in Swiss mice. Twenty-five micrograms of native sPLA2, sPLA2:Q, and pure quercetin were injected into the gastrocnemius muscle. Results were expressed as units of enzymatic activity per liter (U/L). Error bars indicate the SEM. *$P<0.05$ compared to sPLA2 activity.

ities. Treatment with quercetin resulted in modification of the protein secondary structure as observed in circular dichroism assay (Fig. 2b). Similar results were observed by Iglesias et al. [12] with sPLA2 from *C. durissus cascavella* after modification with the flavonoid morin. Although a secondary structure modification could be observed, the results do not allow concluding about tertiary structure modification, once the fluorescence spectrum of quercetin has shown that this compound has a fluorescent peak near the region of the tryptophan, suggesting that the increase in the total fluorescence of the protein might be caused due to the bonding of quercetin.

Treatment with quercetin led to a decrease of about 40% in sPLA2 enzymatic activity, similar to p-bromophenacyl bromide (p-BPB), a PLA2 inhibitor. Docking studies suggest that quercetin binds in the vicinity of the His48 residue, leading to inhibition of enzymatic activity. As shown by Verheij et al. [23], and Scott and Sigler [24], these amino acids are fundamental to enzymatic activity; His48 is involved in the first step of the enzymatic mechanism, and Asp49 is important for binding to Ca^{2+}, a cofactor. It seems that quercetin bound to sPLA2 interferes with the binding between the protein and its substrate. Soares et al. [27] suggested that enzymatic activity is not required for antibacterial activity after chemical modification with BPB and EDTA in a *C. durissus terrificus* sPLA2 isoform, and the same was observed by Diz Filho et al. [28] for *Crotalus durissus ruruima*. However, our results show correlation between the enzymatic and antibacterial activities against Gram-positive bacteria. Treatment of sPLA2 with quercetin decreased both these activities. According to Buckland and Wilton [29], mammalian PLA2 phospholipid hydrolysis is correlated with antibacterial activity because both are calcium dependent. We believe this isoform behaves as a mammalian PLA2 in this respect.

Several studies have investigated the anti-inflammatory effects of flavonoids as well as their effect on both catalytic and pharmacological activities of PLA2 [8,11,12,30,31]. According to these models, quercetin molecules would interact with the enzymatic site thus affecting enzymatic and pharmacological activities. Our results have showed that some pharmacological activities such as inflammatory and neurotoxicity were not inhibited after treatment with quercetin. This result corroborates the proposal by Ohno et al. [32], who suggested the existence of a distinct pharmacological site

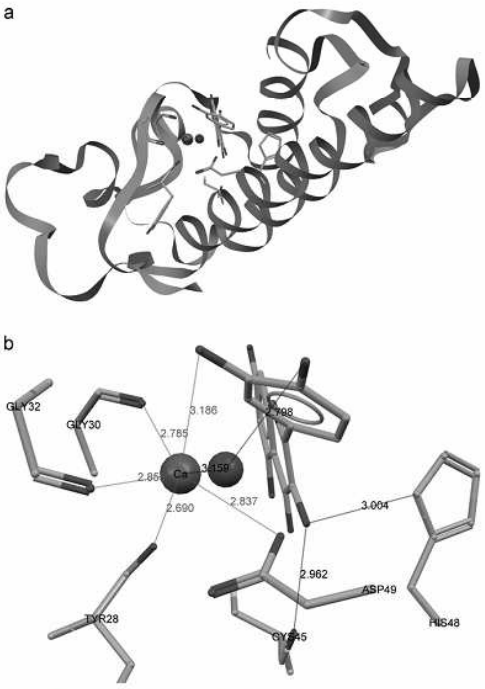

Fig. 6. Molecular docking. (a) Best docking solution for quercetin (stick model) and important interacting residues of the Crotoxin B target, besides Ca^{2+} ion and water oxygen. (b) Detailed view, with hydrogen bonds (distances in Å) shown as lines not involving the Ca^{2+} ion. Hydrogen atoms were omitted for clarity and the lines linked to the Ca^{2+} ion represent the coordination polyhedron.

near the C-terminal region of PLA2 away from the catalytic site. After modifications with 7-hydroxycoumarin, Toyama et al. [31] observed that decreasing the enzymatic activity had not suppressed the pharmacological effect of PLA2, suggesting the presence of a distinct site. Although the inflammatory and neurotoxic effects were unchanged after treatment with quercetin, the myotoxic and platelet aggregation effects were decreased by 40% and 55%, respectively, indicating dependency on the enzymatic activity. We believe that perhaps both the myotoxic and the platelet aggregation sites are located in a second pharmacological site near the calcium binding loop as proposed by Valentin and Lambeau [33]. Thus, after binding to the protein near that region, quercetin affects this site more directly leading to considerable changes in these activities.

Molecular docking has been used during the last decades due to its importance in elucidating, through computational approaches, the best matches for protein–ligand interactions [34]. In this study, molecular docking was an important tool in evaluating the interactions between sPLA2 and quercetin. Our result agrees with that observed by mass spectrometry, showing that one molecule of quercetin is binding to the protein. Additionally, molecular docking was useful in elucidating some important interactions between sPLA2 and quercetin, which allowed better understanding of the molecular reasons for the effects of quercetin as well as other structurally similar flavonoids that could be studied in the future.

The results shown here led us to conclude that quercetin binding to the protein decreased the catalytic activity of sPLA2 from *C. durissus terrificus*. This sPLA2 probably has two distinct pharmacological sites: one responsible for effects such as platelet aggregation, myotoxicity, and antibacterial activities; and another responsible for inflammatory and neurotoxic effects. We believe the first one is located near the calcium-binding loop region and consequently near the catalytic site where quercetin binds to the protein, thus leading to a loss of activities dependent on this site. The second one, we believe is located near C-terminal region, away from where quercetin binds, and is not directly or indirectly affected by the quercetin binding to the catalytic site. Other flavonoids with very similar chemical structures have a potential to interact with sPLA2 from serpents. Further docking studies with new molecules of this class could be useful in predicting how potent these compounds could be as sPLA2 inhibitors. Although our study clarifies some aspects of the chemical and pharmacological interactions between a flavonoid and a sPLA2, further studies are necessary to better understand the action of flavonoids on sPLA2 activities.

Conflict of interest statement

The authors have no conflict of interest to disclose.

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Pareceres Comitê Ética

DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha dissertação/tese de Mestrado/Doutorado intitulada Modificação estrutural de PLA2 de *Crotalus durissus ruruima* e *Crotalus durissus cumanensis* com p-bromofenacil e cumarinas sintéticas – Caracterização bioquímica e biológica. Estudo da agregação plaquetária e efeito edematogênico

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