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Miotoxinas PLA₂ "like" de *Bothrops pirajai*:
caracterização molecular e funcional.

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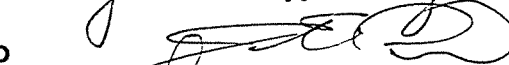
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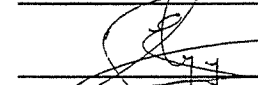
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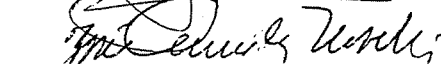
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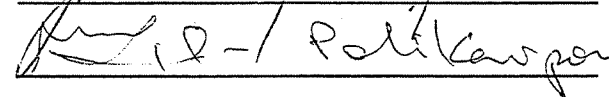
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Lista de Abreviações

I

Lista de Abreviações - Cromatografia

C18	18 High Carbon Load, High activity silica
CLAE	Cromatografia Líquida de Alta Eficiência (HPLC)
CM	Grupo funcional Carboximetil das coluna de troca aniônicas.
CM Sephadex C-50	Coluna de Aniônica com matriz composta de Dextran, Sephadex G50
CM Sepharose CL6B	Coluna de troca Aniônica com matriz composta de Agarose 6%, interligadas.
HPLC	High (Pressure/ Performace) Liquid Chromatography
LC	Sistema cromatografico HPLC de purificação de proteínas biocompatível da Waters, similar ao sistema FPLC da Pharmacia.
PDA	Photodiode Array Detector
Protein-Pak	Serie de Coluna da Waters para Troca Iônica baseadas em polimeros de polimetacrilato , tanto em colunas em vidro como em aço inox polido.
Protein-Pak SP5PW	Trocador polimérico Aniônico forte
RP HPLC	Reverse Phase HPLC
SP	Grupo funcional Sulfopropil das coluna de troca aniônicas
TFA	Ácido Trifluoroacetico
μ-Bondapack C18	Coluna de HPLC com n-octadecyl como base da fase estacionária

Lista de Abreviações - Análise de Aminoácidos, Sequenciamento de Proteínas

AZT	Anilinothiazolina
PICO-TAG	Sistema geral de análise de aminoácidos composto por uma estação de trabalho para hidrólise e derivatização de aminoácidos e por um sistema cromatografico HPLC de separação, detecção e análise dos aminoácidos derivatizados.
PITC	Fenilisotiocianato
PTC	Feniltiocarbamil
PTH	Feniltiohidantoina

Lista de Abreviações - Reagentes, Sais, Tampões

BIS	N,N'- metileno-bis-acrilamida
CAPS	Ácido 3-ciclohexalamino-1 Propanosulfônico
DTT	Ditiotreitol
EDTA	Ácido Etilenediaminetetrácético
EGTA	Ácido etileno glicol tetraacético
HEPES	Ácido N-[2Hidroxietil]piperazine-N'- 2[etanosulfônico]
PAGE	Eletroforese em Gel de poliacrilamida
PBS	Tampão fosfato em salina
PEG	Polietileno glicol
SDS	Dodecil Sulfato de Sódio, Lauril Sulfato de Sódio
TAME	Hidrogenocloreto de ρ -toluenosulfonil-L-arginina-metil éster
TEMED	N,N,N',N',-Tetrametiletilenodiamino
TRIS	Tris[Hidroximetil]aminometano
Tween 20	Polioxietileno Sorbitano Monolaurato
ρ - BPB	Brometo de ρ -Bromofenacil

Lista de Abreviações - Símbolos, Siglas

A₂₂₀	Absorbância em 220 nm
A₂₈₀	Absorbância em 280 nm
BSA	Albumina do soro bovino
Ci	Curie
kDa	Kilo Daltons, 1Da é aproximadamente o peso molecular de um átomo de Hidrogênio
M/V	Porcentagem ou Proporção de massa/ Volume
min	Minuto
Mr	Massa Molecular relativa
MW	Peso Molecular em unidade de massa atômica
n (nano)	Fator de multiplicação 10 ⁻⁰⁹
pl	Ponto Isoelétrico
rpm	Rotações por minuto

s	Segundo
UV	Ultravioleta
V ou v	Volts
V/V	Porcentagem ou Proporção de Volume/Volume
VIS	Visível
μ (micro)	Fator de multiplicação 10^{-6}
ρ (pico)	Fator de multiplicação 10^{-12}

Lista de Abreviações - aminoácidos

Ácido Aspártico	Asp	D
Ácido Glutâmico	Glu	E
Alanina	Ala	A
Arginina	Arg	R
Asparagina	Asn	N
Aspartato/ Asparagina	Asx	
Cisteína	Cys	C
Fenilalanina	Phe	F
Glicina	Gly	G
Glutamato/ Glutamina	Glx	
Histidina	His	H
Isoleucina	Ile	I
Leucina	Leu	L
Lisina	Lys	K
Metionina	Met	M
Prolina	Pro	P
Serina	Ser	S
Tirosina	Tyr	Y
Treonina	Thr	T
Triptofano	Trp	W
Valina	Val	V

Lista de Abreviações - Toxinas, PLA₂, miotoxinas

App 49	PLA ₂ isolada do veneno de <i>Agkistrodon p. piscivorus</i>
BthTX-I	Miotoxina I isolada da peçonha de <i>Bothrops jararacussu</i>
BthTX-II	Miotoxina II isolada da peçonha de <i>Bothrops jararacussu</i>
MOO-1	PLA ₂ isolada do veneno de <i>Bothrops moojeni</i>
MP-III 4R D49	PLA ₂ isolada do veneno de <i>Bothrops pirajai</i>
(PrTX-III)	
PRA-1	PLA ₂ isolada do veneno de <i>Bothrops pradoi</i>
PrTX-I	PLA ₂ isolada do veneno de <i>Bothrops pirajai</i>
PrTX-II	PLA ₂ isolada do veneno de <i>Bothrops pirajai</i>
TFBP I	PLA ₂ I isolada do veneno total de <i>Trimeresurus flavoviridis</i> .
TFPLA₂P2	PLA ₂ P ₂ isolada do veneno total de <i>Trimeresurus flavoviridis</i>
TGPLA₂ - V	PLA ₂ V isolada do veneno total de <i>Trimeresurus gramineus</i>
VRV-PL-VIIIa	PLA ₂ neurotóxica isolada do veneno de <i>Vipera russeli</i>

As serpentes do gênero *Bothrops* incluem várias espécies, que são amplamente distribuídas na América do Sul e do Norte. Entre as proteínas bioativas do veneno de *Bothrops*, as Fosfolipases A₂ (PLA₂, E.C. 3.1.14) e as miotoxinas PLA₂ "like" destacam-se como seus componentes majoritários. Fosfolipases A₂ são enzimas cálcio dependentes que hidrolisam a ligação 2-éster do 1,2 diacil-3sn fosfoglicerídeo (Chang *et al.*, 1994, Shimohigachi *et al.*, 1995, Ogawa, *et al.*, 1996).

Estas enzimas são encontradas em muitos tecidos, principalmente no suco pancreático de mamíferos, no veneno de serpentes e insetos. Enzimas PLA₂ são classificados dentro de quatro grupos (I, II, III e IV) de acordo com sua origem extracelular ou intracelular, de acordo com sua estrutura primária e pontes de sulfeto (Denis *et al.*, 1994).

Nesta tese, trabalhos com o veneno total de *Bothrops pirajai* e suas frações fosfolipases A₂ miotóxicas. Num primeiro instante, nos desenvolvemos uma nova estratégia de purificação destas miotoxinas em HPLC (HPLC de fase reversa, troca iônica e exclusão molecular) e em cromatografia convencional de baixa pressão (CM-Sepharose).

Em nossas condições experimentais, observamos que o HPLC de fase reversa (RP HPLC) tem vantagens sobre o método convencional, em relação ao tempo da corrida cromatográfica, a resolução dos picos e a manutenção da integridade molecular da PLA₂.

O primeiro trabalho feito por Toyama *et al.*, (1995) apresenta os resultados da purificação da principal miotoxina PLA₂ "like" (MPI e MPII) do veneno total de *Bothrops pirajai*, usando uma única etapa cromatográfica em HPLC de fase reversa. Este novo protocolo de purificação restringe a quantidade de proteína a ser colocada na coluna algumas mg. O rendimento final é 35% melhor do que na cromatografia de troca iônica de baixa pressão.

Em Soares *et al.*, (1998), propusemos um uma outra alternativa de purificação das miotoxinas PLA₂ "like" majoritárias usando coluna convencional, uma vez que o RP HPLC não aceita grandes quantidades de amostras. No procedimento convencional, a eluição das miotoxinas PLA₂ "like" era eluída usando acetato de amônia em altas concentrações e altos valor de pH. A troca do acetato de amônio por bicarbonato de amônia permitiu o uso de tampões com baixo pH e baixas concentrações salinas.

Outros venenos de *Bothrops jararacussu*, *Bothrops asper*, *Bothrops atrox*, *Bothrops pirajai*, *Bothrops moojeni*, *Bothrops alternatus* e *Bothrops (Bothriopsis) bilineata* foram fracionados usando procedimentos simplificados baseados na cromatografia em CM-Sepharose a pH 8.0 ou em RP HPLC.

O uso de NH_4HCO_3 ou de acetonitrila como tampões nestes procedimentos cromatográficos descritos acima, tem outras vantagens como a omissão de desalifcação e facilidade na liofilização. Isto permite uma melhor recuperação das proteínas eluídas e redução do tempo de corrida.

O método em CM-Sepharose e RP-HPLC descritos aqui são recomendados para escalas preparativas e analíticas, respectivamente. Nos métodos previamente descritos usam dois ou mais passos cromatográficos para o isolamento de miotoxinas. Em adição as colunas preparativas μ -Bondapack C18 mostraram também ser conveniente para a purificação de PLA_2 s. Ambos os métodos descritos aqui separam os componentes principais do veneno usando unicamente passo cromatográfico.

Os perfis cromatográficos mostraram importantes diferenças no conteúdo de miotoxinas destes venenos. O veneno de *B. alternatus*, *B. atrox* e *Bothriopsis bilineata* não contem a miotoxina principal encontrada em outros venenos. O sequenciamento de aminoácidos dos primeiros 50 resíduos da região N-terminal destas miotoxinas PLA_2 "like" mostram uma homologia de 90 a 96% com outras miotoxinas botrópicas. Todas as miotoxinas isoladas induzem edema de pata, aumentando o nível de creatinina quinase pancreática e indução da mionecrose junto com a infiltração de células polimorfonucleares.

Nesta tese, nos apresentamos o isolamento, purificação e a determinação da estrutura primária de três miotoxinas fosfolipase A_2 "like" do veneno de *Bothrops pirajai*, denominadas como PrTX-I, PrTX-II e MP-III 4R.

PrTX-I ou Piratoxina I foi isolado por Mancuso et al., (1995), e completamente seqüenciada por Toyama et al., (1998). PrTX-I é a principal PLA_2 miotóxica encontrado no veneno total de *Bothrops pirajai*, composto por 121 resíduos de aminoácidos, uma DL_{50} de cerca de 8mg/kg em camundongos e uma dose edematogênica mínima de 39.5 ± 1.8 μg . A modificação química da His 48 da PrTX-I pelo p -BPB praticamente aboliu sua atividade biológica.

PrTX-II ou Piratoxina II foi a segunda miotoxina importante isolada do veneno de *Bothrops pirajai* que foi sequenciada por Toyama et al., (1999, submetido). Tem 121 resíduos de aminoácidos e baixa atividade PLA₂ devido a substituição do Asp49 por Lys49 e alteração do "loop" de ligação do cálcio pela substituição de Gly32 por Leu32 e outras modificações foram importantes para a perda da flexibilidade do sítio de ligação do cálcio. PrTX-II tem somente um único amino ácido modificado (D132 para A132) em relação a PrTX-I, esta mudança confere a PrTX-II um ligeiro caráter básico.

A MP-III 4R foi a terceira miotoxina isolada do veneno de *Bothrops pirajai*. Esta miotoxina PLA₂ "like" tem uma atividade PLA₂ moderada se comparada com outras enzimas encontradas em venenos Crotálicos. MP-III 4R é um raro exemplo de PLA₂ com atividade fosfolipase A₂, anticoagulante e miotóxica. Sua atividade PLA₂ moderada é devido a substituição do resíduo E53 pelo K53 e seu efeito anticoagulante é devido a atividade PLA₂. A miotoxicidade não é devido a sua atividade catalítica.

O alinhamento de amino ácidos da PrTX-I, PrTX-II mostra um alto nível (95%) de homologia sequencial entre esta miotoxina e outras PLA₂ botrópicas. Contudo, estes valores diminuem para 80% para as não botrópicas e para 70-75% para as PLA₂ Asp49. Ambas toxinas foram caracterizadas como miotoxinas potentes e tem um atividade PLA₂ residual. MP-III 4R mostra uma homologia seqüencial de mais de 50% com outras PLA₂ D-49. Mas o alinhamento de aminoácidos da MP-III 4R com PLA₂ K-49 diminui para cerca de 60% de homologia.

PrTX-II e PrTX-III foram cristalizados e difratados usando uma resolução de 2.04 e 2.7 Å. Recentemente, a estrutura tridimensional da PrTX-II foi resolvida e mostrou uma estrutura dimérica.

The genus *Bothrops* comprises several species, which are widely distributed in South and North America. Among the bioactive proteins from *Bothrops* venoms, the phospholipase A₂ (PLA₂, E.C. 3.1.14) and PLA₂-like myotoxin are outstanding as their major components. Phospholipase A₂s are calcium-dependent enzymes which hydrolyze the 2 ester bonds of 1,2 diacyl-3sn phosphoglycerides (Chang *et al.*, 1994, Shimohigachi *et al.*, 1995, Ogawa, *et al.*, 1996).

They are found in most tissues, mainly in the pancreatic juice of mammals, venom of snakes and insects. PLA₂ enzymes are classified onto four groups (I, II, III and IV), according to their extracellular or intracellular origin, their primary structure and disulfide bonding (Denis *et al.*, 1994).

In this thesis, we work with *Bothrops pirajai* whole venom and its myotoxic phospholipase A₂ fractions. At first time, we develop a new strategy of purification of this myotoxin on the HPLC (reverse phase, ion exchange, and molecular exclusion) and in the convention low-pressure chromatography (CM-Sepharose).

In our experimental condition, we observed that Reverse Phase HPLC (RP HPLC) has advantageous on the conventional method about the time of chromatographic run, the resolution of some proteins and preservation of integrity of PLA₂ molecule.

The first work made by Toyama *et al.*, (1995) presents the results of the purification of the main myotoxin PLA₂ "like" (MPI and MPII) from the whole venom of *Bothrops pirajai*, using only chromatographic step on the RP HPLC. This novel purification protocol restricts the amount of protein to be loaded on each column in few mg. The final yield is 35% better than to in low-pressure ion exchange chromatography.

In the Soares *et al.*, (1998), we proposed to increase the purification grade of main PLA₂ "like" myotoxin using conventional column, because the RP HPLC does not accept great amount of samples. In the conventional procedure, the elution of the PLA₂ "like" myotoxin was eluted using ammonium acetate at high salt and pH values. The exchange of the ammonium acetate by ammonium bicarbonate allowed using low pH and ionic salt concentration.

PrTX-II or Piratoxin II was a second important myotoxin from *Bothrops pirajai* that has been sequenced by Toyama (Submitted). It has 121 amino acid residues and has low PLA₂ activity arose of the substitution of Asp49 by Lys49 and alteration of the calcium binding loop sequence by replacement of Gly32 by Leu32 and other modification were important for loss of the flexibility the calcium ion binding site. PrTX-II I have only one amino acid change (D132 to A132) to PrTX-I, this change confer to PrTX-II a slight basic character.

The MP-III 4R was thirty myotoxin isolated from the *Bothrops pirajai* venom. This PLA₂ like myotoxin has a moderate PLA₂ activity if compared to other enzymes found in the Crotalic venom. MP-III 4R is a rare example of PLA₂ with phospholipase A₂, anticoagulant and myotoxic activities. Its moderate PLA₂ activity is due to the replacement of E53 by K53 and its anticoagulant effects is due to that PLA₂ activity. The myotoxicity is not due to the catalytic activity.

The amino acid alignment of PrTX-I, PrTX-II shows a high level (95%) of sequential homology between this myotoxin and other bothropic Lys-49 PLA₂. However, these values fall to 80% for nonbothropic and to 70-75% for the Asp 49 PLA₂s. Both toxins were characterized as very potent myotoxin and have a residual PLA₂ activity. MP-III 4R D49 exhibit a sequence homology with other D-49 PLA₂ up 75%. But the amino acid alignment of MP-III 4R with K-49 PLA₂ falls to around 60% of homology.

PrTX-II and PrTX-III were crystallized and were diffracted at resolution of 2.04 and 2.7 of resolution, respectively. Recently, the three-dimensional structure of PrTX-II was solved and showed a dimeric structure.

Other venoms from *Bothrops jararacussu*, *Bothrops asper*, *Bothrops atrox*, *Bothrops pirajai*, *Bothrops moojeni*, *Bothrops alternatus* and *Bothrops (Bothriopsis) bilineata* were fractionated using a simplified procedure based on ion-exchange chromatography on CM-Sepharose at pH 8.0 or reverse phase HPLC.

The use of NH_4HCO_3 or acetonitrile as the buffer in these chromatographic procedure described above, has other advantages as omission of desalting and easy for freeze-drying. This allows a best recovery of the proteins eluted and reduction of run time.

The CM-Sepharose and the RP-HPLC methods described here are recommended for preparative and analytical purpose, respectively. Previous reported methods use two or more chromatographic steps for the isolation of myotoxins. In addition, the preparative μ -Bondapack C18 column was also useful for the purification of PLA_2 s. Both methods described here allow separating the major components of the venom using an only one chromatographic step.

The resulting elution profiles showed important differences in the myotoxin content of these venoms. The venoms from *B. alternatus*, *B. atrox* and *Bothriopsis bilineata* did not contain the major myotoxin found in the other venoms. The amino acid sequence of the first 50 residues of the N-terminal region of the PLA_2 -like myotoxins showed a homology of 90-96% with other botropic myotoxins. All of the myotoxins isolated induced rat paw edema, increased the level of plasma creatine kinase and produced myonecrosis together with polymorphonuclear cell infiltration.

In this thesis, we present the isolation; purification and determination of primary structure of three phospholipase A_2 "like" myotoxin from the *Bothrops pirajai* snake venom denominated as PrTX-I, PrTX-II and MP-III 4R.

PrTX-I or Piratoxin I was firstly isolated by Mancuso et al., (1995), and full sequenced by Toyama et al., (1998). PrTX-I is the main miotoxic PLA_2 found in the whole *Bothrops pirajai* venom, composed by 121 amino acid residues, a DL_{50} around 8mg/kg in mice and a minimal edematogenic dose of $39.5 \pm 1.8 \mu\text{g}$. The chemical modification of His-48 of PrTX-I by ρ -BPB practically destroyed its biological activity.

1-INTRODUÇÃO

1.1-Aspectos Gerais dos venenos

O veneno das serpentes é composto por uma mistura de proteínas, substâncias orgânicas, sais, íons, açúcares e lipídios. Sendo que o veneno total seco das serpentes é composto por cerca de 80 a 90% de proteínas (Tu, 1977).

Dentre os íons, podemos destacar o cálcio, que é um importante cofator da ação de algumas enzimas proteolíticas e das fosfolipases A₂. O magnésio e o zinco que também são importantes íons para ação das principais metaloproteases do veneno como as "trombinas-likes" (Tu, 1977 e Jia *et al.*, 1996).

O citrato é encontrado em altas concentrações no veneno total de determinadas serpentes e experimentalmente foi demonstrado que o citrato atua como um inibidor de várias enzimas, como as nucleotidases, esterases, proteases e fosfolipases A₂ (Francis *et al.*, 1992). De acordo com estes autores o citrato atuaria como um fator de neutralização endógena da serpente contra enzimas presentes no veneno.

Dentro do veneno total estão presentes várias substâncias orgânicas, denominadas de aminas biogênicas, como a bradicinina, histamina, 4-hidroxitriptamina, N-metil-5-hidroxitriptamina, N'-N'-dimetil-5-hidroxitriptamina e serotonina, que atuam como mediadores da dor provocado pelo veneno das serpentes (Ferreira *et al.*, 1992).. Classicamente estas enzimas podem ser agrupadas em cinco classes principais (Tu, 1977), sendo denominadas de:

Oxidoreductases

O veneno total de serpentes possui duas enzimas responsáveis pela reações redox, as L-aminoácido oxidase (LAO) e lactato desidrogenase. As LAOs convertem o aminoácido livre em um α -cetoácido. Muitos venenos de serpentes possuem LAO muito ativas e são responsáveis pela cor amarela do veneno seco, sua função biológica não é totalmente conhecida. Contudo, descobriu-se que as LAO são capazes, em determinadas condições laboratoriais, de induzirem a agregação plaquetária (Li *et al.*, 1994), e apoptose em determinadas células (Suhr e Kini, 1996).

Mclean et al., (1971) descreveu que o veneno de algumas serpentes catalisam a conversão do lactato à ácido pirúvico. A presença da lactato desidrogenase foi demonstrado em vários venenos, principalmente em serpentes do gênero *Naja* e *Dendroaspis*.

Fosfatases

São enzimas que hidrolisam ligações fosfomonoéster e fosfodiéster. As mais conhecidas são as fosfodiesterases que são largamente utilizadas no seqüenciamento ou caracterização de oligonucleotídeos e polinucleotídeos (Lasckowski, 1971).

A 5'-nucleotidase é uma fosfatase específica encontrada na maioria dos venenos de serpentes, são mais instáveis do que as fosfodiesterases (Mebs, 1970).

Existem também as fosfatases não específicas que são divididas em duas categorias de acordo com o pH ótimo de ação: as fosfatases ácidas, que possuem um pH ótimo em torno de 5.0 e as fosfatases alcalinas com um pH ótimo em torno de 8.5 (Tu e Chua, 1966).

A última classe de fosfatases são as endonucleases específicas para o RNA (Ribonucleases) e para o DNA (Desoxiribonucleases). São enzimas relativamente mais estáveis do que as outras fosfatases (Willians *et al.*, 1961).

Glicosidases

A hialuronidase é uma enzima que catalisa a reação de hidrólise do ácido hialurônico, que é um mucopolissacarídeo presente na pele, nos tecidos conectivos e nos tendões. Esta enzimas teriam a função de facilitar a difusão das toxinas do veneno para dentro do tecido das vítimas (Meyer *et al.*, 1960). Também são encontrados duas outras enzimas as α -amilases e a NAD nucleotidases.

Proteases

As proteases encontradas no veneno das serpentes podem ser agrupadas em dois grupos: as endopeptidases e as exopeptidases (Iwanaga *et al.*, 1976).

As proteases presentes no veneno total não são responsáveis pela sua ação letal, mas podem causar outros tipos de efeitos danosos, como a hemorragia, que é

desencadeada por um conjunto de proteases chamadas de Hemorraginas que são abundantes em venenos de serpentes Crotalidae e Viperidae. Um grande número de metaloproteases tem sido isoladas de diferentes venenos e caracterizados como hemorraginas. As metaloproteases destes venenos são de dois tipos, conforme a especificidade de substrato: enzimas de alta especificidade de substratos, que induzem hemorragia quando injetadas em animais (Mandelbaum *et al.*, 1976; Mori *et al.*, 1987; Sanches *et al.*, 1987 e Tu, 1982) e as enzimas com ampla especificidade de substratos que não induzem hemorragia (Assakura *et al.*, 1985; Sanches *et al.*, 1987 e Tu, 1982).

O veneno de serpentes da família Crotalidae e Viperidae, contém além das Hemorraginas muitas outras proteinases, como aquelas que agem na cascata de coagulação sangüínea (Iwanaga *et al.*, 1979; Seegers & Ouyang, 1979 e Kornalik, 1990). No Brasil, os estudos têm sido realizados principalmente com as peçonhas das serpentes do gênero *Bothrops* e *Crotalus*.

As enzimas isoladas, de venenos de serpentes, que possuem atividade tipo trombina, apresentam a capacidade de atuar sobre a molécula de fibrinogênio encontradas no plasma, transformando-a diretamente em fibrina (Seegers & Ouyang, 1979).

Lipases

As fosfolipases e a acetilcolinesterases, são enzimas mais comumente encontradas nos venenos de serpentes, sendo que as fosfolipases A₂ são as enzimas mais estudadas, não somente pela suas propriedades químicas, mas também por sua importância biológica (Tu, 1977).

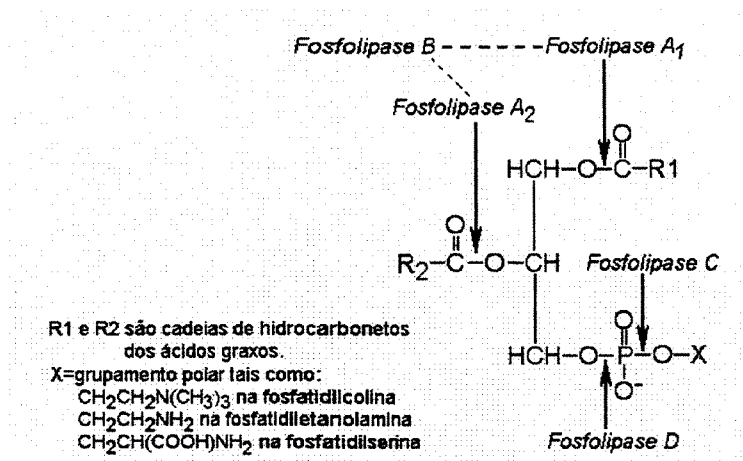
Além das enzimas hidrolíticas, o veneno total também possui outras moléculas de importância sem atividade enzimática, como as desintegrinas e as lectinas. As desintegrinas, possuem massa molecular variada e são proteínas que inibem a interação entre o fibrinogênio e o complexo glicoproteico I Ib - I Ia, na coagulação sangüínea. Possuem em comum uma seqüência de três resíduos de aminoácidos conservados que são responsáveis pela atividade biológica (Musial *et al.*, 1990).

As proteínas denominadas de lectinas tipo C, constituem um grupo de proteínas estruturalmente homólogas, mas com funções distintas. Algumas delas exibem atividade

Lectina, outras induzem efeitos anticoagulantes, ou efeitos na aglutinação e agregação de plaquetas (Kini, 1996).

1.2-Fosfolipase A₂ (PLA₂) - Aspectos Gerais

A degradação dos fosfoglicerídeos é realizada por fosfolipase encontradas em todos os tecidos, suco pancreático e venenos de serpentes. Várias bactérias patogênicas produzem fosfolipases, que dissolvem as membranas e permitem a disseminação da infecção. As fosfolipases são enzimas esterolíticas que hidrolisam glicerofosfolípídios. Estas enzimas são separadas em várias classes de fosfolipases, denominadas A₁, A₂, B, C e D, que é baseada nos seus sítios de hidrólise (Quadro 1).



Quadro 1. Sítio de ação das fosfolipase A₂

As fosfolipases A₂ (PLA₂) hidrolisam fosfolípídeos na posição sn-2 do esqueleto do glicerol, liberando o lisofosfolípídeo e ácido graxo respectivamente. Estas enzimas hidrolisam vários tipos de fosfolípídeos tais como: a fosfatidilcolina, fosfatidiletanolamina, fosfatidilserina, fosfatidilinositol, fosfatidilglicerol, fator de agregação plaquetária.

Estas fosfolipases ocorrem abundantemente na natureza, e são do ponto de vista bioquímico e biológico, as mais estudadas. As fosfolipases A₂, foram as primeiras fosfolipases a serem reconhecidas, sua descoberta foi baseada na observação da ação do suco pancreático e do veneno de serpentes na hidrólise da fosfatidilcolina (Wittcoff, 1951). Estudos subsequentes mostraram que as fosfolipases A₂ são abundantemente

encontradas em venenos de várias serpentes e nas glândulas exócrinas, onde estas enzimas tem um papel importante na digestão de lipídios (Wittcoff, 1951, Dennis, 1983 e Harris, 1991). Nestes últimos anos a pesquisa sobre fosfolipases A₂ tem sido impulsionadas principalmente pelo seu importante papel em várias atividades biológicas (Tabela 1).

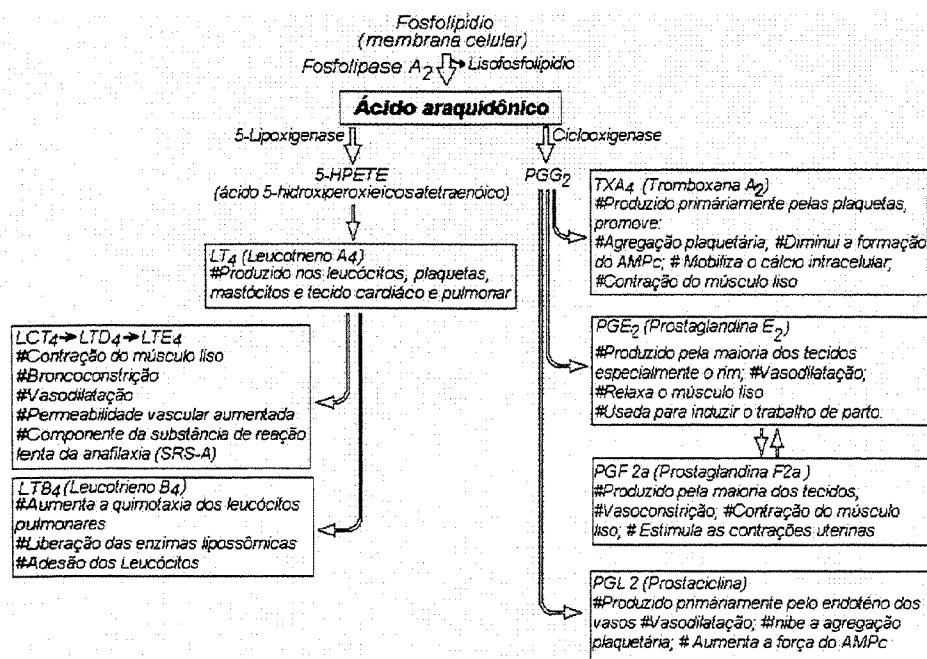
Tabela 1 Efeitos farmacológicos das enzimas PLA₂ isoladas do veneno total de serpentes.

Neurotoxicidade
Neurotoxinas Pré-sinápticas
Neurotoxinas Pós-sinápticas
Miotoxicidade
Mionecrose Local
Miotoxicidade Sistêmica
Cardiotoxicidade
Efeito anticoagulante
Iniciador da agregação plaquetária
Inibidor da agregação plaquetária
Atividade Hemolítica
Hemorragia Interna
Atividade antihemorrágica
Atividade Convulsionante
Atividade Hipotensiva
Atividade Edematogênica
Lesão de órgãos e tecidos.

As fosfolipases A₂ também tem grande importância nos processos de fertilização (Fry 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 enzimas PLA₂ de mamíferos tem um importante papel na manutenção dos depósitos de fosfolipídios e no reparo da membrana plasmática através das vias de deacilação/ reacilação (Verheij et al., 1980; Waite, 1990; Dennis et al., 1991; Kudo et al., 1993 e Dennis, 1994).

As fosfolipases A_2 tem um papel central no metabolismo de lipídios e esta intimamente relacionada com a liberação de ácido araquidônico, que é um precursor de lipídios ativos como prostaglandinas, tromboxanos e leucotrienos (coletivamente chamados de eicosanoídes) (Quadro 2). São compostos extremamente potentes que desencadeiam uma ampla faixa de respostas fisiológicas. Estes compostos têm uma meia vida muito curta, que os torna difíceis de se estudar. Embora tenham sido comparados à hormônios em termo de ações, as prostaglandinas diferem dos hormônios verdadeiros, pois são formadas em quase todos os tecidos, e não em uma glândula específica, e geralmente agem localmente, em vez de serem transportadas pelo sangue à sítios de ação distantes.



Quadro 2. Visão geral da biosíntese de algumas prostaglandinas importantes, leucotrienos e um tromboxano a partir do ácido araquidônico (Mayatepek & Hoffmann, 1995).

As fosfolipases A_2 de mamíferos as PLA_2 do veneno de serpentes possuem além do seu papel de digestão da presa, um grau variado de ações farmacológicas que interferem em processos fisiológicos normais, como mostrado na Tabela 1.

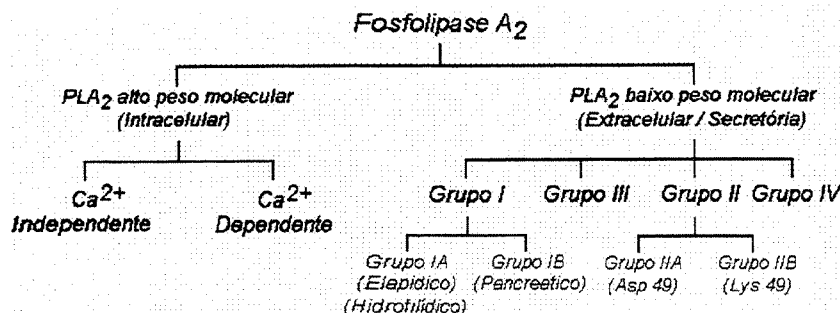
Apesar da gama extensa de atividades farmacológicas desencadeadas por estas PLA₂ de serpentes, estes efeitos aparentemente não tem correlação direta com a atividade catalítica da enzima e não são correlacionáveis com suas diferenças estruturais, pois observa-se uma grande homologia seqüencial destas enzimas, que mostram também grande similaridade estrutural. Desta forma a correlação estrutural e funcional destas enzimas é extremamente difícil. O aspecto catalítico também não explica a gama tão diversa de atividades.

Classificação das Fosfolipases A₂

Existem várias formas de se classificar as PLA₂, de acordo com o seu peso molecular as PLA₂ podem ser divididas em dois grandes grupos: uma de alto peso molecular e uma de baixo peso molecular. As PLA₂ 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 e as que não necessitam (Kini, 1997) (Quadro 3). Dentro do grupo de PLA₂ de alto peso molecular podemos colocar as PLA₂ classe V isoladas do músculo cardíaco de cães, e as PLA₂ da classe IV descritas por Dennis (1994).

De acordo com Kini (1997) as fosfolipases A₂ de baixo peso molecular podem ser subdivididas em quatro subgrupos: Grupo I, Grupo II, Grupo III e Grupo IV. As fosfolipases A₂ do grupo I, englobam as isoladas dos venenos elapídicos, hidrofilídicos e as isoladas do pâncreas. Estas enzimas contêm cerca de 115 – 120 resíduos de aminoácidos e sete pontes de dissulfeto. Enzimas do grupo II, são encontrados nos venenos de serpentes crotalídicas e viperídicas e em células humanas como as plaquetas. Estas enzimas possuem cerca de 120 a 125 resíduos de aminoácidos e sete pontes de dissulfeto. Fazem parte deste grupo as enzimas PLA₂ miotóxicas Asp 49 (D49) e Lys 49 (K49), que possuem uma atividade enzimática baixa ou residual. As fosfolipases do grupo I podem ser subdivididas em 5 subgrupos, e as do grupo II são divididos em 6 subgrupos, estas subdivisões levam em conta aspectos particulares de cada subgrupo, e as pontes de dissulfeto presentes, basicamente. As do grupo III englobam aquelas isoladas de abelhas, diferentemente das outras classes de PLA₂ estas são glicoproteínas, contendo de 130 a 135 resíduos de aminoácidos. Sua homologia com as outras PLA₂ é baixa.

As PLA₂ do grupo IV podem ser considerado o mais recente dentro das PLA₂, do ponto de vista estrutural. Estas PLA₂ tem 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).



Quadro 3 Classificação das enzimas PLA₂

De acordo com Dennis (1994) as fosfolipases A₂ pertencentes ao grupo I, II e III são as menores e as mais simples PLA₂, contudo todas as PLA₂ de baixo peso molecular hidrolisam os fosfolípidos através de mecanismos similares. Pois todas estas enzimas de baixo peso molecular mostram inibição pelo inibidor MG-14 (McIntosh et al., 1995; Yuan et al., 1990; Jain et al., 1992 e Bayburt et al., 1993). Já as PLA₂ de alto peso molecular promovem a hidrólise do fosfolípido usando um outro mecanismo de catálise, que pode ser dependente ou não de cálcio.

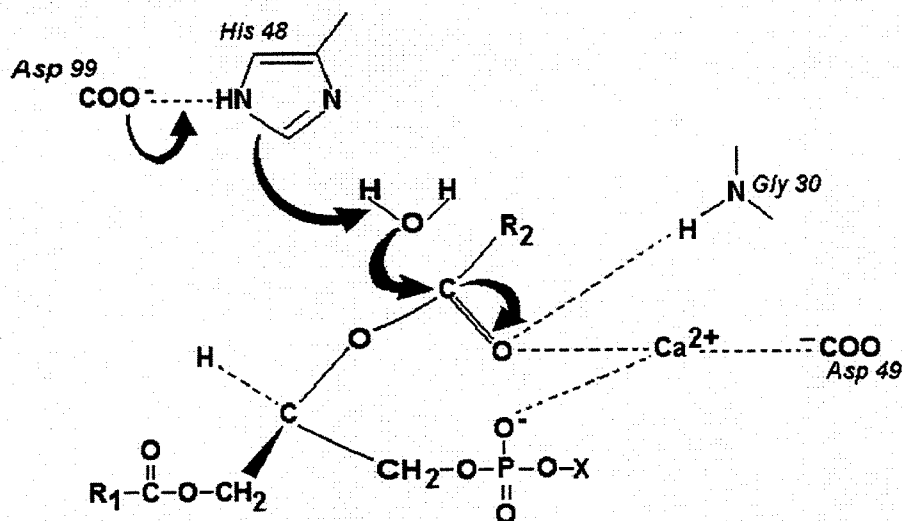
Nos grupos I, II e III, os resíduos envolvidos no mecanismo catalítico ocupam especialmente as mesmas posições, ocorrendo uma sobreposição destes três resíduos nas três classes (Scott et al., 1990). De acordo com o modelo de classificação proposto por Henrickson (Henrickson et al., 1977) as fosfolipases dos grupos I e II se originaram de um grupo ancestral comum. Agora com avanço das técnicas de biologia molecular, de clonagem e seqüenciamento automático de nucleotídeos possibilitaram o refinamento da classificação das várias PLA₂ em grupos e subgrupos. De acordo com essas análises a origem dos genes codificadores das PLA₂ dos grupos I e II é oriundo da duplicação de um gene comum. Após a duplicação e inserção de introns neste gene poderiam ter originado os genes codificadores das PLA₂ do grupo II (Dense et al., 1997).

Fosfolipases A₂ - Catálise

As fosfolipases A₂ são enzimas que catalisam a quebra do fosfolipídeos em um lisofosfolipídeo e um ácido graxo respectivamente, e podem atuar em substratos monoméricos ou agregados em micela ou em uma bicamada. A atividade enzimática está relacionada com as propriedades físicas do agregado, tais como densidade, temperatura, fase de transição, curvatura da micela e presença de detergentes.

Em 1980 foi proposto um mecanismo de catálise das PLA₂ baseado na comparação estrutural com as serino proteases (Verheij et al., 1980). No modelo de ação das PLA₂, a atividade catalítica compreende os resíduos de His 48, Asp99 e uma molécula de água, seu funcionamento é similar ao mecanismo das serino proteases.

O íon cálcio, que é um elemento importante na catálise, está ligado pelos oxigênio da Tyr28, Gly30, Gly32 e pelo oxigênio da cadeia lateral do Asp 49. No mecanismo proposto o cálcio teria uma função dupla que seria a fixação do grupamento fosfato do fosfolipídeos e pela estabilização da carga negativa sobre o oxigênio carboxil da ligação éster na posição sn-2. A amida NH da Gly30 também foi sugerida como um fator importante na estabilização do estado de transição (Verheij et al., 1980) (Quadro 4).



Quadro 4. A figura acima mostra de forma esquemática o modelo do mecanismo de catálise das fosfolipases A₂ de forma geral (Yang, 1994).

As fosfolipases A₂, não dependentes de Ca²⁺ são de forma geral ativadas e estabilizadas pela presença de ATP. Basicamente, o ATP modularia a polimerização destas PLA₂, o que o tornaria cataliticamente ativo e estável (Dennis, 1994).

Fosfolipase A2 - Efeitos Biológicos

As fosfolipases A₂ de serpentes exibem uma ampla variedade de efeitos farmacológicos, apesar de sua similaridades, na estrutura primária, secundária e terciária.

Isoformas de PLA₂ de um mesmo veneno também exibem diferentes efeitos farmacológicos. Dentro destes como já citamos os mais comuns seriam a neurotoxicidade, miotoxicidade e efeito anti coagulante. Mesmo com o avanço da compreensão estrutural, funcional e do mecanismo de ação destas PLA₂, muitos dos aspectos do mecanismo dos eventos farmacológicos ainda permanecem desconhecidos.

Um exemplo, uma enzima PLA₂ que afeta a agregação plaquetária podem induzir efeitos cardiotoxícos e edematogênicos simultaneamente por vias completamente distintas. Estas atividades não podem ser explicadas pela simples correlação entre estrutura e função (Kini, 1997).

Muitas das PLA₂ do veneno de serpentes necessitam de proteínas associadas para expressão de sua atividade farmacológica, estes fatores podem estar ligados a proteína de forma covalente ou não covalente.

Um exemplo desta complexa interrelação estão as β-neurotoxinas, que são potentes neurotoxinas que bloqueiam a transmissão neuromuscular. Estas β-neurotoxinas podem ser encontradas em duas formas básicas: na forma monomérica ou na forma multimérica (Bon, 1997).

Estas neurotoxinas multimericas, são feitas de duas, três ou cinco subunidades polipeptídica, estruturalmente são homólogas às PLA₂, contudo algumas destas enzimas podem não apresentar atividade enzimática. Estas subunidades estão associadas por interações não covalentes como no caso da crotoxina, formado pela associação de uma subunidade ácida (crotapotina) e uma básica (PLA₂). Outras neurotoxinas estão associadas por pontes de sulfeto de forma similar ao inibidor do tipo Kunitz (Bon, 1997).

Os vários efeitos farmacológicos promovidos pelas fosfolipase A₂ podem ser explicados também pela presença de sítios alvos específicos, que estão localizados na

superfície das células ou do tecido alvo (Quadro 5). Estes sítios alvos seriam reconhecidos por sítios farmacológicos localizados na superfície das PLA₂, 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 os sítios farmacológicos e o sítio alvo em termos de cargas, interações hidrofóbicas e interações de van der Waals.

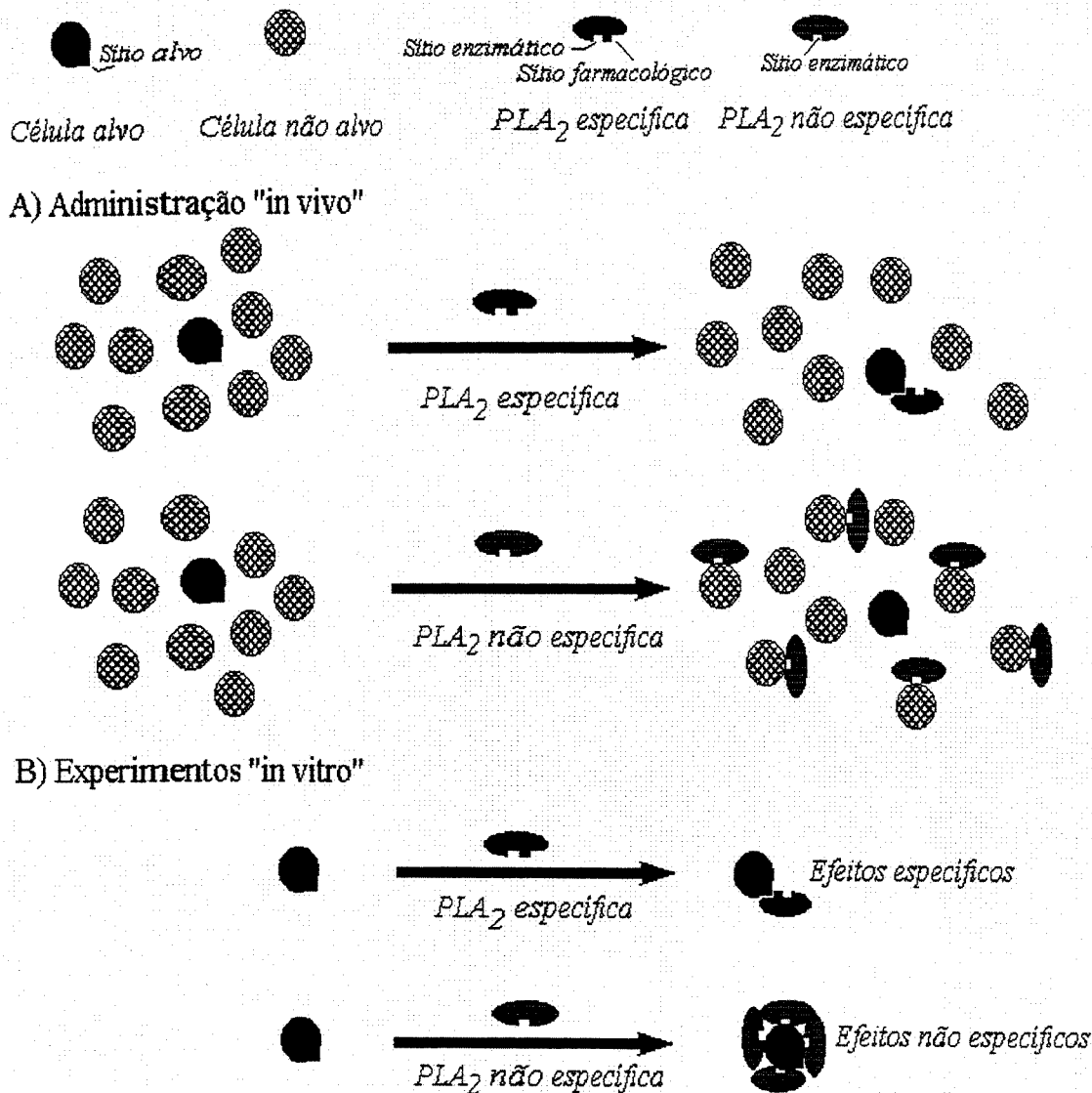
De acordo com Kini e Evans (1989) a natureza química deste sítio de ligação entre as PLA₂ e as células ou tecido alvo poderia estar entre um lipídio ou uma proteína (glicoproteínas). Devido à alta especificidade das interações pode-se supor que as glicoproteínas podem estar diretamente relacionadas com o reconhecimento, contudo lipídios próximos ao sítio de ligação também podem contribuir para o aumento da especificidade.

Muitos dos receptores para estas PLA₂ já foram identificados e caracterizados tanto ao nível estrutural como funcional. Um dos mais bem caracterizados e conhecidos está a proteína do canal de potássio (Kini, 1997).

PLA₂ "like".

As PLA₂ "like", podem ser definidas como aquelas proteínas, que possuem uma estrutura molecular similar às das PLA₂ cataliticamente ativas. Estas proteínas PLA₂ "like" são proteínas desprovidas de ação catalítica, portanto para desempenharem "suas atividades biológicas" dependem de outros mecanismos, que sejam independentes da quebra do ácido araquidônico (Maraganore *et al.*, 1984; Pedersen *et al.*, 1994; Gutiérrez & Lomonte, 1995; Arni & Ward, 1996 e Selistre de Araújo *et al.*, 1996).

De acordo com Gutiérrez & Lomonte (1995) estas PLA₂ "like" poderiam se ligar a determinados sítios, que poderiam ser proteínas carregadas negativamente, sendo um sítio de ancoragem para as PLA₂ "like". Uma vez aderido à membrana estas proteínas poderiam causar a desorganização da membrana plasmática, que levaria à alteração de sua permeabilidade ao meio, levando à destruição celular.



Quadro 5. Diagrama esquemático do modelo de interação das PLA₂ com as células alvos e com os tecidos e desencadeamento da atividade farmacológica. A base deste modelo está na capacidade de certas PLA₂ interagirem especificamente com determinadas células, via "sítios alvos". As PLA₂ não específicas podem interagir de forma aleatória com todos tipos celulares inclusive com as "células alvos". De acordo com este modelo os efeitos farmacológicos produzidos pela interação de PLA₂ não específicas é menos efetiva e potente do que numa interação específica.

Esta destruição seria mais pela desorganização celular do que pela toxicidade da proteína em si. Um outro fator que ajudaria esta desorganização provocada pelas PLA₂ "like" seria a própria fluidez da membrana. Pedersen et al., (1994), não fala com clareza sobre a presença de sítios proteicos como propõe Gutiérrez & Lomonte, (1995) e sim uma ligação ao próprio lipídio da membrana.

De acordo com Pedersen et al., (1994), muitos dos trabalhos realizados com as PLA₂ "like" não são conclusivos devido a um ponto crucial: o grau de pureza das amostras usadas, uma vez que muitas das preparações realizadas com PLA₂ "like" estavam ou podiam estar contaminadas com uma pequena percentagem de PLA₂ cataliticamente ativas.

De acordo com Fletcher & Jiang, (1995) e Shimohigashi, et al., (1995), existiria uma outra hipótese, onde estas PLA₂ "like" cataliticamente inativas seriam capazes de hidrolisar os fosfolípidos da membrana, liberando o ácido araquidônico independentemente do mecanismo de ação destas PLA₂ dependeria de cálcio.

Existe ainda uma terceira hipótese que tenta explicar a ligação destas PLA₂ com a membrana, que seria a auto acilação destas PLA₂ "like". Estas PLA₂ "like" são capazes de incorporar em sua estrutura ácidos graxos livres de forma covalente. Esta reação de auto acilação teria um papel de âncora para a interação destas PLA₂ "like" sobre as membranas (Lugtigheid et al., 1993 e Pedersen et al., 1994).

"PLA₂ like myotoxins"

As miotoxinas, incluem diferentes grupos de peptídeos isolados dos veneno de serpentes e abelhas, que poderiam causar a mionecrose. Dentro dos vários grupos podemos destacar três em particular, pela sua importância: as PLA₂ miotóxicas; os peptídeos citolíticos não enzimáticos e os peptídeos não citolíticos, não enzimáticos (Fletcher, et al., 1996).

As fosfolipases A₂ miotóxicas são macromoléculas compostas por cerca de 110 a 135 resíduos de aminoácidos e possuem uma estrutura básica das fosfolipases A₂ cataliticamente ativas. As miotoxinas PLA₂ podem ser divididas em dois grandes grupos. As PLA₂ miotóxicas Asp 49 (D49), que possuem o resíduo de Asp 49 conservado, essencial para o seu pleno funcionamento. O outro grupo de miotoxinas são

caracterizadas por terem no lugar de Asp 49, Lys 49, levando a perda da ligação do Ca^{2+} , diminuindo substancialmente ou reduzindo a atividade hidrolítica sobre substratos purificados ou sobre a lecitina de ovo (Fletcher et al., 1996).

O modo de ação das PLA_2 miotóxicas Lys 49 e das Asp 49 sobre o músculo, são geralmente do mesmo tipo, tanto as miotoxinas Lys 49 e Asp 49 podem causar lise rápida do sarconema, levando a um rápido estado de mionecrose. O papel da atividade catalítica, levado a cabo por estas miotoxinas é um ponto não esclarecido e controverso, já que as miotoxinas, PLA_2 "like" Lys 49 não possuem uma atividade lipolítica, portanto outros mecanismos devem estar envolvidos na expressão da atividade miotóxica (Fletcher et al., 1996).

Na América Latina, de modo geral a maioria dos acidentes ofídicos são causados por serpentes do gênero *Bothrops*. A picada destas serpentes leva geralmente a um quadro de mionecrose local, provocadas pela presença das miotoxinas PLA_2 "like", que são proteínas estruturalmente semelhantes as PLA_2 ativas, contudo sem ou com atividade enzimática residual (Gutiérrez et al., 1984).

Várias miotoxinas isoladas nestes últimos anos, tem sido caracterizados como proteínas básicas de massa molecular em torno de 13 kDa, estas miotoxinas PLA_2 "like" também pelo seu forte caráter associativo em dímeros, que é a forma mais comum de polimerização encontrada para as PLA_2 miotóxicas de modo geral e eventualmente em tetrameros.

Todas as miotoxinas isoladas até o presente momento, tem na cromatografia de troca iônica, um dos passos obrigatórios para sua purificação. Em muitos casos, as miotoxinas de caráter básico, são os últimos componentes a serem eluídos em cromatografia de troca catiônica usando gradientes de sais neutros, como fosfato, Tris-HCl, Acetato de Amônia (Homsí-Branderburgo et al., 1988; Gutierrez, et al., 1995 e Nakai et al., 1995).

O veneno botrópico bem como o veneno de outras espécies de serpentes tem mostrado a presença de isoformas, tanto para diferentes indivíduos de uma mesma espécie, como para o mesmo indivíduo. Sabe-se que serpentes, possuem vários genes que codificam várias isoformas, contudo a expressão e a síntese das diferentes isoformas

permanece ainda desconhecido. A separação destas isoformas só tem sido possível ao uso de HPLC de fase reversa (Bruses et al., 1993).

As miotoxinas isoladas, até o presente momento, são moléculas compactas, extremamente estáveis às variações de temperaturas, pH, concentração salina altas e na presença de solventes orgânicos.

Devido a estas características, as PLA₂ miotóxicas não se desnaturam em condições usuais de trabalho, durante os processos de purificação e testes biológicos (Gutierrez et al., 1995 e Toyama et al., 1995).

As miotoxinas de Bothrops, tem sido classificadas como PLA₂ do grupo II, extracelulares com uma massa molecular de 13 a 15 kDa e podem ser subdivididas em dois subgrupos distintos: 1) as PLA₂ D49 que se caracterizam por possuírem em seu sítio catalítico a presença de Ca²⁺ e alta atividade enzimática e 2) as PLA₂ K49 que possuem uma atividade catalítica baixa ou ausente, devido a presença do resíduo de lisina na posição 49 (K49) promovendo um impedimento espacial e estereoquímico de ligação do Ca²⁺. As PLA₂ K49 apesar de não possuírem o Ca²⁺ como cofator de sua atividade podem eventualmente promover ruptura da bicamada da membrana de lipossomos, através de um mecanismo independente da atividade enzimática, contudo ainda não se conhece com precisão o mecanismo deste evento (Gutiérrez e Lomonte 1995).

As PLA₂ miotóxicas tem sido objetos de muitos estudos em razão de três motivos principais: 1) são frações importantes, 2) de fácil isolamento e 3) representam cerca de 20 a 30% do veneno total. Muitas miotoxinas já foram isoladas e parcialmente estudadas do ponto de vista bioquímico e farmacológico (Tabela 2).

O mecanismo de ação das miotoxinas PLA₂ "like" ainda permanece obscuro de acordo com Harris (1991); Gutierrez & Lomonte, (1995) e Selistre de Araújo et al., (1996). A hipótese sobre o mecanismo de ação das miotoxinas indicam que as PLA₂ miotóxicas se ligam à determinados receptores para intermediar a ação miotóxica. Em linhas gerais esta hipótese teria os seguintes eventos: 1) a miotoxina liga-se a receptores de membrana, 2) ocorre uma alteração dos fluxos iônicos de sódio e potássio, 3) ocorre um aumento da concentração de fluido para dentro da célula, 4) inicia-se uma vacuolização 5) inicia-se o processo de desorganização das fibras musculares, 5) perda acentuada da integridade celular.

As PLA₂ tanto D49 como as K49 podem estar ou não na forma agregada, mais comumente na forma dimérica (Arni & Ward, 1996). Estas neurotoxinas necessitam para levar a cabo o evento miotóxico de resíduos carregados positivamente tais como Lisina, Arginina e Histidina. De acordo com Selistre de Araújo et al., (1996) as PLA₂ ácidas de *Bothrops jararacussu* não causam a miotoxidade, portanto aminoácidos carregados positivamente são extremamente importantes para a expressão da miotoxidade.

Tabela 2 Miotoxinas botropicadas.

Espécie	Nome da toxina	KDa	pI	PLA₂	Referências
<i>B. asper</i>	Miotoxina I	10,7	Nd	+	Gutiérrez et al., (1984)
<i>B. asper</i>	Miotoxina II	13,3	Nd	—	Lomonte & Gutierrez, (1989)
<i>B. asper</i>	Miotoxina III	13,9	>9,5	+	Kaiser et al., (1990)
<i>B. asper</i>	Miotoxina IV	15,5	Nd	—	Diaz et al., (1995)
<i>B. asper</i>	Miotoxina PLA ₂	14,1	Nd	+	Mebs e Samejima, (1986)
<i>B. mummifer</i>	Miotoxina	16,0	Nd	—	Gutierrez et al., (1993)
<i>B. mummifer</i>	Miotoxina pico IV	15,0	~10,6	—	Bruses et al., (1993)
<i>B. jararacussu</i>	Bothropstoxin I	13,8	~8,2	—	Homsi-Brandeburgo et al., (1988); Cintra et al., (1993)
<i>B. jararacussu</i>	Bothropstoxin II	15,7	~7,7	+	Homsi-Brandeburgo et al., (1988)
<i>B. insularis</i>	Miotoxina	15,0	Nd	+	Selistre et al., (1990)
<i>B. atrox</i>	Miotoxina	13,4	Nd	+	Lomonte et al., (1990)
<i>B. moojeni</i>	Miotoxina I (IV*)	13,4	Nd	—	Lomonte et al., (1990)
<i>B. moojeni</i>	Miotoxina II (V*)	13,4	Nd	—	Lomonte et al., (1990)
<i>B. moojeni</i>	MOO-1	15,0	Nd	—	Moura da Silva et al., (1991)
<i>B. pradoi</i>	PRA-1	15,0	Nd	+	Moura da Silva et al., (1991)
<i>B. godmani</i>	Miotoxina I	13,3	~8,2	+	Díaz et al., (1992)
<i>B. godmani</i>	Miotoxina II	13,4	~8,9	—	Díaz et al., (1992)
<i>B. pirajai</i>	PrTX-I	~13,5	~8,3	—	Mancuso et al., (1995) Toyama et al., 1995
<i>B. pirajai</i>	PrTX-II,	~13,5	~8,2	—	Mancuso et al., (1995) Toyama et al., (1995)
<i>B. pirajai</i>	PrTX-III	~13,5	~8,0	+	Mancuso et al., (1995) Toyama et al., (1995)

Díaz-Oreiro e Gutierrez (1997) demonstraram que modificações químicas realizadas nos resíduos de lisina inibem substancialmente a atividade miotóxica e anticoagulante, mostrando a importância dos resíduos de lisina. De acordo com os trabalhos de Arni e Ward (1996) e Selistre de Araújo et al., (1996) estas lisinas e outros

aminoácidos carregados são comuns nas fosfolipases A_2 miotóxicas. Estes resíduos carregados positivamente ajudariam as PLA_2 miotóxicas a se posicionarem sobre um ou mais sítios receptores localizados na membrana celular das fibras musculares. De acordo com Gutiérrez e Lomonte (1995) e Selistre de Araújo (1996) existem evidências da existência de receptores de alta afinidade. Este receptor poderia ser de acordo com os trabalhos de Fletcher et al., (1996), receptores ligados aos canais de sódio ou o próprio canal poderia estar envolvido.

2 - OBJETIVOS

1) Purificação e Isolamento de miotoxinas de *Bothrops pirajai* através de métodos cromatográficos convencionais em colunas de Sephadex G75 e troca iônica em SP-Sephadex C25

2) Purificar e isolar miotoxinas e toxinas de *Bothrops pirajai* através técnicas em HPLC de Exclusão molecular, Troca Iônica e Fase Reversa.

3) Desenvolver e Simplificar as metodologias de purificação de venenos botropicos utilizando técnicas de HPLC e realizar estudos comparativos com outras metodologias de cromatografia de resinas convencionais.

4) Realizar os estudos moleculares através da determinação da seqüência das cadeias polipeptídicas, com o objetivo de localizar os sítios catalíticos e as regiões moleculares envolvidas nos mecanismos de ação. Realizar estudos de homologia seqüencial.

5) Obtenção dos cristais de proteína para estudos de difração de raios X, usando como fonte de energia a Luz Sincotron.

3-Trabalhos Decorrentes do Projeto (Aceitos / Submetidos)

A Quick Procedure for the isolation of dimeric piratoxins I and II, two myotoxins from *Bothrops pirajai* snake venom, N-Terminal Sequencing.

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A QUICK PROCEDURE FOR THE ISOLATION OF DIMERIC
PIRATOXINS-I AND II, TWO MYOTOXINS FROM *BOTHROPS PIRAJAI*
SNAKE VENOM. N-TERMINAL SEQUENCING

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SUMMARY: Two myotoxins, MP-I and MP-II, from *Bothrops pirajai* snake venom, have been purified by a quick high performance liquid chromatography (HPLC) procedure. Based on the HPLC coelution profile, amino acid composition, N-terminal sequence, polyacrylamide gel electrophoresis (PAGE) migration, as well as lack of phospholipase-A₂ (PLA₂) and proteolytic activities, MP-I and MP-II were identified as piratoxin-I (PrTX-I) and II (PrTX-II), respectively. This procedure affords, aside the reduced operation time, a high yield (35% of the applied sample in terms of A_{280nm}) of MP-I, which is the major myotoxin of the venom. The N-terminal sequences of MP-I, MP-II, PrTX-I and PrTX-II, up to the 51st, 41st, 46th and 39th residues, respectively, have been determined, revealing MP-I (and hence PrTX-I) as a Lys-49 PLA₂-like myotoxin. Both MP-I and MP-II have been shown, by SDS-PAGE, to occur in dimeric isoforms.

INTRODUCTION

The genus *Bothrops* embodies many species of snakes distributed in South America from Mexico to Argentina. *Bothrops pirajai* is a species geographically restricted to the south region of Bahia State, in Brazil (1). Envenomation symptoms are characterized by a local pain, swelling, haemorrhage and necrosis, producing muscular alteration which may result in permanent injury and even death of local tissue (2, 3). Many myotoxins isolated from snake venoms are phospholipases A₂ (4). Other myotoxins components are structurally related to phospholipase A₂, but lack this enzymatic activity, as

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bothropstoxin-I (5, 6) and myotoxin II (7). Both are Lys-49 myotoxic phospholipase-like proteins with low phospholipase activity. Enzymatic activity of porcine PLA₂ was shown to depend on the presence of the Asp-49 residue which contributes to calcium binding. When Asp-49 was replaced by Lys-49 through site-specific mutation, the mutant showed reduced binding (8). The residual phospholipase A₂ activity, which was attributed to a slight contamination level (3%), could be removed by further purification.

This paper describes a quick high performance liquid chromatography (HPLC) procedure for the isolation, from *B. pirajai* snake venom, of a Lys-49 myotoxin (MP-I) and a highly homologous myotoxin (MP-II) both of which, after rechromatography, proved to be pure enough to show a single N-terminal amino acid residue in every step of the sequential degradation up to the 51st and 41st residues, respectively. These myotoxins showed to be identical to SIV-SP₅ piratoxin-I (PrTX-I) and SIV-SP₄, piratoxin-II (PrTX-II) respectively, isolated by Mancuso et al. (9) from the same venom by a sequence of purification steps involving gel filtration and ion-exchange chromatography.

MATERIALS AND METHODS

Venom and reagents: *Bothrops pirajai* desiccated venom was a gift from Instituto Butantan, São Paulo. Chemicals, solvents and all reagents used in this work were of analytical, HPLC or sequal grade.

Reverse Phase HPLC: Both myotoxic proteins were purified from *Bothrops pirajai* venom by reverse phase high performance liquid chromatography.

Ten mg of the desiccated whole venom were treated with 200 µl of 0.1% (v/v) trifluoroacetic acid (solvent A). The resulting dispersion was clarified by centrifugation and the clear supernatant was applied on top of a 0.78 cm x 30 cm µ Bondapack C-18 column (Waters 991-PDA system) Elution proceeded with a linear gradient from zero to 66% (v/v) acetonitrile (solvent B) in 0.1% (v/v) trifluoroacetic acid, at a flow rate of 2.0 ml/min. Absorbances were monitored at 280 nm. Fractions MP-I and MP-II were rechromatographed in the same conditions, alone and mixed with PrTX-I and PrTX-II, respectively, and the chromatographic profiles were compared with those of PrTX-I and PrTX-II.

Phospholipase A₂ and proteolytic activities: Phospholipase A₂ activity was measured by potentiometric titration as described by De Haas et al. (10). The substrate used in the enzymatic assays was egg yolk emulsion in the presence of sodium and calcium desoxycholate. Fatty acids released by enzymatic activity were measured by titration with 0.1027 N NaOH. One enzymatic unit of phospholipase A₂ is defined as the amount (µg) of enzyme that releases 1 µmol

of fatty acid per minute at 37°C in the conditions of the method. Proteolytic activity was measured as previously reported (9).

Electrophoresis: SDS-poliacrylamide gel electrophoresis (SDS-PAGE) was performed basically as described by Shapiro et al. (11) and by Weber and Osborn (12). Reduction was carried out by addition of 1M dithiothreitol till 0.65 M final concentration. All samples were heated at 100°C for 3 min. and then run at 15 mA for 5 hr in a 12.5% gel.

Amino acid composition: Amino acid analysis was carried out on a PICO-TAG amino acid analyzer (Waters System) as described by Henrikson and Meredith (13). One nmol of the purified sample was hydrolyzed with 6 M HCl/1% phenol at 106°C for 24 hr. Hydrolyzates reacted with 20 µl of fresh derivatization solution (v/v, 7:1:1:1; ethanol: triethylamine: water: phenylisothiocyanate) for 1 hr at room temperature. After pre-column derivatization, PTC amino acids were identified in a reverse phase HPLC-column according to their retention times compared to those of the standard PTC amino acids. Cysteine residues were quantified as cysteic acid. Tryptophan was determined by PICO-TAG after 24h hydrolysis with methanesulfonic acid according to Simpson *et al.* (14).

N-terminal amino acid sequence: Non reduced myotoxins MP-I and MP-II were used to determine the N-terminal amino acid sequence through direct sequencing by automated Edman degradation in a model 477 A device (Applied Biosystem Sequencer). The PTH amino acids were identified in a model 120A PTH amino acid analyzer (Applied Biosystems), according to their retention times as before. In order to compare these sequences with those of piratoxin I and piratoxin-II, both toxins were sequenced as well along the N-terminal region.

RESULTS

Fig. 1 shows the chromatographic profile of *Bothrops pirajai* venom where two fractions, with retention times corresponding to 58% and 62% of solvent B, respectively, are indicated.

MP-I was repurified in the same conditions and is indicated by the arrow in Fig. 2. It represents the major fraction of the venom (35% of the applied sample). In non reducing conditions, MP-I ran as a diffuse band with an apparent molecular mass of 28,5 kDa. After reduction with DTT, a single band of 15.3 kDa appeared (Fig. 3B).

The second myotoxin, named MP-II, represents only 4 ~ 5% of the total applied protein. This fraction was repurified in the same conditions and is indicated by the arrow in Fig. 3A. As MP-I, it ran as a diffuse band in non reducing conditions with an apparent molecular mass of 28,5 kDa. In reduction

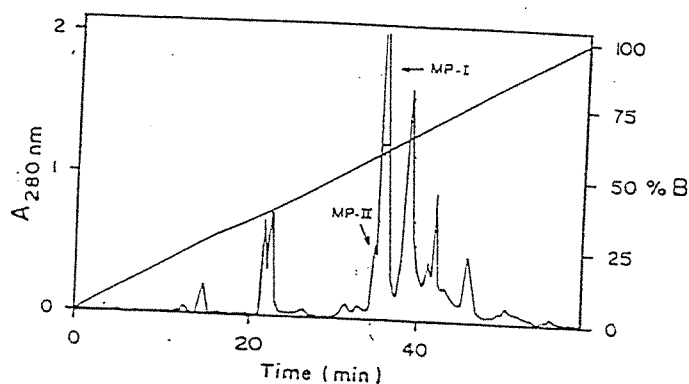


Fig. 1: Elution profile of *Bothrops pirajai* snake venom from a HPLC 0.78 cm x 30 cm μ Bondapack C-18 column. Elution was performed using a linear gradient from 0 to 66% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid at room temperature. Absorbance was recorded at 280 nm. Flow rate = 2.0 ml/min.

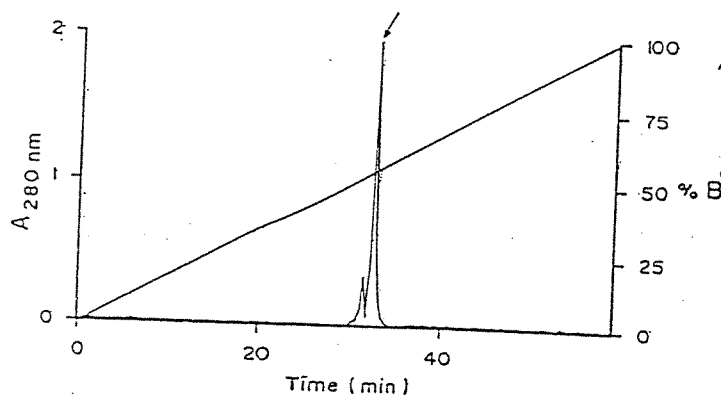


Fig. 2: Rechromatography of MP-I from Fig. 1. Rechromatography was performed in the same conditions of Fig. 1. SDS-

conditions MP-II shows a single band of 15,3 kDa in SDS-PAGE (Fig. 3B). None of the isolated myotoxins showed any significant PLA₂ or proteolytic activity. Their amino acid composition is shown in Table 1.

Comparative HPLC profiles of MP-I, MP-II, PrTX-I and PrTX-II are shown in Fig. 4 and 5. The elution times for MP-I and MP-II fit perfectly with those for PrTX-I and PrTX-II, respectively. Their amino acid composition is reported in Table 1.

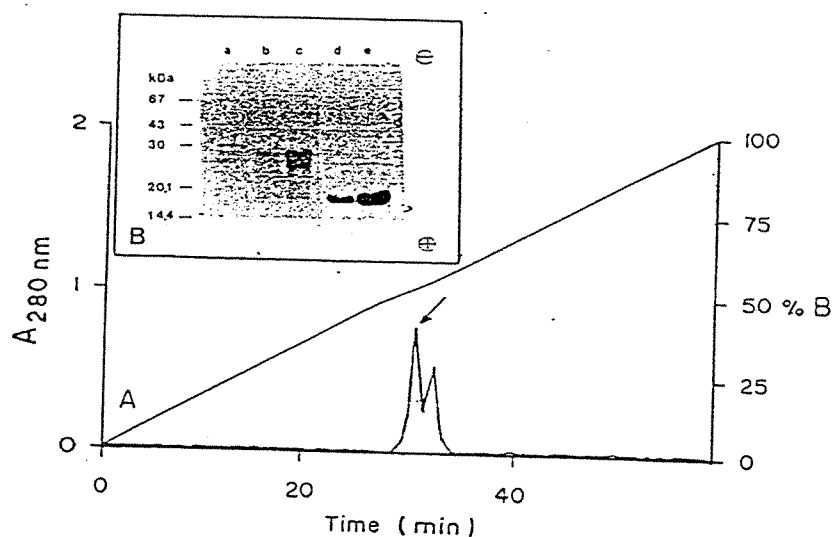


Fig. 3: Rechromatography of MP-II from Fig. 1 (3A) and SDS-PAGE of the repurified fractions MO-I and MP-II, in the absence and presence of dithiothreitol (3B).

Rechromatography was carried out as in Fig. 1. Lane a: standards; b: native MP-II; c: native MP-I; d: reduced MP-II; e: reduced MP-I. The amount of protein applied to the lanes was not quantified.

The sequences of MP-I, MP-II, PrTX-I and PrTX-II, up to the 51st, 41st, 46th and 39th residues, respectively, are shown in Fig. 6. As seen, they are identical along these N-terminal regions. These partial sequences are also identical to that of myotoxin II from *Bothrops asper* (7). No significant differences could be detected between the amino acid composition of MP-I and MP-II.

DISCUSSION

Myotoxins or myonecrotic toxins constitute a group of venom proteins with a specific action on skeletal muscle, affecting only muscle fibers and leaving other tissue structures such as connective tissue, nerves and vessels essentially unharmed (15). These proteins have a phospholipase (PLA₂) structure, although not necessary PLA₂ activity, usually 120-140 amino acid residues and six to eight disulfide bridges. They are omnipresent components of typical South-American bothropic venoms (5), together with thrombin-like

Table 1: Amino acid composition of MP-I, PrTX-I, MP-II and PrTX-II

Amino Acid	MOLAR RATIO			
	MP-I	PrTX-I	MP-II	PrTX-II
Asx*	15	15	14	14
Thr	6	7	6	6
Ser	4	4	6	7
Glx*	9	8	9	9
Pro	7	7	6	5
Gly	11	11	11	11
Ala	7	7	7	7
1/2 Cys**	14	14	14	14
Val	4	4	4	5
Met	1	1	1	1
Ile	3	3	5	5
Leu	12	12	11	11
Tyr	10	10	8	8
Phe	2	2	3	3
Lys	15	15	14	14
His	2	2	3	3
Arg	6	6	6	5
Trp***	1	1	1	1
TOTAL	129	129	129	129

* Asx, Glx: Asp + Asn, Glu + Gln, respectively.

** Determined as cysteic acid after performic acid oxidation and 24h hydrolysis with 6 M HCl at 106°C.

*** Determined after 24h hydrolysis with 3M methanesulfonic acid at 106 °C

enzymes (16), hemorrhagins, edema-inducing toxins (17), bradykinin potentiating peptides (18) and anticoagulant proteins (9).

Making use of the high resolution capacity and high flow rate of the HPLC system, two myotoxins, here named MP-I and MP-II, could be isolated from *B. pirajai* snake venom in a relatively short time. MP-I, which is the major toxin in this venom, can be prepared even in a one-step chromatography.

The identity between MP-I and PrTX-I or between MP-II and PrTX-II was strongly suggested by the HPLC coelution profile (Figs. 4 and 5).

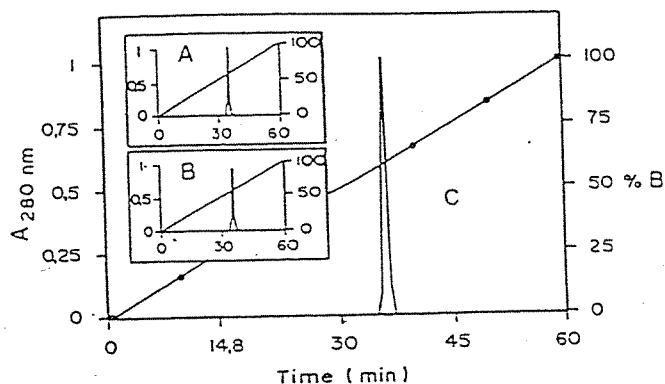


Fig. 4: Coelution profile of MP-I and PrTX-I.
A: MP-I; B: PrTX-I; C: MP-I + PrTX-I. Conditions as in Fig. 1.

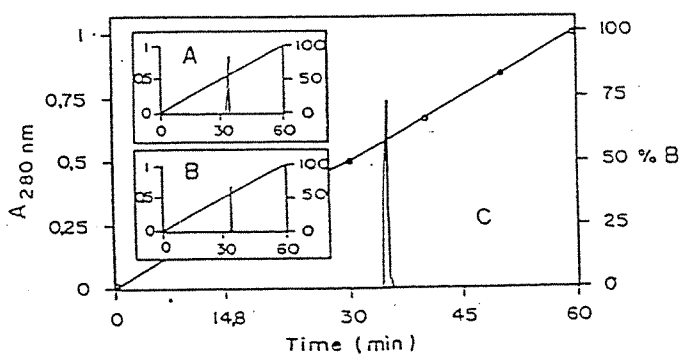


Fig. 5: Coelution profile of MP-II and PrTX-II.
A: MP-II; B: PrTX-II; C: MP-II + PrTX-II. Conditions as in Fig. 1.

Although the present procedure restricts the amount of protein to be loaded on each column to a few mg, the final yield of 35% for MP-I, when compared to 19.7 % as obtained by the original method, represents an advantageous strategy when larger amounts of the myotoxin are not a required condition. For MP-II there was not any significant difference in the corresponding yields.

As shown in Fig. 6, MP-I is a Lys-49 phospholipase A₂-like protein. Its lack of PLA₂ activity is therefore not surprising, as was the case of myotoxin-II from *Bothrops asper* venom (7).

According to Dijkstra et al. (19), the essential calcium ion in PLA₂s is located in the active site and is stabilized by one carboxyl group as well as by a peptide loop. Nothing however may be anticipated with regard to position

	10	20	30	40	50
MP-I	SLFELGKMIL	QETGKNPAKS	YGAYGCNCGV	LGRGKPKDAT	DRCCYVHKCC
MP-II	SLFELGKMIL	QETGKNPAKS	YGAYGCNCGV	LGRGKPKDAT	D.....
PrTX-I	SLFELGKMIL	QETGKNPAKS	YGAYGCNCGV	LGRGKPKDAT	DRCCYV.....
PrTX-II	SLFELGKMIL	QETGKNPAKS	YGAYGCNCGV	LGRGKPKDA..	

Fig. 6: Partial sequences of MP-I, MP-II, PrTX-I and PrTX-II.

The native samples were used for N-terminal sequencing by automated Edman degradation.

49 of MP-II since the lack of, or only residual PLA₂ activity does not necessary imply the absence of Asp-49 but rather a general disorganization of the Ca²⁺-binding loop or active site due to the presence of inadequate amino acid residues in strategical positions.

In non-reducing conditions, MP-I and MP-II, as obtained above, migrate as diffuse 28.5 kDa bands in SDS-PAGE. A careful inspection of Fig. 3B points to microheterogeneity, very probable due to the presence of isoforms (which are, among phospholipases, the rule rather than an exception). Similar diffuse band was obtained for the dimeric myotoxin II from *Bothrops asper* (Francis et al., 1991). The pale lower bands in the same Figures are sharper and match the reduced 15.3 kDa monomers, thus indicating an equilibrium between the monomeric and dimeric forms. The Figures also suggest that the monomers, which are indistinguishable from one another by SDS-PAGE, produced a set of compact dimers with different molecular sizes but apparent mol.weights lower than twice that of the monomers. In typical phospholipases the transition monomer → dimer is suggested (20) and found to enhance PLA₂ activity up to 200-fold. This is however not the case for MP-I and MP-II, whose PLA₂ activity is near zero.

Although myotoxins are deprived of any proteolytic activity (and MP-I/MP-II are not an exception), the synergistic effect of proteolytic enzymes in snake venoms upon the necrotic action should not be disregarded since many of them, including venom proteinases, as well as papain, trypsin and chymotrypsin, can induce necrosis (21).

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A Rapid procedure for the isolation of the Lys-49 myotoxin II from *Bothrops moojeni* (Caissaca) venom: Biochemical Characterization, Crystallization, Myotoxic and Edematogenic Activity.

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A RAPID PROCEDURE FOR THE ISOLATION OF THE LYS-49 MYOTOXIN II FROM *BOTHROPS MOOJENI* (CAISSACA) VENOM: BIOCHEMICAL CHARACTERIZATION, CRYSTALLIZATION, MYOTOXIC AND EDEMATOGENIC ACTIVITY

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A. M. Soares, V. M. Rodrigues, M. I. Homsi-Brandeburgo, M. H. Toyama, F. R. Lombardi, K. Arni and J. R. Giglio. A rapid procedure for the isolation of the Lys-49 myotoxin II from *Bothrops moojeni* (caissaca) venom: Biochemical characterization, crystallization, myotoxic and edematogenic activity. *Toxicon* 36, 503–514, 1998.—*Bothrops moojeni* snake venom was fractionated on a CM-Sepharose column which was previously equilibrated with 0.05 M ammonium bicarbonate buffer at pH 8.0 and subsequently eluted with an ammonium bicarbonate concentration gradient from 0.05 to 0.5 M at constant pH (8.0) and temperature (25°C). The fraction which eluted last (M-VI) showed, after direct lyophilization, a single band by polyacrylamide gel electrophoresis (PAGE) and SDS-PAGE, indicating an approximate M_r of 14 000 and 27 000, in the presence and absence of dithiothreitol, respectively. Its amino acid composition revealed a high level of hydrophobic and basic amino acids as well as 14 half-cystine residues. Its isoelectric point and extinction coefficient ($E_{1.0}^{1.0 \text{ mg/ml}}$ at 278 nm and pH 7.0) were 8.2 and 1.170, respectively. M-VI was devoid of phospholipase A₂ (PLA₂) activity on egg yolk, as well as of hemorrhagic, anticoagulant and coagulant activities, but could induce drastic necrosis on skeletal muscle fibres as well as rapid and transient edema on the rat paw. Its N-terminal sequence: SLFELGKMIL-QETGKNPAKSYGVYGCNCGVGGRGKPKDATDRCCYVHKCCYK... revealed high homology with other Lys 49 PLA₂-like myotoxins from other bothropic venoms. Orthorhombic crystals of M-VI, which diffracted to a maximal resolution of 1.6 Å, were obtained and indicated the presence of a

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Keywords: *Bothrops moojeni*, myotoxin-II, myonecrosis, crystallization

INTRODUCTION

Phospholipases A₂ (PLA₂s, E.C. 3.1.1.4) are largely distributed in nature and are found both intra and extracellularly (Bosch, 1980). Extracellular PLA₂s are abundant in pancreatic secretion and in snake and arthropod venoms.

Snake venoms PLA₂s are able to induce several biological effects such as pre or post-synaptic neurotoxicity, cardiotoxicity, myotoxicity, platelet aggregation inhibition, edema, hemolysis, anticoagulation, convulsion and hypotension (Kini and Iwanaga, 1986a,b).

During the last fifteen years, there has been considerable interest in the study of the venom components responsible for myonecrosis and their modes of action. As a result, several myotoxins from bothropic venoms have been isolated and characterized (Gutiérrez and Lomonte, 1995). Myotoxic PLA₂s from bothropic venoms may be divided into two major classes: those with enzymatic activity on artificial substrates, the Asp 49 myotoxic PLA₂s, and those lacking this catalytic activity, the Lys 49 myotoxins (Maraganore *et al.*, 1984; Homsí-Brandeburgo *et al.*, 1988; Gutiérrez and Lomonte, 1995; Mancuso *et al.*, 1995; Arni and Ward, 1996). Additionally, natural occurring PLA₂ homologous have been isolated which possess Ser (Krizaj *et al.*, 1991) or Ala (Liu *et al.*, 1991) at position 49, both of these substitutions resulting in catalytically inactive proteins.

Lomonte *et al.* (1990) have reported a two-step chromatographic procedure for the isolation and partial characterization of two basic myotoxins from *Bothrops moojeni* snake venom, referred to as myotoxins-I and -II. The present paper describes a simplified, rapid, dialysis-free, one-step procedure for the isolation of the most basic myotoxin from this venom, myotoxin-II, its biochemical characterization, myotoxic activity, crystallization and N-terminal sequence up to the 53rd amino acid residue.

MATERIALS AND METHODS

CM-Sepharose was purchased from Pharmacia (Uppsala, Sweden). Molecular weight markers, polyethylene glycol 400 (PEG-400) and *N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulphonic acid (HEPES) were from Sigma Chem. Co. Highly purified, lyophilizable ammonium bicarbonate was synthesized as previously described (Sampaio *et al.*, 1983). All other reagents were of analytical or sequanal grade.

Purification procedure

Desiccated *B. moojeni* venom (200 mg, from snakes of the State of São Paulo, Brazil) was dispersed in 2.5 ml of 0.05 M ammonium bicarbonate buffer at pH 8.0 and cleared by centrifugation at 480 × *g* for 10 min at room temperature. An aliquot of this solution was used for measurement of the total absorbance at 280 nm (*A*_{280 nm}) and total protein estimation by the microbiuret method of Itzhaki and Gill (1964). The clear solution was applied on a CM-Sepharose column (2.0 × 20 cm) which was previously equilibrated with the same buffer. Elution started with this low buffer concentration followed by a concentration gradient from 0.05 to 0.5 M at pH 8.0 and 25°C. Fractions of 3 ml/tube were collected at a flow rate of 20 ml/h using an LKB fraction collector connected to a U.V. detector device. Absorbances at 280 nm were also read in a Beckman spectrophotometer and conductivities of the collected fractions were monitored with a Beckman RC-16C conductimeter.

Biochemical characterization

Fraction M-VI was assayed for purity by PAGE, following the method of Reisfeld *et al.* (1962) for basic proteins and by SDS-PAGE according to Laemmli (1970).

The extinction coefficient ($E_{1.0\text{ cm}}^{1.0\text{ mg/ml}}$) at $\lambda = 278\text{ nm}$, pH = 7.0, was evaluated at different protein concentrations (0.25 to 2.0 mg/ml). In addition, the following determinations were carried out for the isolated protein as previously detailed (Honsi-Brandeburgo *et al.*, 1988): amino acid composition (Spackman *et al.*, 1958), isoelectric point (Vesterberg, 1972), N-terminal amino acid residue (Gray, 1972) and amino terminal sequence, up to the 53rd amino acid residue of the reduced and carboxymethylated toxin, using an Applied Biosystem Sequencer, model 477A (Toyama *et al.*, 1995).

Enzymatic and biological assays

The edematogenic activity was assayed as described by Lloret and Moreno (1993) using 180–200 g Wistar rats. Aliquots of 50 μg of the purified toxin were diluted with 100 μl saline (0.9%, w/v, NaCl) and injected in the subplantar region of the right paw ($n = 6$), whereas in the left paw the same volume of saline alone was injected as a control. The edema formation was measured with the use of a plethysmograph, computerized at several time intervals (0, 0.5, 1, 2, 4 and 24 h). Edematogenic activity was expressed in percent of volume increase of the right paw as compared to the left one.

The myotoxic activity was assayed on the basis of the morphologic alterations induced by i.m. injections of 50 μg toxin in the right gastrocnemius skeletal muscle of Swiss mice ($n = 6$). After 24 h, the animals were sacrificed by deep anesthesia with ethyl ether and a small section of the central region of the muscle was excised and soaked in fixing solution [95% (v/v) ethanol–40% (w/v) formaldehyde–glacial acetic acid– H_2O , 3:1:1:5 (v/v)]. The material was then dehydrated by increasing concentrations of ethanol and processed for inclusion in glycol metacrylate. The resulting blocks were sliced in 2.5 μm thick sections, stained with 0.25% (w/v) toluidine blue and examined under a light microscope.

The PLA_2 activity was determined by potentiometric titration using an egg yolk emulsion, which contained phosphatidylcholine, as a substrate, as described by De Haas *et al.* (1968). Other additional activities assayed were: anticoagulant, using citrated bovine plasma (Alvarado and Gutiérrez, 1988); hemorrhagic, through i.d. injections in the back of mice (Nikai *et al.*, 1984) and coagulant, using citrated bovine plasma or purified fibrinogen (Selistre and Giglio, 1987; Assakura *et al.*, 1992).

Crystallization

Multiple crystallization trials were set up in Linbro tissue culture plates by the micro-vapour diffusion hanging drop method (Ducrix and Geige, 1992). A stock protein solution was prepared by dissolving the lyophilized sample in distilled water at room temperature (20°C) to a concentration of 15.0 mg/ml. Initially, the crystallization conditions were screened using the sparse matrix sampling techniques (Jancarik and Kim, 1991) in which 0.5 μl of the protein solution suspended on siliconized microscope-slide coverslips were equilibrated against 0.5 ml of the reservoir solution. The best crystals were obtained from 0.1 M HEPES, 2.0 M ammonium sulphate and 2.0% (v/v) PEG400 (pH 5.5). Once the optimal conditions for crystallization were determined, the volume of the protein was increased to 4 μl . The crystals were then mounted in thin-walled glass capillaries along with a small quantity of mother liquor to prevent dehydration. A native X-ray diffraction data set was collected from a single crystal on a Rigaku R-AXIS IIC imaging plate detector and X-rays were generated by a Rigaku RU200 rotating anode X-ray generator operating at 50 kV and 100 mA, utilizing a graphite monochromator and a 0.3 mm collimator. The detector-to-imaging plate distance was set to 58.4 mm and the swing angle at 0°. Two still images were recorded. A complete X-ray diffraction data set was collected to 1.6 Å. The cell parameters were then refined further and the data processed utilizing the software package PROCESS (Higashi, 1990). The integrated intensities for both full and partial reflections were obtained using profile fitting in which both temperature and intensity factors were used for scaling ($R_{\text{merge}} = 5.5\%$). The reflection files were then examined.

RESULTS

Figure 1(A) illustrates the chromatographic profile of the venom fractionation on CM-Sepharose. The main protein fractions were pooled and named M-I_a + b, M-II_a + b, M-III, M-IV_a + b, M-V and M-VI. PLA_2 , coagulant and hemorrhagic activities are indicated in Table 1. Table 1 presents also the recovery in absorbance and total protein. Fraction M-VI (myotoxin II) accounts for 7% (w/w) of the desiccated venom. The ($E_{1.0\text{ cm}}^{1.0\text{ mg/ml}}$) of M-VI at 278 nm and pH 7.0 was 1.170.

PAGE of the whole venom and M-VI is shown in Fig. 1(B). The toxin, basic in character, appeared as a single electrophoretic band. Manual identification of the

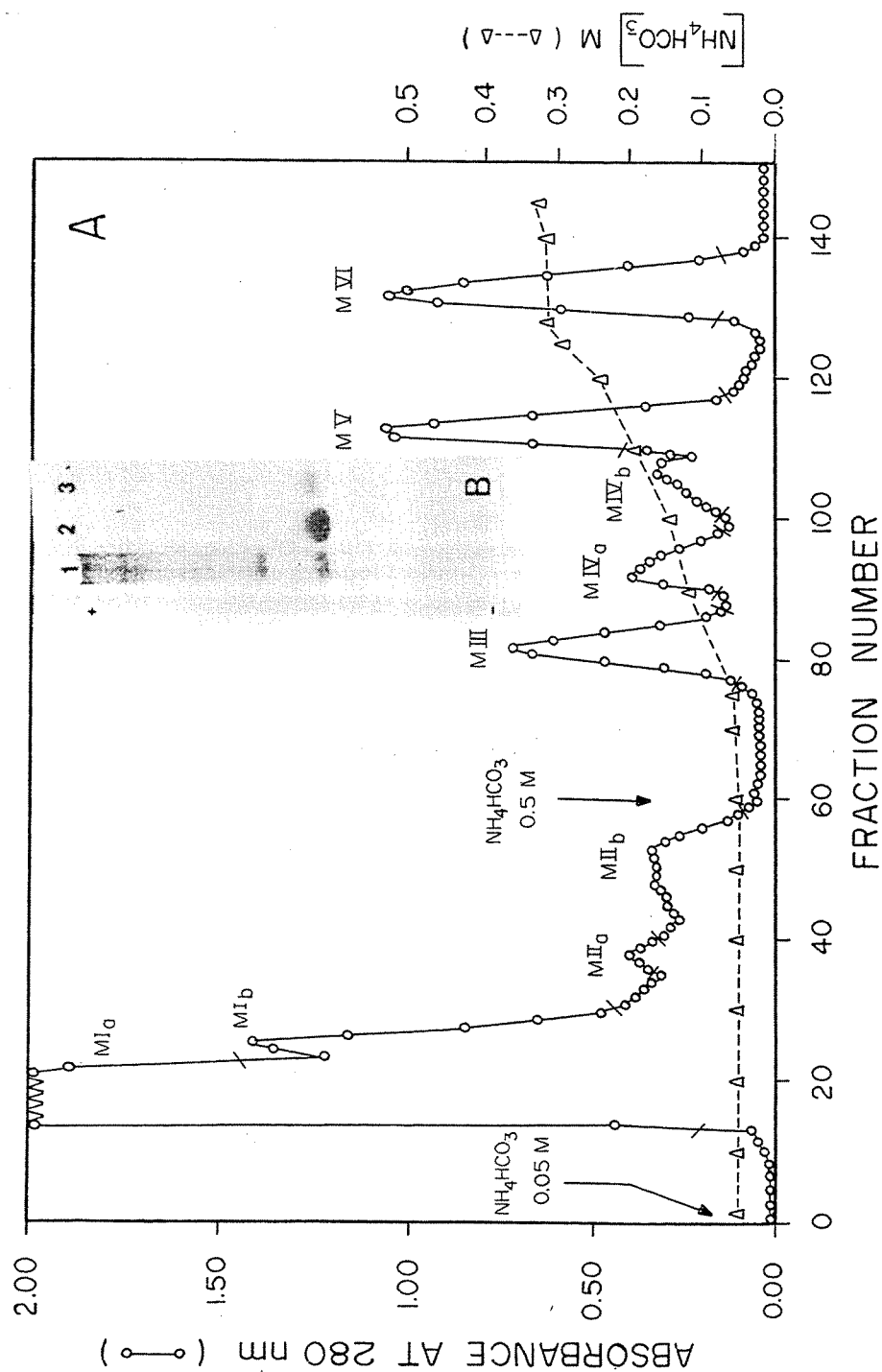


Fig. 1. (A) Chromatography of *B. maojoni* venom (200 mg) on a 2.0×20 cm column of CM-Sephacrose which was previously equilibrated with 0.05 M, pH 8.0 NH_4HCO_3 buffer. Elution started with this buffer, followed by a continuous concentration gradient from 0.05 to 0.5 M at constant pH and 25°C . Flow rate: 20 ml/h. [B (insert)] PAGE on a 10% (w/v) gel in β -alanine buffer at pH 4.5, 15 mA, for 2 h. Lane 1: *B. maojoni* venom; lane 2: fraction M-V; lane 3: fraction M-VI.

Table 1. Percent recoveries (%Rec) and enzymatic activities of the several fractions from *B. moojeni* venom

Fraction	Protein		$A_{280\text{ nm}}$		PLA ₂ activity (U/mg)	Clotting activity MCD ^a (μg)	Hemorrhagic activity MHD ^b (μg)
	mg	%Rec	U	%Rec			
Whole venom	170.0	100.0	262.6	100.0	84.0	13.8	8.4
M-Ia	71.8	42.2	157.5	60.0	168.9	—	6.0
M-Ib	10.2	6.0	18.4	7.0	14.1	residual	residual
M-IIa	2.6	1.5	4.7	1.8	14.6	residual	—
M-IIb	10.5	6.2	12.0	4.6	14.8	16.6	residual
M-III	11.6	6.8	12.0	4.6	residual	20.8	—
M-IVa	14.0	8.2	7.3	2.8	residual	2.0	—
M-IVb	4.7	2.8	7.0	2.6	10.6	2.6	—
M-V	8.6	5.1	13.0	4.9	—	—	—
M-VI	11.4	6.7	16.6	6.3	—	—	—
Total	145.4	85.5	248.5	94.6			

^aMCD: Minimum coagulant dose, μg of dry whole venom or fractions that clots 0.2 ml bovine citrated plasma in 1 min.

^bMHD: Minimum hemorrhagic dose, minimum amount of venom or fractions that produces in mice a hemorrhagic spot of 10 mm diameter.

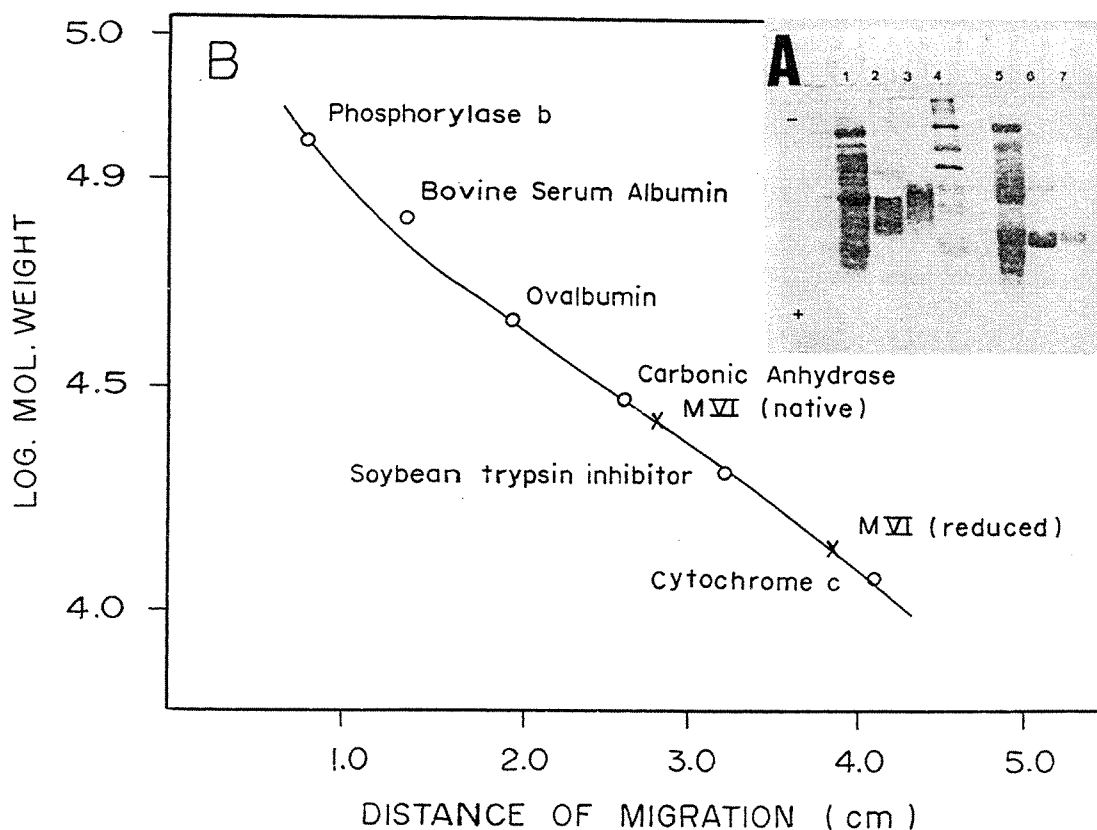


Fig. 2. [A (insert)] SDS-PAGE on a 15% (w/v) gel, at 15 mA, for 2 h 30 min. Lane 1: *B. moojeni* venom; lane 2: fraction M-V; lane 3: fraction M-VI; lane 4: mol. weight markers; lane 5: *B. moojeni* venom; lane 6: fraction M-V; lane 7: fraction M-VI. Fractions 1 to 3: native; fractions 4 to 7: dithiothreitol reduced. (B) Determination of the M_r of native and reduced M-VI.

Table 2. Amino acid composition of M-VI after hydrolysis with 5.3 N azeotropic HCl solution for 30 h at 110°C

Amino acid residue	M-VI
Asp	15
Thr	6
Ser	5
Glu	7
Pro	5
Gly	10
Ala	5
Half-Cys ^a	14
Val	5
Met	1
Ile	3
Leu	9
Tyr	10
Phe	2
Lys	19
His	1
Arg	5
Trp	n.d. ^b
Total	122 + Trp
Mol. weight	13 887 + Trp

^aDetermined as cysteic acid after oxidation with performic acid and hydrolysis as above.

^bNot determined.

N-terminal residue revealed Ser as the only amino acid at this position. The N-terminal sequence as determined by automatic Edman degradation was found to be ¹SLFELGKMI¹⁰LQETG-KNPA²⁰KSYGVYGCNC³⁰GVGGRGKPKD⁴⁰ATDRCCYV-HK⁵⁰CCYK... The data from SDS-PAGE (Fig. 2) indicate an M_r of around 14 000 in reducing conditions but 27 000 in the native state.

As seen from the amino acid composition data presented in Table 2, M-VI is rich in basic and hydrophobic amino acid residues including 14 half-cystine residues/mol. The pI of M-VI was 8.2 as determined by isoelectric focusing (Fig. 3).

No enzymatic activity on the assayed substrates was detected in this toxin. However, drastic myotoxic activity (Fig. 4) and a transient edema formation (Fig. 5) was observed.

Under the conditions described, M-VI crystallized in an orthorhombic lattice (Fig. 6). The parameters from the crystal structure are shown in Table 3. Large single crystals with dimensions of 0.5 × 0.5 × 0.3 mm were obtained. The crystal system was deduced to have cell parameters $a = 64.18 \text{ \AA}$, $b = 88.71 \text{ \AA}$ and $c = 51.09 \text{ \AA}$ (all angles = 90°). The crystal diffracted strongly to 1.6 Å and was determined to belong to the space group P2₁2₁2₁.

DISCUSSION

The procedure described above for the isolation of fraction M-VI from the venom of *B. moojeni* is relatively simple, quick and efficient. It avoids the use of high buffer concentration for elution as well as dialysis and rechromatography. On basis of the data of Lomonte *et al.* (1990), who isolated myotoxins-I and -II from the same venom using

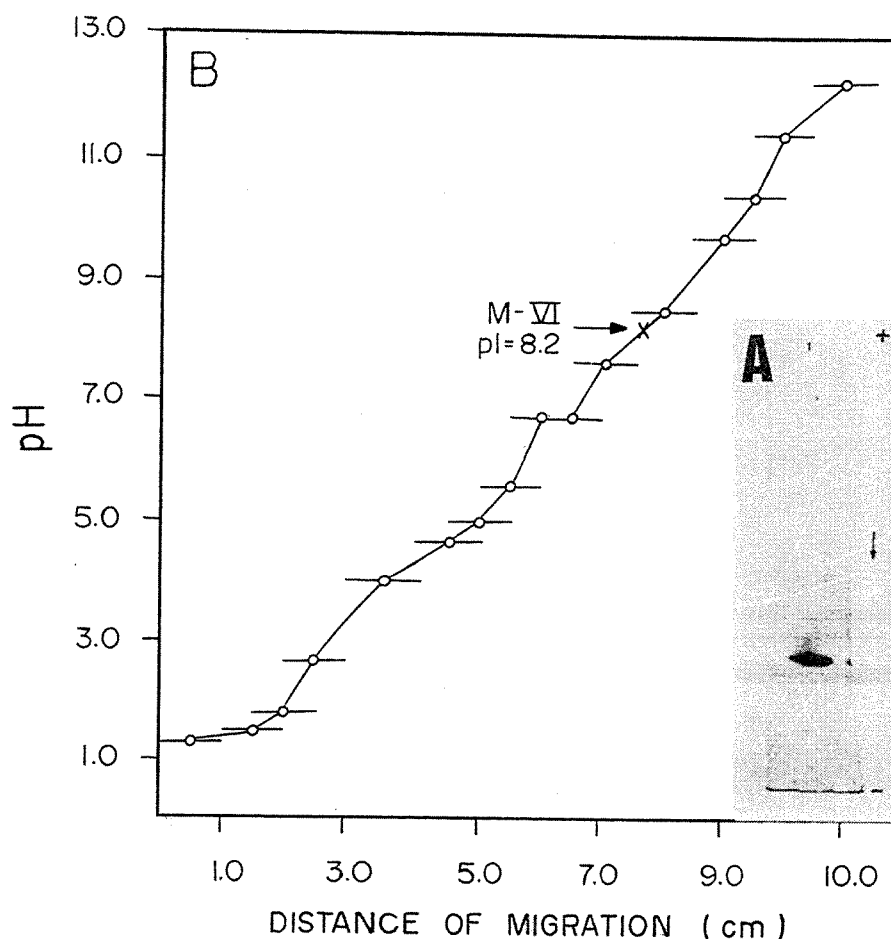


Fig. 3. [A (insert)] Electrofocusing of M-VI on polyacrylamide gel. A pre-focusing was performed at 100 V and 40 mA for 30 min, followed by focusing at 250 V and 40 mA for 30 min, 750 V (40 to 2 mA) for 2 h 15 min and finally at 1550 V (5 to 0 mA) for 1 h 15 min. (B) pH gradient from the isoelectric focusing. M-VI focused at pH 8.2.

0.75 M Tris-HCl buffer at pH = 7.0 and CM-Sephadex C-25, fraction M-VI (this work) was identified as myotoxin-II.

Raising the pH to 8.0 facilitated the elution of several fractions so that M-VI could be eluted at an ionic strength = 0.35 (0.35 M NH_4HCO_3) instead of 0.75 (0.75 M Tris-HCl). In addition, NH_4HCO_3 is lyophilizable, permitting us to omit dialysis. Highly purified NH_4HCO_3 , prepared by direct synthesis from $\text{NH}_3 + \text{CO}_2$ (Sampaio *et al.*, 1983), was used to buffer the system leaving no residue after lyophilization.

The last eluted fraction, M-VI, passed several purity tests, focused at pH 8.2 as a single band and produced a single amino acid residue after each step of Edman degradation up to the 53rd residue. Therefore no rechromatography was necessary. It is possible that the change from CM-Sephadex to CM-Sephadex contributed to higher resolution.

As seen in Table 1, PLA_2 , hemorrhagic and coagulant activities are present in different fractions of the venom, thus suggesting the presence of different molecular structures expressing the same kind of activity or even strong molecular interaction not disrupted

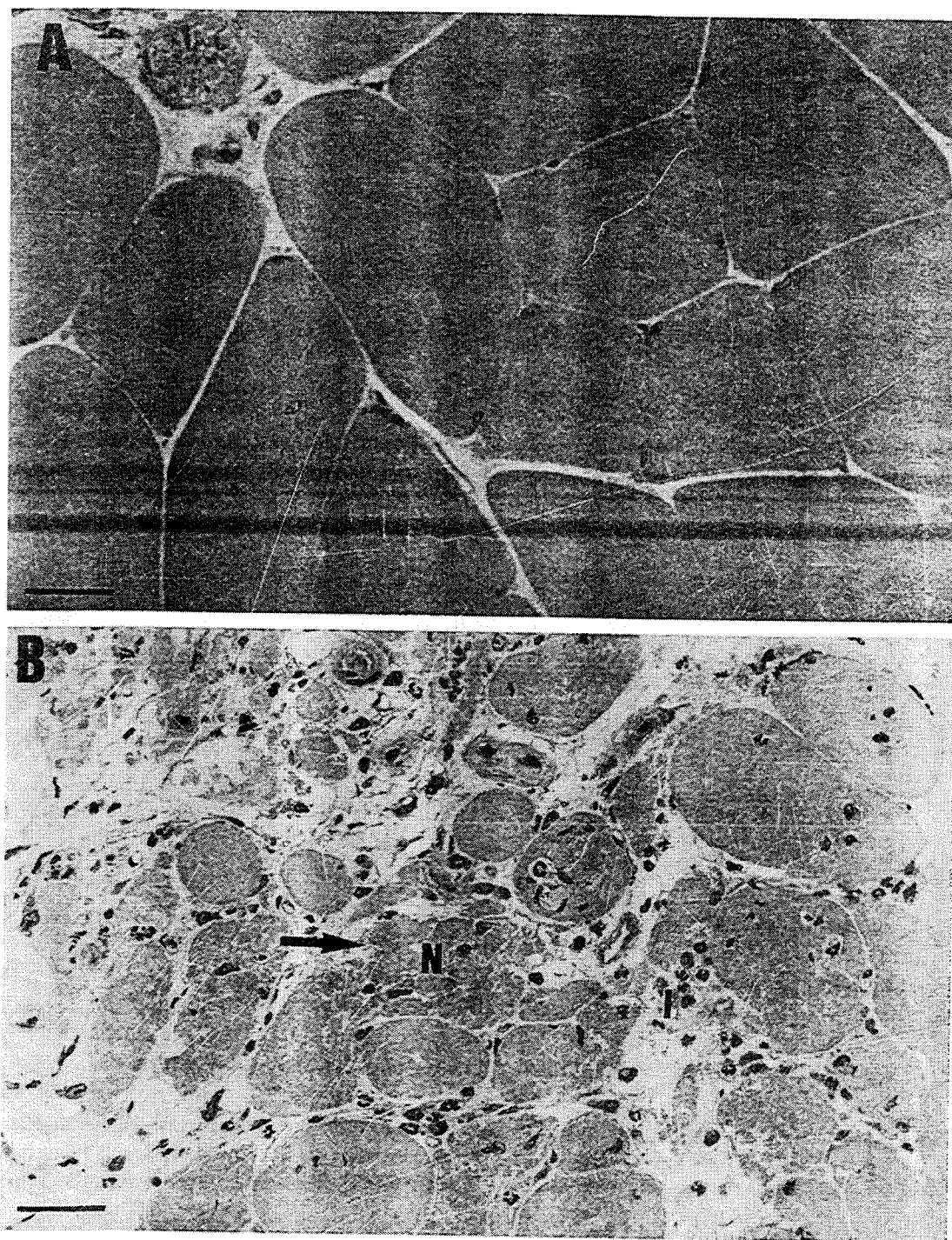


Fig. 4. Photomicrograph of gastrocnemius muscle slices ($2.5\ \mu\text{m}$ thickness) from the right limb of 18–22 g male mice. (A) control; (B) fraction M-VI ($50\ \mu\text{g}$). Staining with toluidine blue. The bar represents $50\ \mu\text{m}$. N: necrosis of the muscle fibre; I: leucocitary infiltration.

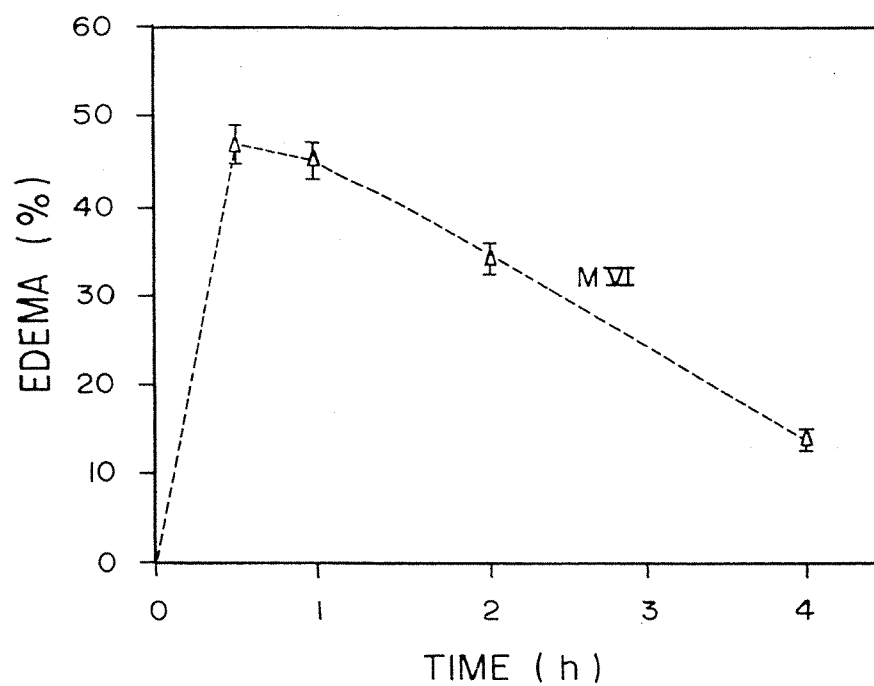


Fig. 5. Time-dependent edema induced by fraction M-VI ($50 \mu\text{g}$) in the paw of 180–200 g male Wistar rats.

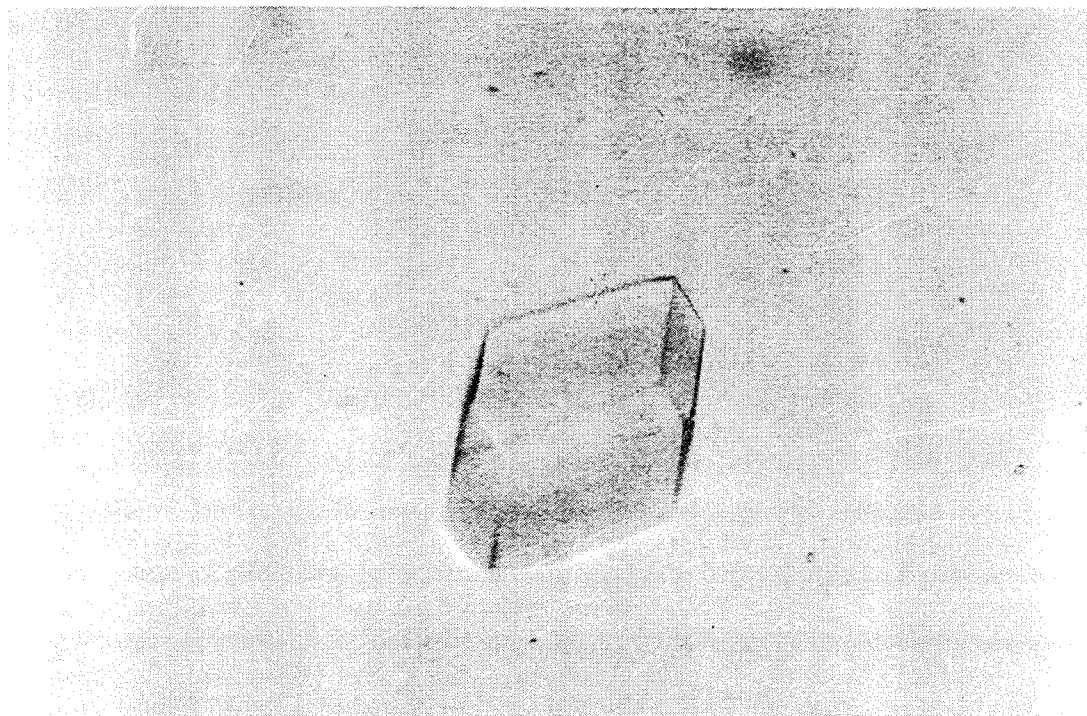


Fig. 6. Photomicrograph of a crystal of M-VI (0.3 mm) for the X-ray diffraction studies.

Table 3. Crystallographic parameters for *Bothrops moojeni* M-VI

X-ray diffraction data	M-VI
Crystal size (mm)	0.5 × 0.5 × 0.3
Crystal system	orthorhombic
Space group	P2 ₁ 2 ₁ 2 ₁
Cell dimensions	$a = 64.18 \text{ \AA}$ $b = 88.71 \text{ \AA}$ $c = 51.09 \text{ \AA}$
	$\alpha = \beta = \gamma = 90^\circ$
Volume per Dalton ($\text{\AA}^3$)	2.7
Molecules in the asymmetric unit	2
Max resolution ($\text{\AA}$)	1.6
R_{MERGE}	5.5%
Number of days for growing	3–4

^a $R_{\text{MERGE}} = \sum |I_i| - |I_{\text{mean}}| / \sum |I_{\text{mean}}|$, where I_i is the intensity of the i th observation of the reflection, and I_{mean} is the mean of these intensities.

by the ion-exchange process. As inferred from PAGE (not shown), all these fractions look still heterogeneous, except for M-VI [Fig. 1(B)], which is homogenous and lacks catalytic activity.

When in solution, this fraction dimerizes indicating a molecular mass of 27 kDa but, under reduction conditions, it dissociates to a monomeric form of 14 kDa as estimated by SDS-PAGE. Dimeric toxins have been encountered in other bothropic venoms. Examples are: myotoxin-II from *B. asper* (Francis *et al.*, 1991) and pyratoxins-I and -II from *B. pirajai* which showed diffuse bands around 28.5 kDa (Toyama *et al.*, 1995).

M-VI has a high content in Lys, Leu, Tyr and fourteen half-Cys residues. Its whole amino acid composition (Table 2) is very close to that of myotoxin-II (Lomonte *et al.*, 1990), strongly indicating that it is the same toxin. The absence of Cys at position 11 identifies M-VI as a class II PLA₂, in common with other myotoxins from bothropic venoms (Díaz *et al.*, 1995). The high content in Lys (~15% of the total residues) may be involved with a Lys-rich heparin-binding region which includes residues 115–129 (Gutiérrez and Lomonte, 1995). The heparin-binding ability of this region is believed to be enhanced by the close vicinity of Lys-36 and Lys-38 in similar myotoxins (Arni *et al.*, 1995).

The isoelectric point 8.2 is also very close to those of other myotoxins, such as bothropstoxins (Honsi-Brandeburgo *et al.*, 1988) and piratoxins (Mancuso *et al.*, 1995).

Fraction M-VI induced quick and drastic edema (45%/50 μg) with a maximum activity at 30 min, disappearing within 24 h. Myotoxin-II from *B. asper*, which is also devoid of PLA₂ and anticoagulant activity under the same conditions, is also able to induce a dose-dependent edema of 30%/38 μg (Lomonte and Gutiérrez, 1989).

The drastic necrosis produced by 50 μg of fraction M-VI was accompanied by a leucocyte infiltration of mainly polymorphonuclear leucocytes, demonstrating the intense inflammatory reaction caused by the toxin. No blood vessels were damaged, according to the hypothesis that the specific muscular injury induced by these toxins occurs directly via membrane alteration of the muscle cell (Gutiérrez and Lomonte, 1995).

A comparison between the N-terminal sequence of M-VI and those of other bothropic myotoxins with or without PLA₂ activity shows that its first 53 residues are highly homologous (85–95%) to those of the enzymatically inactive Lys 49 myotoxins but only 50–60% to those active Asp 49 PLA₂s. Residues Ser-1 and Glu-4 are believed to be highly conserved. Glu-4 forms an intra-helix hydrogen bond with the hydroxyl group of Ser-1 in the crystal structures of Lys 49 myotoxins from *Agkistrodon piscivorus pisci-*

vorus (Scott *et al.*, 1992). The proper conservation of the N-terminal sequence of these myotoxins seems to be fundamental for the binding of the toxin to the biomembrane (Díaz *et al.*, 1994). Since both Ser-1 and Glu-4 are present, it was expected that the whole sequence would reveal the substitution of one or more of the so considered strategic residues which forms the Ca^{2+} loop for PLA_2 activity, mainly Lys 49 for Asp 49. This provision was confirmed by the sequence data up to residue 53.

The crystal structure of M-VI, which is currently being determined, shows an orthorhombic form containing two toxin molecules in its asymmetric unit, thus characterizing a dimeric form. This dimeric form withstands solubilization and appears in solution under non-reducing conditions.

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Amino Acid Sequence of Piratoxin-I, a Myotoxin from *Bothrops pirajai* Snake Venom, and Its Biological Activity After Alkilation with ρ -Bromophenancil Bromide.

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Amino Acid Sequence of Piratoxin-I, a Myotoxin from *Bothrops pirajai* Snake Venom, and Its Biological Activity After Alkylation with *p*-Bromophenacyl Bromide

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The complete sequence of the 121 amino acid residues of piratoxin-I (PrTX-I), a phospholipase A₂ (PLA₂)-like myotoxin from *Bothrops pirajai* snake (*Bahia jararacussu*) venom, is reported. From the sequence, an *M_r* of 13,825 and an approximate *pI* of 8.3 were calculated. PrTX-I shows a high sequence homology with Lys-49 myotoxins from other bothropic (~95%) and nonbothropic (~80%) venoms, but only 70–75% homology when aligned with the catalytically active Asp-49 PLA₂s. When compared with bothropstoxin-I from *Bothrops jararacussu*, which is morphologically almost identical to *B. pirajai*, only two changes out of 121 total amino acid residues have been observed. The approximate minimal lethal dose *LD*₅₀ (mice, i.p., 24 hr) of PrTX-I was 8 (6.8–9.1) mg/kg, and the minimal edematogenic dose (MED) in a rat paw model was 39.5 ± 1.8 ug. After alkylation of His-48 with *p*-bromophenacyl bromide, the MED was 40.1 ± 1.9 ug, but up to 4 *LD*₅₀ were unable to cause death in any of a group of eight mice after 72 hr. Therefore the edematogenic activity was retained and apparently did not involve His-48, suggesting that at least two biologically active sites are present in PrTX-I.

KEY WORDS: Piratoxin-I; *Bothrops pirajai*; Lys-49 myotoxin; *p*-bromophenacyl bromide; lethality; edema.

1. INTRODUCTION

The genus *Bothrops* comprises several venomous snake species which are widely distributed from South to North America (Hoge and Romano Hoge, 1978/79). Regarding the bioactive proteins from bothropic venoms, the phospholipases A₂ (PLA₂, EC 3.1.1.4) and PLA₂-like myotoxins are outstanding as major components. PLA₂s specifically catalyze the hydrolysis of phospholipids at position sn-2 (van Deenen and de Haas, 1963).

Snake venom PLA₂s are usually distributed into two major groups: those found in (I) the Elapidae and Hydrophidae and (II) the Crotalidae and Viperidae fam-

ilies. Those in group II include the catalytically active Asp-49 and the inactive or little active Lys-49 PLA₂s (Selistre de Araujo *et al.*, 1996). In addition to catalytic activity, the PLA₂s may show neurotoxic, myotoxic, pro or anti-platelet aggregating, hemolytic, anticoagulant, cardiotoxic, and edematogenic activities (Kini and Evans, 1989). The myotoxic PLA₂s are responsible for the intense myonecrosis produced by the bothropic venoms. Some of them have anticoagulant and/or edematogenic activity. Besides the muscle cells, other tissues, such as macrophages, fibroblasts, and neurons, are apparently additional *in vitro* targets of these toxins (Gutiérrez and Lomonte, 1995). The specific receptor on the plasma membrane of the muscle cell is unknown (Fletcher *et al.*, 1996).

In previous papers we have reported the isolation (Mancuso *et al.*, 1995), crystallization (Canduri *et al.*, 1998), and the crystal structure (de Azevedo *et al.*, n.d.) of piratoxin-I (PrTX-I), a PLA₂-like myotoxin from

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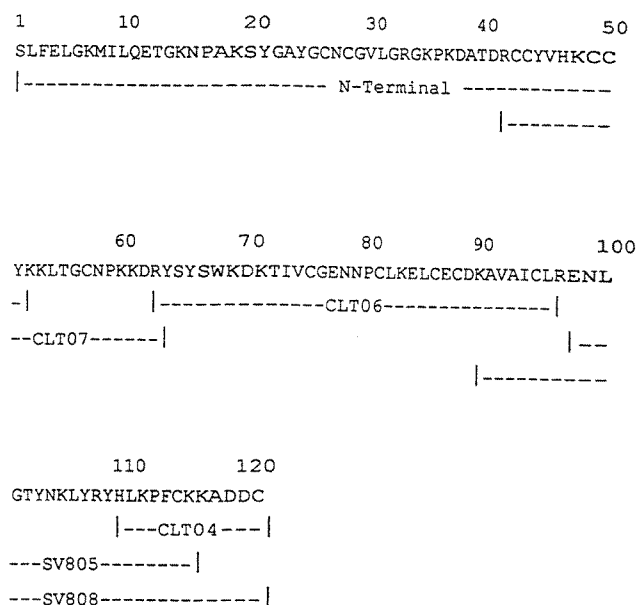


Fig. 1. The primary structure of the Lys-49 PLA₂-like myotoxin piratoxin-I (PrTX-I), showing the alignment of the peptides from the *Staphylococcus aureus* V8 protease (SV8) and from the clostripain (Clt) digest. The first 51 residues were directly sequenced. Note that the numbering does not show the deletions which appear in Fig. 4.

Bothrops pirajai snake venom. This paper reports its amino acid sequence and the effects of alkylation by *p*-bromophenacyl bromide upon its biological activity.

2. MATERIALS AND METHODS

2.1. Materials

Bothrops pirajai snake venom was a kind gift from CEPLAC/CEPEC (Ministry of Agriculture, Itabuna, Bahia, Brazil). All reagents used in this work were of analytical or sequence grade. *p*-Bromophenacyl bromide (p-BPB) was purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. Purification Procedure, Reduction, and Carboxymethylation of PrTX-I

PrTX-I was purified (Mancuso *et al.*, 1995) and rechromatographed by high-performance liquid chromatography (HPLC) (Toyama *et al.*, 1995) as previously described. The toxin was then dissolved in 6 M guanidine containing 0.4 M Tris and 2 mM EDTA, final pH 8.15, reduced with dithiothreitol (DTT), and carboxy-

methylated with ¹⁴C-iodoacetic acid (Frangione *et al.*, 1983). Desalting was performed by gel filtration of the sample on a Sephadex G-25 column in 1 M acetic acid at 25°C.

2.3. Digestion of the Toxin and Purification of the Resulting Peptides

Reduced and carboxymethylated PrTX-I (RC-PrTX-I) was digested with *Staphylococcus aureus* protease V8 for 16 hr at 37°C, using a 1:30 enzyme to substrate molar ratio and stopping the reaction by lyophilization (Houmard and Drapeau, 1972). RC-PrTX-I was also digested with clostripain for 8 hr at 37°C and lyophilized (Cintra *et al.*, 1993).

Both protease V8 and clostripain digests were fractionated by reverse-phase HPLC (RP-HPLC) using a Waters PDA 991 System and a C-18 μ Bondapak column under a concentration gradient of acetonitrile in 0.1% (v/v) trifluoroacetic acid.

2.4. Sequencing Procedure

Direct sequencing from the N-terminal end of the toxin was carried out with the native protein followed by those of the isolated peptides from the RC-PrTX-I, using an Applied Biosystem 477 automatic sequencer. The phenylthiohydantoin (PTH) amino acids were identified according to their retention time compared with those of the 20 standard PTH-amino acids. Cys was identified as PTH-¹⁴C-carboxymethylated Cys and confirmed by radioactive counting.

2.5. Alkylation of PrTX-I and Biological Assays

Alkylation with p-BPB (Díaz-Oreiro and Gutiérrez, 1997) was performed with 3 mg of PrTX-I dissolved in 1 ml of 0.1 M ammonium bicarbonate buffer, pH 8.0, plus 125 μ l of a solution containing 1.5 mg of p-BPB/ml of ethanol. After 24 hr of incubation at 25°C, the sample was centrifuged at 7000 $\times$ g for 10 min and the excess reagent was removed by successive washings with the same buffer in an Amicon ultrafiltration system using a YM-3 membrane. A control experiment was run as above, using however 125 μ l of ethanol replacing the p-BPB solution.

Assays for the lethal (*LD*₅₀) (Homsí-Brandeburgo *et al.*, 1988), myotoxic, and edematogenic activities (Soares *et al.*, 1998) of the native and alkylated toxin were then performed as previously described.

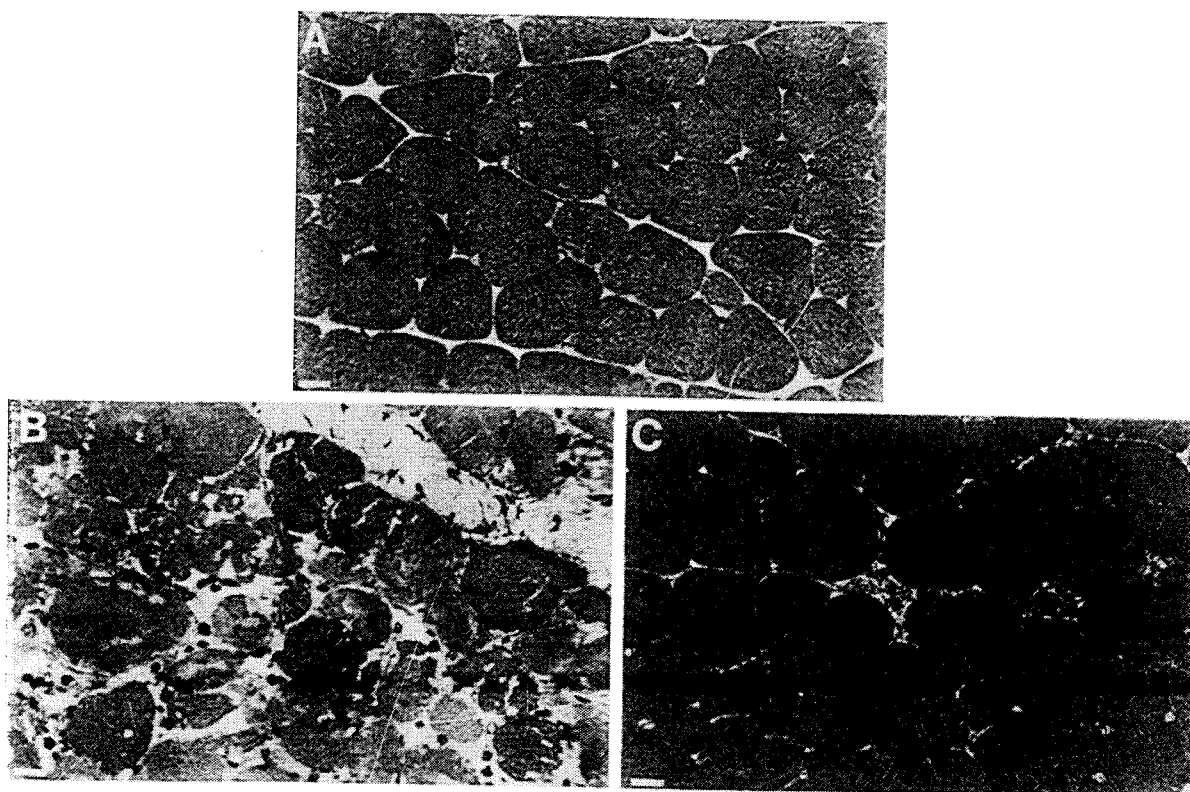


Fig. 2. The myotoxic activity of PrTX-I in mice. (A) saline control; (B) native PrTX-I; (C) p-BPB-alkylated PrTX-I: apparent complete loss of the myotoxic activity. Bar: 50 μ m.

3. RESULTS

The RP-HPLC profile of PrTX-I revealed a single and symmetric major peak corresponding to 97% of the total eluted protein (not shown). This highly purified toxin was then directly sequenced up to the 51st residue (Fig. 1). The subsequent sequencing of the isolated peptides from the protease V8 and clostripain digests made possible the complete alignment of the 121 amino acid residues of the toxin (Fig. 1). The calculated M_r was 13,825 and the estimated pI derived from this sequence was 8.26.

Akylation of PrTX-I with p-BPB resulted in a loss of the lethal and myotoxic (Fig. 2), but not of the edematogenic activity (Fig. 3). For the native toxin, the MED (μ g of toxin able to induce 30% edema in the rat paw) was $39.5 \pm 1.8 \mu$ g; for the alkylated toxin it was $40.1 \pm 1.9 \mu$ g. The LD_{50} was 8 mg (6.8–9.1mg)/kg (mice, i.p., 24 hr) for the native toxin. No death was observed, however, 72 hr after injecting up to 4 LD_{50} of alkylated toxin in a group of eight animals. The amino acid composition (not shown) of alkylated PrTX-I revealed a single free His/mole.

No detectable difference between the pI_e of native and alkylated PrTX-I and no difference in cross-reaction against an antiothropic serum could be observed (results not shown).

4. DISCUSSION

Geographic confinement of some species of snakes belonging to a single genus may induce morphological and biochemical alterations which differentiate them to the point of constituting a subspecies. This is the case of *B. pirajai*, which is found only in southern Bahia State, Brazil, and is morphologically almost identical to *B. jararacussu*, hence its popular name "Bahia Jararacussu." The fractionation of its venom was previously reported (Mancuso *et al.*, 1995), pointing to the similarities with that from *B. jararacussu*. PrTX-I is the *B. pirajai* myotoxin corresponding to *B. jararacussu* bothropstoxin-I (BthTX-I), whose amino acid sequence was also already reported (Cintra *et al.*, 1993).

The present results show a high level (95%) of sequential homology between PrTX-I and other bothropic

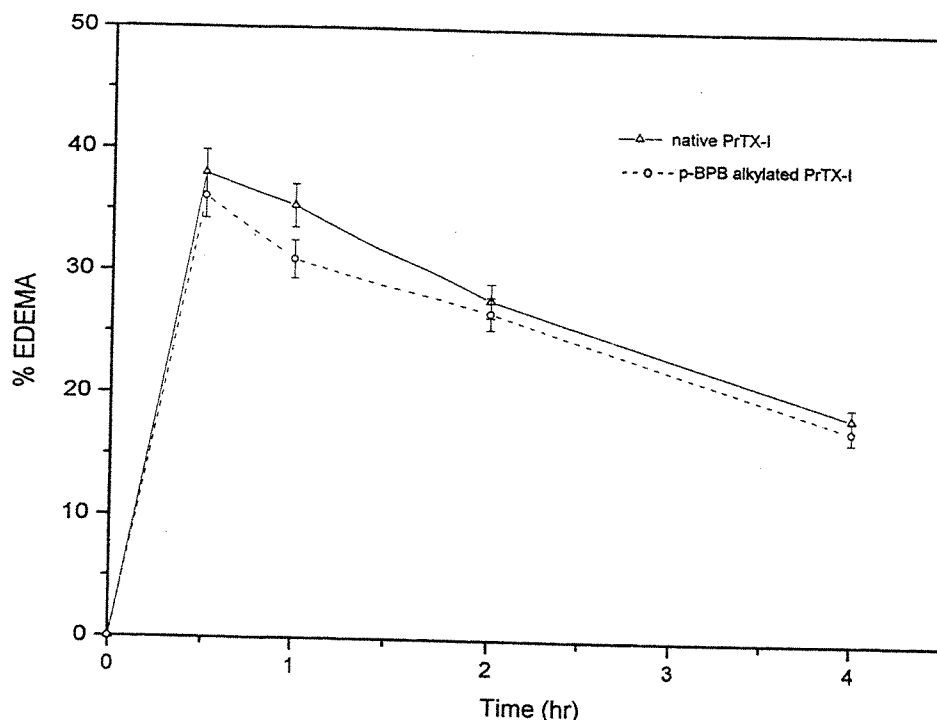


Fig. 3. Induction of time-dependent edema (50 μ g) by native PrTX-I and p-BPB-alkylated PrTX-I: no significant difference is visible.

Lys-49 PLA₂s. However, this value falls to 80% for the nonbothropic and to 70–75% for the Asp-49 PLA₂s. Asp-49 is considered vital, although not sufficient (Pereira *et al.*, 1998), for the catalytic activity. Therefore, the presence of Lys-49 can explain the absence of PLA₂ activity in PrTX-I. The first 11 residues of these toxins form the N-terminal α -helix and unusual changes along this segment can also produce loss of catalytic activity (Heinrikson, 1991). This was not the case with PrTX-I. As a rule, Q-4, F-5, and I-9 are conserved residues in the D-49 PLA₂s, but the K-49 variants have L-5 for F-5.

Inspection of Fig. 4 shows that in the D-49 PLA₂s highly conserved positions are Y-25, G-26, C-27, Y-28, C-29, G-30, G-32, G-33, P-37, D-39, D-42, R-43, C-44, C-45, H-48, D-49, C-50, C-51, and Y-52. Among them, the segments 44–50 and 28–32 represent the regions more directly involved with the catalytic function (Arni and Ward, 1996). In PrTX-I we have N-28 for Y-28, L-32 for G-32, and K-49 for D-49. This last substitution was decisive for the loss of PLA₂ activity, further aggravated by the extra changes. The D-49 PLA₂s have G-32 and G-33.

Alignment of PrTX-I with BthTX-I (Fig. 4) shows only two mutations in the peptide chains, namely L-107

$\leftrightarrow$ K-107 and D-122 $\leftrightarrow$ P-122. The presence of L-107 and D-122 in PrTX-I substituting for K-107 and P-122 of BthTX-I should confer a slightly higher basic character to BthTX-I. This slight difference in basicity, however, is not enough to produce any significant difference between the corresponding electrophoretic mobilities (Mancuso *et al.*, 1995). Surprisingly, however, this is the only example where L-107 and D-122 appear at these positions (Fig. 4). Consequently, our previous finding that PrTX-I has a very low anticoagulant activity (Mancuso *et al.*, 1995) may now be partially attributed to the absence of Lys-107, since the Lys-rich heparin-binding region corresponding to the last 20 residues of the PLA₂ chains is believed to be related to this activity (Lomonte *et al.*, 1994). Finally, the presence of D-122 substituting for the P-122 would contribute still more to the loss of anticoagulant activity due to a further decrease of the cationic character of the C-terminal region.

The chemical modification of His-48 of PrTX-I by p-BPB practically destroyed its myotoxic and lethal activities, but did not apparently change its edematogenic activity, thus suggesting distinct pharmacological sites. The biochemical characterization of native and alkylated PrTX-I, however, did not show any significant difference regarding the pI , M_r , and cross-reaction against

	1	10	20	30	40
PrTX-I	SLFELGKMILQETG-KNPAKSYGAYGCNCVGLGRGPKDAT				
BthTX-I	SLFELGKMILQETG-KNPAKSYGAYGCNCVGLGRGPKDAT				
Myotoxin II	SLFELGKMILQETG-KMTAKSYGAYGCNCVGLGRGPKDAT				
App K49	SVLELGKMILQETG-KNAITSYGSYGNCVGLGRGPKDAT				
Myotoxin III	SLIEFAKMILEETK-RLPFYPPYGCYCGWGGQGPQKDAT				
VRV-PL-VIIIa	SLLEFGKMILEETG-KLAIPSYSSYGCGWGGKGTQKDAT				
TFPA2P2	HLMQFENMIKKVTG-RSGIWWYSGYGCYCGKGEGRPQDPS				
TGPLA2-V	SVIELGKMIFQETG-KNPATSYGLYGCNCVGGRRPKDAT				
TFBP I	SLVQLWKMI FQETG-KEAAKNYGLYGCNCVGGRRGPKDAT				

	50	60	70	80
PrTX-I	DRCCYVHKCCYKKLTGCPNPKKDRYSYSWKDKTIVCGENNP-			
BthTX-I	DRCCYVHKCCYKKLTGCPNPKKDRYSYSWKDKTIVCGENNP-			
Myotoxin II	DRCCYVHKCCYKKLTGCPNPKKDRYSYSWKDKTIVCGENNP-			
App K49	DRCCFVHKCCYKKLTDCNPKKDRYSYSWKNAIICEKNP-			
Myotoxin III	DRCCFVHDCCYCKLSNCKPKTDYRSYRKSGVILCEGTP-			
VRV-PL-VIIIa	DRCCFVHDCCYGNLPCNPKSDRYKYRVNGAIVCEKGT-			
TFPA2P2	DRCCFVHDCCYGVNCGNPKKAVYIYSLNGDIVCGDDP-			
TGPLA2-V	DRCCYVHKCCYKKLTDCDPIKDRYSYSWVNAIVCGEDNP-			
TFBP I	DRCCYVHKCCYKKVTGCDPKMDSYSYSWKNAIVCGEKNP-			

	90	100	110	120
PrTX-I	CLKELCECDKAVAICLRENLTGTYNKLRYHLKPFCK-KKADDC			
BthTX-I	CLKELCECDKAVAICLRENLTGTYNKLRYHLKPFCK-KKADPC			
Myotoxin II	CLKELCECDKAVAICLRENLTGTYNKLRYHLKPFCK-KKADAC			
App K49	CLKEMCECDKAVAICLRENLTGTYNKLRYHLKPFCK-KKPDTC			
Myotoxin III	CEKQICECDKAAAVCFRENLTGTYNKLRYHLKPFCK-KKPAEC			
VRV-PL-VIIIa	CENRICECDKAAAVCFRENLTGTYNKLRYHLKPFCK-KGELKC			
TFPA2P2	CRKEVCECDKAAAVCFRENLTGTYNKLRYHLKPFCK-KGELKC			
TGPLA2-V	CLKEMCECDKAVAICLRENLTGTYNKLRYHLKPFCK-KKADTC			
TFBP I	CLKQVCECDKAVAICLRENLTGTYNKLRYHLKPFCK-KKADTC			

Fig. 4. Alignment of PrTX-I with other PLA₂-like toxins. BthTX-I (Cintra *et al.*, 1993), myotoxin-II (Francis *et al.*, 1991), App K-49 (Maraganore and Heinrichson, 1986), myotoxin-III (Kaiser *et al.*, 1990), VRV-PL-VIIIa (Gowda *et al.*, 1994), TFPA2P2 (Ogawa *et al.*, 1992), TGPLA₂-V (Nakai *et al.*, 1995), and TFBP-I (Yoshizumi *et al.*, 1990).

bothropic antiserum. The amino acid composition of alkylated PrTX-I revealed that His-48 of native PrTX-I was alkylated. Therefore, the single remaining His which was found after alkylation is His-112 since native PrTX-I has only 2 His/mole.

Recently (Díaz-Oreiro and Gutiérrez, 1997) showed that alkylation of His-48 by pBPB abolished the PLA₂, myotoxic, and anticoagulant activities of myotoxins-I from *B. godmani* and *B. asper*, while the ability to rupture liposomes was only partially decreased. This apparent independence was also manifested by PrTX-I, since our results point to the existence of at least two distinct biologically active sites, the former responsible for the myotoxic/lethal and the latter for the edematogenic activity.

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Crystallization and preliminary X-ray diffraction studies of piratoxin II, a phospholipase A_2 isolated from the venom of *Bothrops pirajai*.

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Crystallization and preliminary X-ray diffraction studies of piratoxin II, a phospholipase A₂ isolated from the venom of *Bothrops pirajai*

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Abstract

The phospholipases A₂ (PLA₂, E.C. 3.1.1.4, phosphatide *sn*2 acylhydrolases) are the major components of the venom of several snakes. They are responsible for several important pharmacological effects observed in ophidian incidents. PLA₂ piratoxin II from *Bothrops pirajai* has been crystallized by the vapour-diffusion technique. X-ray diffraction data have been collected to 2.04 Å resolution (90.2% complete, $R_{\text{merge}} = 0.070$). The space group is $P2_1$ and the cell parameters are $a = 46.19$, $b = 60.36$, $c = 58.74$ Å and $\beta = 96.05^\circ$. The structure has been solved by molecular replacement using the crystallographic structure of PLA₂ from *Bothrops asper* (PDB code 1CLP) as a search model.

1. Introduction

The phospholipases A₂ (PLA₂, E.C. 3.1.1.4, phosphatide *sn*2 acylhydrolases) are present in the venom of several snake species (Mebs & Samejima, 1986). These enzymes specifically hydrolyse the *sn*2 ester bond of phospholipids (van Deenen & de Haas, 1963), having an enhanced activity towards phospholipids in a micellar or lamellar aggregate rather than freely diffusible single phospholipid molecules (Slotboom *et al.*, 1981).

A large number of different PLA₂s have been described in several different organisms in nature, and they have been traditionally classified as intracellular and extracellular (Dennis, 1994). The extracellular PLA₂s are small proteins of 119–143 amino acids, with molecular weights varying from 12 to 15 kDa. They have been separated into three different classes according to amino-acid sequence and disulfide-bonding pattern (Heinrikson *et al.*, 1977; Renetseder *et al.*, 1985). Class I enzymes are present in *Elapidae* and *Hydrophiidae* snake venoms and mammalian pancreatic juice. Class II enzymes have been isolated from snake venoms of *Crotalidae* and *Viperidae* species and, more recently, they have been detected in mammalian non-pancreatic tissues (Johansen *et al.*, 1992; Kudo *et al.*, 1993). Finally, there are the class III enzymes, which have been isolated from lizard and bee venoms. Crystallographic experiments showed that the distinct classes have different three-dimensional structures. Classes I and II have a similar overall folding pattern, whereas class III PLA₂s adopt a different folding pattern, where the only conserved structural motif between this class and class I/II is the calcium-binding site (White *et al.*, 1990).

The class II PLA₂s are of special importance since they constitute a large part of the venoms of the most dangerous species of snake, such as rattlesnakes and vipers (Mebs &

Samejima, 1986). The enzymes belonging to this class have been subdivided into two subgroups: (i) enzymatically active D49 PLA₂s and (ii) K49 PLA₂s, which have little or no enzymatic activity (Maraganore *et al.*, 1984). The difference in enzymatic activity is due to the substitution of the residue responsible for the binding of a calcium ion, which is an essential cofactor. Crystal structures have revealed that the calcium ion-binding site that is in D49 is occupied by N ζ in K49, hence resulting in a loss of catalytic activity (Holland *et al.*, 1990; Scott *et al.*, 1992; Arni *et al.*, 1995). Despite the low or non-existent enzymatic activity, K49 PLA₂s are capable of disrupting liposomes (Díaz *et al.*, 1991; Rufini *et al.*, 1992). They are also responsible for muscular necrosis and oedema formation (Lomonte *et al.*, 1994). All these effects are independent of catalytic activity, suggesting the presence of an additional motif in the enzyme, additional to the active site, that interacts with some substrates (Gutiérrez & Lomonte, 1995).

Although there are 20 PLA₂ crystallographic structures deposited in the Protein Data Bank (as of March, 1998), the reason for calcium-independent activity is still unknown. New PLA₂ structures might help to shed some light on the reason for this activity. Piratoxin II (PrTX-II) is a PLA₂ isolated from the venom of *Bothrops pirajai*, a *Crotalidae* snake geographically restricted to the southern region of Bahia state in Brazil. Although highly homologous to the abundant piratoxin I (PrTX-I) present in the same venom, PrTX-II could be separated and analysed (Toyama *et al.*, 1995). Under physiological conditions, it exists as a ~26 kDa homodimer and is present in the venom as a minor fraction (less than 10%) when compared to PrTX-I (Toyama *et al.*, 1995).

Here we report the crystallization conditions and preliminary data on the crystals of PrTX-II, and its molecular-replacement solution.

2. Methods and results

Isolation of PrTX-II from the *Bothrops pirajai* venom by semi-preparative reverse-phase HPLC has been described previously (Toyama *et al.*, 1995).

Initial screening of the crystallization conditions has been performed using a sparse-matrix screen at room temperature (301 K) (Crystal Screen I and II, Hampton Research). The hanging-drop vapour-diffusion technique was used for all conditions. Drops containing 1 µl of precipitant solution and 1 µl protein solution were prepared on siliconized glass coverslips and suspended over a reservoir containing 500 µl of the same precipitant solution. The first attempts, performed

Table 1. *Data-collection statistics*

Crystal data		
Space group	$P2_1$	
Cell parameters	$a = 46.19$, $b = 60.36$, $c = 58.74$ Å; $\beta = 96.05^\circ$	
Data collection		
	Crystal 1	Crystal 2
Resolution (Å)	2.65	2.04
Last resolution shell	2.74–2.65 Å	2.11–2.04 Å
Number of observations	24255	23809
Number of unique reflections	8531	6934
R_{merge} (%)	10.8	7.0
R_{merge} , last resolution shell (%)	31.4	26.0
Completeness (%)	90.2	90.2
Completeness, last resolution shell (%)	93.4	72.5

with a protein dissolved in water at a concentration of 10 mg ml^{-1} , showed no crystal formation in any of 96 tested conditions. New trials were performed with protein solution concentrated to 20 mg ml^{-1} , using the same conditions at 277 K. After approximately 20 d, small crystals ($\sim 0.05 \text{ mm}$) appeared at condition 17 of the Crystal Screen I kit (30% PEG 4000, 0.1 M Tris-HCl pH 8.5, 0.2 M lithium sulfate), but their morphology suggested that they were twinned. Slightly modified crystallization conditions resulted in better crystals. They grew within 40 d at 277 K in 28% PEG 3350, 0.25 M lithium sulfate and 0.1 M Tris-HCl pH 8.5. These were flat crystal plates with approximate dimensions $0.1 \times 0.1 \times 0.02 \text{ mm}$.

Crystals were mounted in quartz capillaries and data collection was performed at the Protein Crystallographic (PCr) beamline (Polikarpov, Oliva *et al.*, 1998; Polikarpov, Peries *et al.*, 1998) at the Laboratório Nacional de Luz Síncrotron (LNLS) located in Campinas, Brazil. The synchrotron radiation wavelength was set to 1.38 Å and all diffraction images were collected on a MAR 345 image plate. The image plate was operated in the 300 mm scanning mode and the crystal-to-detector distance was set to 200 mm. Two data sets were collected. The first data set was collected from a single crystal to 2.65 Å at 277 K. A total of 78 scanning-oscillation images were recorded (steps of 1.8° , total of 133.2° rotation) and processed with *DENZO* and *SCALEPACK* (HKL program suite; Otwinowski, 1993). The second data collection resulted in a higher resolution data set. 58 images were recorded in steps of 1.5° , totalling 87° rotation. This data set was collected from another crystal which diffracted to 2.04 Å resolution. During data collection this crystal was also chilled to 277 K. Images were processed with *DENZO* and *SCALEPACK*. Statistics for both data sets are given in Table 1.

Assuming a molecular mass of 13 kDa, we estimated the number of molecules in the asymmetric unit using the Matthews method (Matthews, 1968). There were two possible solutions: one ($V_m = 2.11 \text{ Å}^3 \text{ Da}^{-1}$) indicating three molecules per asymmetric unit and the other ($V_m = 3.17 \text{ Å}^3 \text{ Da}^{-1}$) suggesting two molecules per asymmetric unit. With this information, we solved the crystal structure by molecular-replacement methods using the *AMoRe* program (Navaza, 1994). We used as a search model a monomer of the PLA₂ dimer of *Bothrops asper* (PDB code 1CLP), including all amino-acid residues and their side chains (Arni *et al.*, 1995).

The two most significant unique rotation-search solutions have correlation coefficients of 23.6 and 21.6%, whereas all other solutions were below 11.1%. These two solutions were used in the translation-function search and the best solution was subjected to ten cycles of rigid-body refinement against data between 10 and 3.3 Å resolution (fitting function of *AMoRe*). The fitting yielded a solution with a correlation coefficient of 63.4% and an *R* factor of 38.9%. The high V_m for the two-molecule solution can, therefore, be explained by the relatively high solvent content ($\sim 61\%$) of the crystal. The crystal packing was inspected using the program *O* (Jones & Kjeldgaard, 1993). Using the same PLA₂ from *Bothrops asper* (1CLP) as search model, but now as a dimer, we also performed self-rotation, translation and rigid-body refinement functions (*AMoRe*) in the resolution range 3.3–10 Å, to verify if the monomeric PrTX-II crystals have similar interactions to the 1CLP dimeric model. After rigid-body refinement, the final correlation coefficient was 45.6 and the final *R* factor was 46.1%, suggesting that the interaction of the monomers in the PrTX-II crystal are different to those described by Arni *et al.* (1995). This was subsequently confirmed by inspection of the monomer-monomer interactions using the program *O* (Jones & Kjeldgaard, 1993). Further steps of model building and refinement are in progress.

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Crystallization and preliminary X-ray diffraction studies of piratoxin III, a D-49 phospholipase A₂ from the venom of *Bothrops pirajai*.

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Crystallization and preliminary X-ray diffraction studies of piratoxin III, a D-49 phospholipase A₂ from the venom of *Bothrops pirajai*

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Piratoxin III (PrTX-III) is a phospholipase A₂ (PLA₂, E.C. 3.1.1.4, phosphatide *sn*-2 acylhydrolase) isolated from *Bothrops pirajai*. Crystals of PrTX-III were obtained using the vapour-diffusion technique and X-ray diffraction data have been collected to 2.7 Å resolution. The enzyme was crystallized in the space group C2 with unit-cell parameters $a = 60.88$, $b = 100.75$, $c = 48.19$ Å, $\beta = 123.89^\circ$. A molecular-replacement solution of the structure has been found using bothropstoxin I from the venom of *B. jararacussu* as a search model.

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1. Introduction

The genus *Bothrops* comprises several species which are widely distributed in South and North America. Among the bioactive proteins from *Bothrops* venoms, phospholipases A₂ appear as a major component. Phospholipases A₂ (PLA₂, E.C. 3.1.1.4) are calcium-dependent enzymes which are responsible for the cleavage of the *sn*-2 ester bond of phospholipids (Deenen & de Haas, 1963). They are found in most animal tissues, mainly in the pancreatic juices of mammals and the venoms of snakes and insects. The PLA₂ enzymes are believed to participate in cellular functions and cell signalling (Kudo *et al.*, 1993), as well as in the formation of several important metabolic precursors (*e.g.* inflammatory response mediators) derived from cleavage of the phospholipids (Siraganian, 1988). These enzymes are classified into four groups, according to their extracellular or intracellular origin, primary structure and disulfide-bond pattern (Heinrikson, 1990; Dennis, 1994).

Class II PLA₂s constitute a large part of the venom of many species of snake, such as rattlesnakes and vipers (Mebs & Samejima, 1986). The enzymes belonging to this class can be subdivided into two distinct groups, according to enzymatic activity: the inactive group and the active group (Maraganore *et al.*, 1984). The enzymatically inactive PLA₂s (K-49 PLA₂, PLA₂-like myotoxins) are PLA₂s which exhibit low or no phospholipid cleavage activity. This is thought to arise from a substitution of the aspartate residue at position 49, the side chain of which is important in the binding of calcium ions (an essential cofactor), by a lysine residue (Holland *et al.*, 1990; Scott *et al.*, 1992; Arni *et al.*, 1995). However, despite the low or lack of enzymatic activity, K-49 PLA₂s exhibit several different pharmacological activities, such as post-synaptic neurotoxicity, oedema formation (Gutiérrez &

Lomonte, 1995), myotoxicity (Lomonte *et al.*, 1994) and liposome and membrane disruption (Días *et al.*, 1991; Ruffini *et al.*, 1992). The enzymatically active PLA₂s (D-49 PLA₂) hydrolyse the *sn*-2 ester bond of 1,2-diacyl-*sn*-phosphoglycerides. The D-49 PLA₂s require calcium ion and are almost all at least 100 times more active when the substrate is condensed in micelles (Pieterse *et al.*, 1974) or lamellar aggregates such as monolayers, vesicles and membranes (Slotboom *et al.*, 1981).

In this work, we present the crystallization and preliminary diffraction data of piratoxin III (PrTX-III) myotoxin. PrTX-III is a D-49 PLA₂ with moderate PLA₂ activity, myotoxic and anticoagulant activity. It was isolated from *B. pirajai* snake venom (Toyama *et al.*, 1998) which was a kind gift from CEPIAC/CEPE (Ministry of Agriculture, Bahia, Brazil). The enzymatically inactive K-49 PLA₂ piratoxin I (PrTX-II) from *B. pirajai* has previously been crystallized in our group and is currently in the process of structural refinement (Lee *et al.*, 1998). Comparison of two (active and inactive) phospholipases from the same organism will provide us with more information on the structural differences and shed light on the activity of these proteins.

2. Protein purification

PrTX-III was isolated and purified from the whole venom of *B. pirajai* by reverse-phase and cation-exchange HPLC. 20 mg of whole venom were dissolved in 250 µl of 0.1% (v/v) trifluoroacetic acid. The resulting sample was centrifuged and the supernatant was applied to a 0.78 × 30 cm µ-Bondapak C-18 column (Waters 991 PDA system).

The purification of the venom was performed with a linear gradient of 0–66% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid at a flow rate of 2.0 ml min⁻¹.

Table 1

Crystal data and data-collection statistics.

Crystal data	
Space group	C2
Unit-cell parameters (Å, °)	$a = 60.88$, $b = 100.75$, $c = 48.19$, $\beta = 123.89$
Data collection	
Resolution (Å)	2.70
Last resolution shell (Å)	2.79–2.70
Number of observations	15881
Number of unique reflections	5514
R_{merge} (%)	11.4
Last resolution shell (%)	26.4
Completeness (%)	84.5
Last resolution shell (%)	81.2

and was monitored at 280 nm. The PrTX-III fraction was lyophilized and dissolved in 0.05 M ammonium bicarbonate pH 7.4, centrifuged and applied to a 0.39×7.8 cm Protein-Pack SP 5PW cation-exchange column, which had previously been equilibrated with the same buffer. Elution of PrTX-III was performed using a 0.05–1.0 M ammonium bicarbonate (pH 7.4) linear gradient. The chromatographic run was performed at a flow rate of 1.0 ml min^{-1} and was monitored at 280 nm. The purified sample was lyophilized and used for crystallization trials.

3. Crystallization and data collection

Preliminary screening of the crystallization conditions was performed using a sparse-matrix screen at 291 K (Crystal Screens I and II, Hampton Research). Lyophilized PrTX-III was initially dissolved to a concentration of 10 mg ml^{-1} in water and used in the screening procedure. Small crystals were found in condition number 40 of the Crystal Screen I kit (20% 2-propanol, 20% PEG 4000, 0.1 M sodium citrate pH 5.6). A search for refined crystallization conditions was then performed. New crystals were grown at 291 K using the hanging-drop vapour-diffusion technique by mixing equal volumes ($1 \mu\text{l}$) of a protein solution concentrated to 5 mg ml^{-1} with a reservoir solution which contained 19% 2-propanol, 20% PEG 4000 and 0.1 M sodium citrate pH 5.5. Plate-like crystals measuring $0.1 \times 0.1 \times 0.02 \text{ mm}$ appeared in 10–15 d.

X-ray diffraction data were collected at the protein crystallography beamline (Polikarpov, Oliva *et al.*, 1998; Polikarpov, Perles *et al.*, 1998) at the Laboratório Nacional de

Luz Síncrotron (LNLS), Campinas, Brazil. The images were recorded using a MAR 345 image plate and synchrotron radiation of wavelength 1.38 Å . 110 oscillation images were collected corresponding to a total rotation of 132° . The collected images were processed and scaled with *DENZO* and *SCALEPACK* (Otwinowski, 1993). The crystals belong to the space group C2 with unit-cell parameters $a = 60.88$, $b = 100.75$, $c = 48.19 \text{ Å}$, $\beta = 123.89^\circ$. Data-set statistics are given in Table 1.

Calculations using the Matthews coefficient (Matthews, 1968) suggested the presence of two molecules per asymmetric unit ($V_m = 2.84 \text{ Å}^3 \text{ Da}^{-1}$). The crystal structure of PrTX-III was solved by the molecular-replacement method using the program *AMoRe* (Navaza, 1994). Several molecular-replacement search models were tested. The best solution was found with the most homologous PLA₂ available in the main databases (all non-redundant GenBank CDS translations, PDB, Swissprot, PIR and PRF), bothropstoxin I from *B. jararacussu*, which displayed 65% primary sequence identity with PrTX-III. The atomic coordinate file of bothropstoxin I was kindly provided by the authors (Da Silva-Giotto *et al.*, 1998). The two most significant rotation-search solutions [correlation coefficients (CC) of 23.5 and 21.9%] were used for the translation search using reflections in the resolution range 10–2.7 Å. The best solution of the translation search (CC = 43.1%; R factor = 49.2%) was subjected to ten cycles of rigid-body refinement against all data between 10 and 2.7 Å resolution (fitting function of *AMoRe*). The fitting yielded a solution with a correlation coefficient of 49.8% and an R factor of 48.1%. The crystal packing was inspected using the program *O* (Jones & Kjeldgaard, 1993) and did not show any crystallographic or non-crystallographic clashes. Initial refinement steps were performed using the maximum-likelihood method as implemented in the program *REFMAC* (Murshudov *et al.*, 1997). At present, the R factor of the model is 31.3% and R_{free} is 38.6%. Further model-building and refinement steps are under way.

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Amino Acid Sequence of piratoxin-II, a myotoxic Lys49 phospholipase A₂ homologue from *Bothrops pirajai* venom.

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Biochimie

(Submetido e aceito para publicação)

BIOCHIMIE

Subject area: Structure of Macromolecules

**Amino acid sequence of piratoxin-II, a myotoxic Lys49 phospholipase A₂
homologue from *Bothrops pirajai* venom**

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Abbreviations: PLA₂, phospholipase A₂; PrTX-II, piratoxin II from *Bothrops pirajai*;

SDS, sodium dodecyl sulphate; CK, creatine kinase.

Abstract

The complete amino acid sequence of the 121 amino acid residues of piratoxin II, a phospholipase A₂ like myotoxin from *Bothrops pirajai* venom, is reported. PrTX-II is a basic protein with a $M_r = 13,740$, a calculated $pI = 9.03$, but an experimental $pI = 8.4 \pm 0.2$, showing sequential similarity with other bothropic (90-99 %) or non bothropic (~80 %) Lys49 PLA₂-like myotoxins. This similarity falls to ~70 % when this sequence is aligned with those of Asp49 PLA₂s. Due to the substitution of Asp49 by Lys49 and alterations in the calcium binding loop structure, as the replacement of Gly32 by Leu32, piratoxin-II shows no PLA₂ activity when assayed on egg yolk. Piratoxin-II showed the same primary structure as piratoxin-I, except that it has Lys116 for Leu116. Despite this slightly higher basicity at the C-terminal region, piratoxin-II showed to be less myotoxic than piratoxin-I. The change Leu → Lys induced an alteration of the molecule surface shape and probably of the environment charge high enough to slightly decrease the myotoxic activity. When aligned with *B. jararacussu* bothropstoxin-I and with *B. asper* Basp-II, piratoxin-II revealed a single (position 132) and a quintuple (positions 17, 90, 111, 120 and 132) amino acid substitution, respectively, suggesting a common evolutionary origin for these three myotoxins.

Keywords: myotoxins, phospholipase A₂, piratoxin-II, *Bothrops pirajai*, primary structure, molecular model.

1. Introduction

Snakes belonging to the genus *Bothrops* are largely distributed from South to North America [1]. Among the bioactive proteins from *Bothrops* venoms, the phospholipases A₂ (PLA₂, E.C. 3.1.1.4) and PLA₂-like myotoxins are outstanding as major components. Phospholipases A₂ are calcium-dependent enzymes which hydrolyze the 2nd ester bond of 1,2 diacyl-3 phosphoglycerides [2, 3].

Snake venom PLA₂s have molecular weights of 12,000-14,000 and are classified as class I (Elapidae and Hydrophidae) or class II (Crotalidae and Viperidae) PLA₂s based on the disulphide bond pattern and the presence of short insertions of 3-7 amino acids in two loop regions [4, 5]. In addition to their catalytic activity, many class I and II PLA₂s from snake venoms exhibit several pharmacological effects [4 – 7].

Myotoxic PLA₂-homologues which are catalytically inactive upon artificial substrates have been isolated from the venoms of several species of *Bothrops* [6, 8 – 10] and the amino acid sequences revealed several substitutions in the Ca⁺⁺ binding loop region [11 – 14]. This article reports the amino acid sequence of piratoxin-II (PrTX-II), a myotoxin from *Bothrops pirajai* snake venom.

2. Materials and Methods

Isolation and biochemical characterization: Piratoxin II was purified as previously described [15, 16]. Homogeneity of the toxin was assayed by SDS-PAGE [17], PAGE for basic proteins [18] and isoelectric focusing [19]. The myotoxic activity was determined on basis of the plasma creatine kinase releasing from the mouse gastrocnemius injected muscle [20].

Amino Acid Sequencing: Reduced and carboxymethylated PrTX-II (RC PrTX-II) was digested with *Staphylococcus aureus* protease V8 for 16 h at 37°C [21], or

clostripain for 8h at 37°C [12]. Both protease V8 and clostripain digests were fractionated by reverse phase (RP) HPLC using a Waters PDA 991 system and a C18 μ Bondapak column under a concentration gradient of acetonitrile in 0.1% (v/v) trifluoroacetic acid [22]. Direct sequencing from the N-terminal side of the toxin was carried out with the native protein using an Applied Biosystem 477A automatic sequencer. The Cys residues were identified as PTH- ^{14}C carboxymethylated Cys and confirmed by radioactive impulse counting.

Molecular Modelling: - - - - - completar - - - - - PrTX-II was crystallized by the vapour diffusion method, using a sparse matrix screening. The best crystallization condition was determined and diffraction data were collected [23]. The structure was solved using the molecular replacement method and refined to an R-factor of 17.6 %.

3. Results and Discussion

The complete amino acid sequence (Fig. 1) was deduced by alignment of overlapping resulting peptides and from the N-terminal sequence. PrTX-II has 121 amino acid residues. An M_r and pI of 13,744 and 9.03 were calculated, respectively, from the amino acid sequence, but a $pI \sim 8.4$ was experimentally determined.

The final amino acid sequence was initially aligned with other Lys49 PLA₂s (Fig. 2) and showed a sequentail similarity of 90 – 99 % . When compared with Asp49 PLA₂s, these values were around 65 %. PrTX-II has no PLA₂ activity due to the absence of Asp49 which was replaced by Lys49. Asp49 is necessary for enzymatic activity, but is not determinant of a high activity. *B. jararacussu* bothropstoxin-II [24] for example is a basic Asp49 with a specific activity of 37 U/mg compared with 109 U/mg for the acidic PLA₂s from the same venom (Andrião-Escarso, S.H. et al., submitted).

An inspection of Fig. 2 shows that in the Asp49 PLA₂ there is a sequence of Gly residues, namely Gly26, Gly30, Gly32 and Gly33. In PrTX-II and other PLA₂-like myotoxins, this sequence is incomplete. The replacement of Gly32 for Leu32 is the most common mutation noted. The presence of those Gly residues and of Tyr28 is important to the conformation flexibility that structures the calcium ion binding site [4]. Tyr28 is usually replaced by Asn28 in catalytically inactive PLA₂s of Fig. 2.

At the C-terminus (residues 115-129), a set of basic and hydrophobic amino acids plays a relevant role in cytotoxic and myotoxic activities [25 – 27]. PrTX-II has Lys116, compared with Leu116 in PrTX-I. Despite displaying a higher basicity at the 115-129 segment, PrTX-II showed a lower myotoxic activity when assayed by creatine kinase releasing (Fig. 3). The hypothesis that additional positive charge in PrTX-II might have induced a conformational change which resulted in a myotoxic effect lower than that of PrTX-I was then further investigated.

Crystallographic studies were performed in order to reveal the structural significance of the Leu → Lys 116 substitution. Preliminary analysis showed that the Leu → Lys substitution occurred on the C-terminal loop region, in a highly accessible position. The solvent accessible area of Lys side chain is up to 147 Å², increasing the contact surface in this part of the molecule, when compared to PrTX-I. This mutation certainly increases the cationic character of this region of the molecule and is consistent with the differential electrophoretic mobility of PrTX-I and PrTX-II that has been observed (not shown).

As mentioned before, this lysine-rich region that comprises 115-129 is believed to be responsible for the cytotoxic activity. Heparin-binding cytotoxicity neutralization experiments have been successfully performed using both *B. asper* myotoxin II and a synthetic peptide of residues 115-129 [25 – 27]. This suggested that the heparins

neutralize myotoxin II by binding to a region directly involved in its cytotoxicity. Since this binding is believed to be a consequence of the cationic character of this region, a new lysine presumably should enhance its heparin-binding ability and its myotoxicity [25]. However, PrTX-II presented a lower myotoxicity when compared to PrTX-I. As showed in the Fig. 4, the Leu $\rightarrow$ Lys substitution leaded to a change in the molecule surface shape. The protusion of a bulky lysine side chain might cause a steric hindrance between this region and its target. Alternatively the substitution of a hydrophobic residue by a charged could possibly have altered the environment charge necessary for an optimal myotoxicity (Fig. 4a, b).

The amino acid alignment of PrTX-II and PrTX-I shows that Leu116 $\leftrightarrow$ Lys116 is the only amino acid substitution. The presence of Lys116 should confer a slightly basic character to PrTX-II. PrTX-II when compared with *B. jararacussu* BthTX-I, shows also a single amino acid residue change (Asp132 $\leftrightarrow$ Ala132) and compared with *B. asper* Basp-II showed five amino acid substitutions (Asn17 $\leftrightarrow$ Met17; Pro90 $\leftrightarrow$ Ser90; Gly111 $\leftrightarrow$ Asn111; His120 $\leftrightarrow$ Tyr120 and Asp132 $\leftrightarrow$ Ala132).

Geographic confinement of some species of snake belonging to a single genus may induce morphological and biochemical alterations able to differentiate them on the point of constituting a subspecies [9, 28]. This is the case of *Bothrops pirajai*, which is confined in Southern Bahia State, Brazil, and is morphologically almost indistinguishable from *B. jararacussu*, hence its popular name “Bahia’s jararacussu”. *Bothrops pirajai* PrTX-I and PrTX-II, *B. jararacussu* BthTX-I and *B. asper* Basp-II have around 97% of sequential similarity (Tab.1), and have very probably arisen from a common encoding ancestral gene.

Acknowledgements

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Table 1. Homology comparison of PrTX-II with Lys49 and Asp49 PLA₂s

Protein	Source	*Database: Accession no.	% Identity	% Identity + % Conserved	References
PrTX-I	<i>B. pirajai</i>	-	99	99	[14]
godMT-II	<i>C. godmani</i>	P81165	78	86	[30]
BthTX-I	<i>B. jararacussu</i>	-	98	98	[12]
Basp-II	<i>B. asper</i>	PA22_BOTAS	95	95	[11]
ACLMT	<i>A. c. laticinctus</i>	P49121	76	83	[31]
App K49	<i>A. p. piscivorus</i>	PA2H_AGKPI	76	83	[32]
Basp-III	<i>B. asper</i>	PA21_BOTAS	59	69	[33]
Bp PLA ₂	<i>Bos taurus</i>	PA2_BOVIN	34	43	[34]

*Accession numbers in the SwissProt.

Legends for Figures

Fig. 1. Complete amino acid sequence of PrTX-II from *Bothrops pirajai*. Overlapping peptide fragments generated by enzymatic cleavages are indicated. Fragments were produced by cleavages with *Staphylococcus aureus* protease S8 (SV) and clostripain (Clt). Fragments numbers are related to the order of peak elution from HPLC.

Fig. 2. Amino acid sequence alignment of PrTX-II from *Bothrops pirajai* with other PLA₂s sequences. The alignment numbering scheme follows that of Renetseder et al. [29]. PrTX-I, piratoxin I from *B. pirajai* [14]; godMT-II, a myotoxin II from *Cerrophidion (Bothrops) godmani* [30]; BthTX-I, bothropstoxin I from *B. jararacussu* [12]; Basp-II, a myotoxin II from *B. asper* [11]; ACLMT, a myotoxin from *Agkistrodon contortrix laticinctus* [31]; App k49, a myotoxin from *Agkistrodon piscivorus piscivorus* [32]; BthTX-II, bothropstoxin II from *B. jararacussu* [22]; Basp-III, a myotoxin III from *B. asper* [33]; Bp PLA₂, a phospholipase A₂ from bovine pancreatic [34].

Fig. 3. Myotoxic activity of *Bothrops pirajai* PrTX-I and II in mice. Plasma creatine kinase (CK) increased 3 h after the intramuscular injection of 100 µg / 50 µl of PBS. Results are presented as means ± SD (n=6).

Fig. 4 (A): Surface potential pattern. Notice the change in the positive charge when Leu (up) is substituted by Lys (down) in the position 116.

(B): The marked region depicts the change in the surface shape due to Leu → Lys substitution. **Up:** Leu in the position 116. **Down:** Lys in the position 116.

Figure 1.

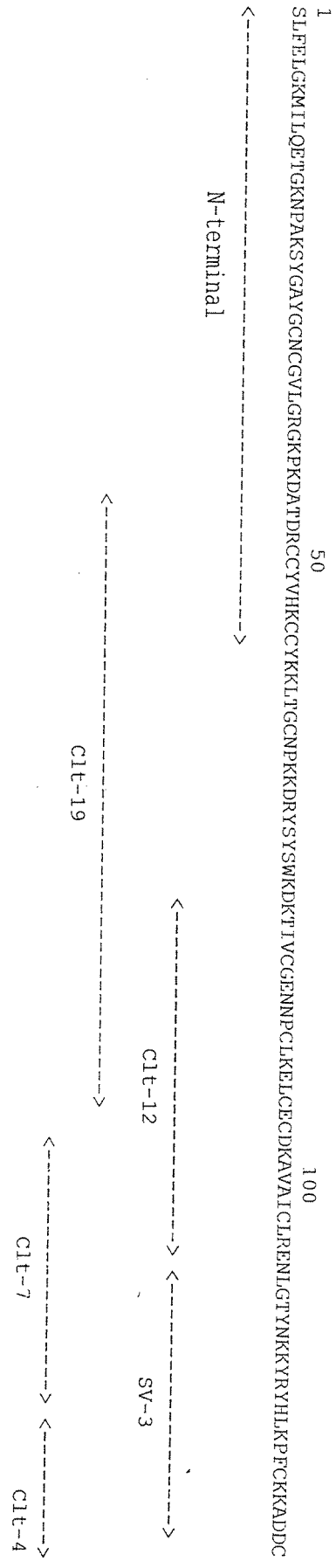


Figure 2.

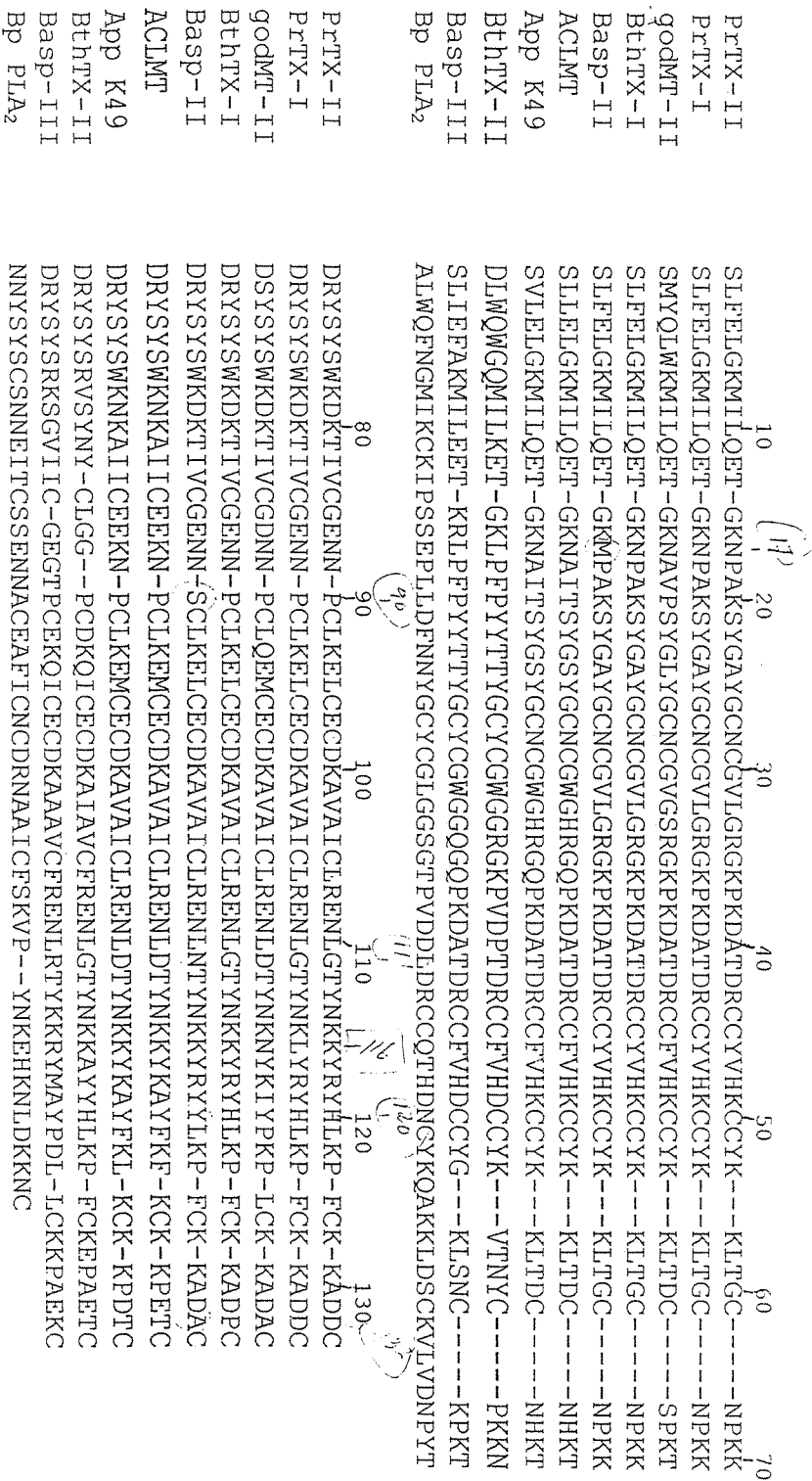
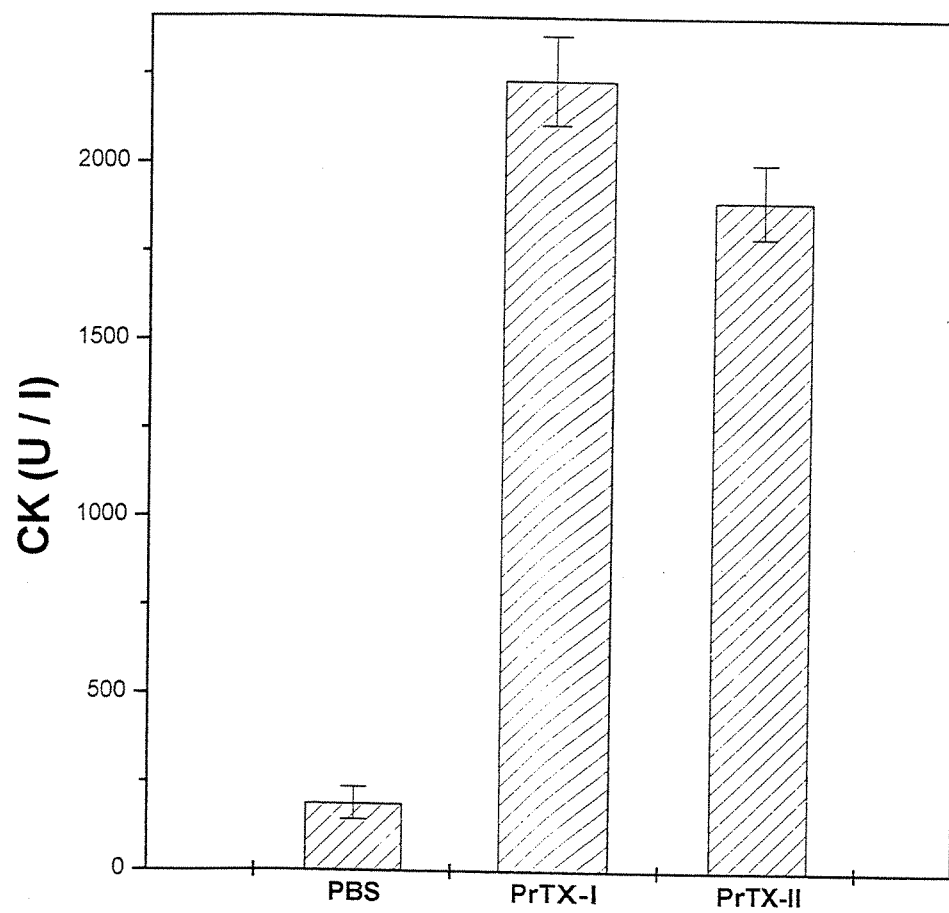


Figure 3.



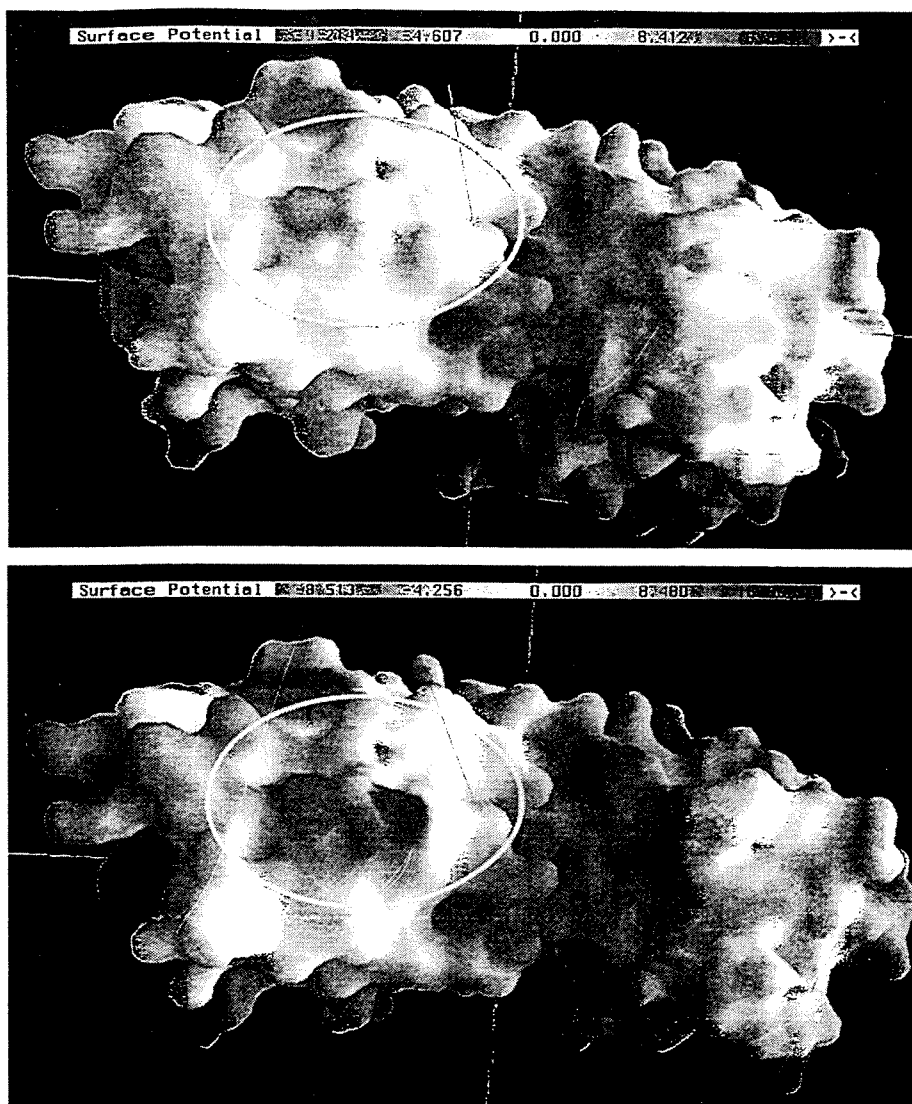


Figure 2: Surface potential pattern. Notice the change in the positive charge when Leu (up) is substituted by Lys (down) in the position 116.

Lee W.-H., M.C. Gonalez, R.M.F. Ramalheira, P.R. Kuser, M.H. Toyama, B. Oliveira, J.R. Giglio, S. Marangoni & I. Polikarpov. "Crystallisation and preliminary X-ray diffraction studies of Piratoxin-II, a phospholipase A₂ isolated from the venom of *Bothrops pirajai*" *Acta Cryst. D54*, 1437-1439 (1998).

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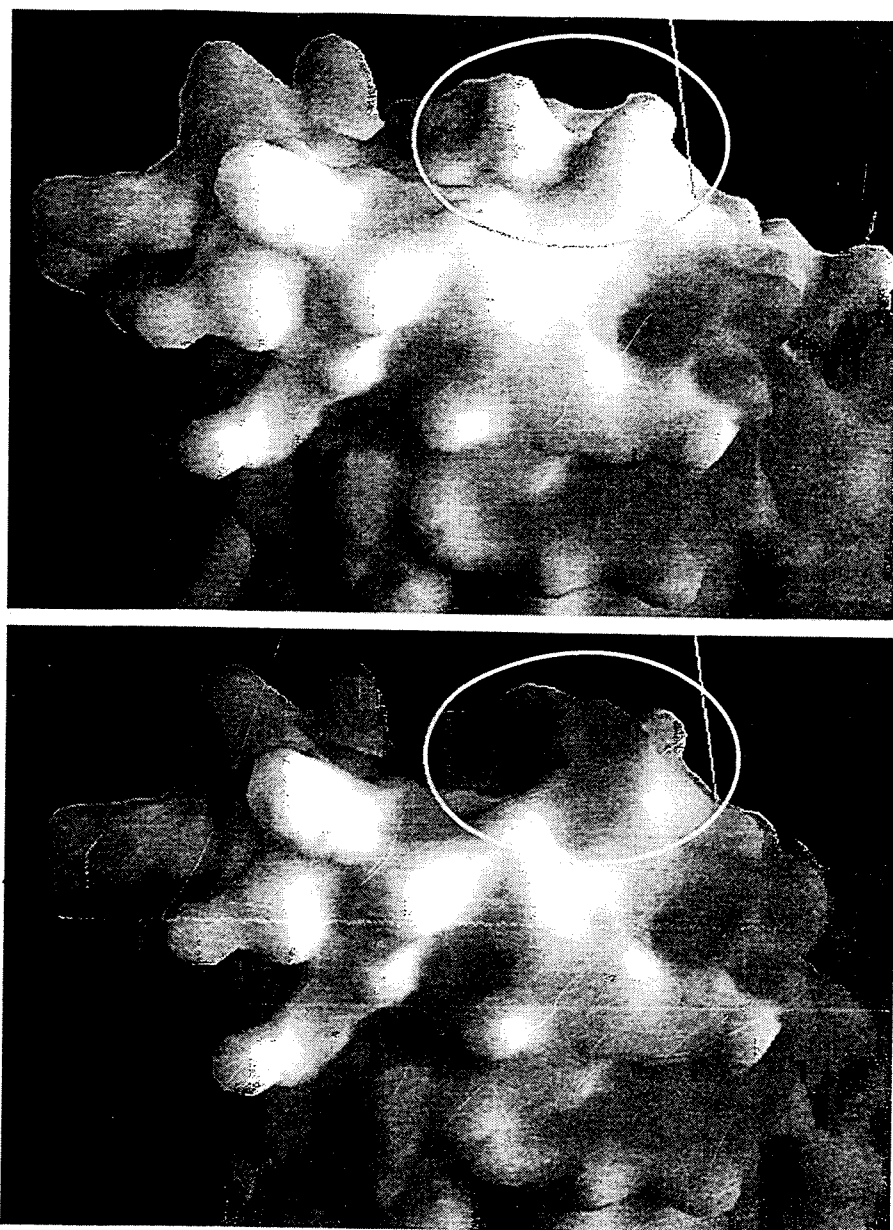


Figure 1: The marked region depicts the change in the surface shape due to Leu $\rightarrow$ Lys substitution. **Up:** Leu in the position 116. **Down:** Lys in the position 116.

Purification and amino acid sequence of MP-III 4R D49 phospholipase A_2 from *Bothrops pirajai* snake venom, a toxin with moderate PLA_2 and anticoagulant activities and high myotoxic activity.

Marcos Hikari Toyama, Patricia Dourado Costa, Jose Camillo Novello, Benedito Oliveira, Jose Roberto Giglio, Maria Alice da Cruz-Höfling and Sergio Marangoni

Journal Protein Chemistry, 18(3): 371-378

**Purification and amino acid sequence of MP-III 4R D49
phospholipase A₂ from *Bothrops pirajai* snake venom, a toxin with
moderate PLA₂ and anticoagulant activities and high myotoxic
activity**

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ABSTRACT

MP-III 4R PLA₂ was purified from the venom of *Bothrops pirajai* venom (Bahia's jararacussu) after three chromatographic steps, which started with RP-HPLC.

The complete amino acid sequence of MP-III 4R PLA₂ from *Bothrops pirajai* was determined by amino acid sequencing of reduced and carboxymethylated MP-III 4R and the isolated peptides from clostripain and protease V8 digestion.

MP-III 4R is a D49 PLA₂ with 121 amino acid residues with a molecular weight estimated at 13800, 14 half cysteines. This protein showed moderate PLA₂ and anticoagulant activity. This PLA₂ does not have a high degree of homology with other bothropic PLA₂-like myotoxin (~75%) and non-bothropic myotoxins (~60%).

MP-III 4R is a new PLA₂, which was isolated using exclusively analytical and preparative HPLC methods. Based on the N-terminal sequence and biological activities, MP-III 4R was identified as similar to piratoxin-III (PrTX-III) which was isolated by conventional chromatography based on molecular exclusion, ion exchange chromatography.

Clinical manifestations indicate that at the site of toxin injection, there may be pain of variable intensity because animals remain licking the limb. No clinical sign indicating general toxicity was noticed.

Myotoxicity was observed in gastrocnemius muscle cells after exposure to MP-III 4R, with a high frequency (70%) of affected muscle fibers.

KEY WORDS: *Bothrops pirajai*, D49 PLA₂, anticoagulant PLA₂, amino acid sequence, PrTX-III, MP-III 4R.

INTRODUCTION

The genus *Bothrops* comprises several species which are widely distributed in South and North America (Hoge and Romano – Hoge, 1978/79). Among the bioactive proteins from *Bothrops* venoms, the phospholipase A₂ (PLA₂, E.C. 3.1.14) and PLA₂-like myotoxin are outstanding as its major components. Phospholipase A₂s are calcium-dependent enzymes which hydrolyze the 2 ester bonds of 1,2 diacyl-3sn phosphoglycerides (Chang *et al.*, 1994, Shimohigachi *et al.*, 1995, Ogawa, *et al.*, 1996).

They are found in most tissues, mainly in the pancreatic juice of mammals and venom of snakes and insects. PLA₂ enzymes are classified onto four groups (I, II, III and IV), according to their extracellular or intracellular origin, their primary structure and disulfide bonding (Denis *et al.*, 1994).

Snake venom PLA₂s are usually distributed into two major groups: I (Elapidae and Hydrophidae) and II (Crotalidae and Viperidae). Group II comprises the catalytic active D49 and the inactive or low activity K49 PLA₂ (Selistre de Araújo, *et al.*, 1996). In addition to the catalytic, the PLA₂s may have neurotoxic, myotoxic, pro- or anti-platelet aggregation, hemolytic, anticoagulant, cardiotoxic and edematogenic activities (Kini and Evans, 1989; Arni and Ward, 1996).

The myotoxic PLA₂s are responsible for the intense myonecrosis produced by the Bothropic venoms. Some of them have anticoagulant and or edematogenic activity (Mancuso *et al.*, 1995; Soares *et al.*, 1998). Besides the muscle cells, other cells such as macrophages, fibroblasts and neurons are apparently additional *in vitro* targets of these toxins (Gutiérrez and Lomonte, 1995). The specific receptors on the plasma membrane of the muscles are unknown (Gutiérrez and Lomonte, 1995; Fletcher *et al.*, 1996).

In this paper we described the procedure of MP-III 4R purification from *Bothrops pirajai* snake venom, which started with RP-HPLC on a preparative μ -Bondapack C18 column, followed by cation ion exchange HPLC and a last repurification step on the analytical RP-HPLC. A previous paper (Mancuso *et al.*, 1995) described the purification and the partial characterization of PrTX-III by a conventional method using molecular exclusion and cation ion exchange chromatography. MPIII-4R was isolated from the *Bothrops pirajai* venom by using exclusively the HPLC method and the fraction was shown to be the fraction is the same PrTX-III as previously described by Giglio's group. MPIII-4R is one of the few examples of bothropic PLA₂ that has three different kinds of biological activities, a moderate anticoagulant and PLA₂ and high myotoxic activities. In addition this work presents the complete amino acid sequence of this new PLA₂ and establishes correlation between its structure and biological activities, PLA₂, anticoagulant and myotoxic. The examination of pathological changes induced in mice skeletal muscle cells was performed *in vivo*.

Material and Methods

Venom, Chemicals and Reagents

Bothrops pirajai snake venom was a gift from CEPLAC, CEPEC (Ministry of Agriculture, Itabuna, Bahia, Brazil). All chemicals and reagents used in this work were of analytical or sequence grade.

Reverse Phase HPLC

Purification of BP-III was made according to a method described by Toyama *et al.*, (1995). Twenty mg of the desiccated whole venom were dissolved with 750 μ l of 0.1% (v / v) trifluoroacetic acid (Solvent A). The resulting solution was clarified by centrifugation and the supernatant was applied on top of a 0.78 cm x 30 cm μ -Bondapack C-18 column (Waters 991-PDA system). Elution of peaks were proceeded with a linear gradient from zero to 66.5% (v / v) acetonitrile (Solvent B) in 0.1% (v / v) trifluoroacetic acid, at a flow rate of 2.0 ml / min. Absorbances were monitored at 280 nm. Fractions were collected, lyophilized and stored at -20 ° C.

Ion exchange HPLC chromatography

BP-III fraction was dissolved in 250 μ l of 0.05M Ammonium bicarbonate, pH 7.8 (Buffer A). After homogenization, MP-III solution was clarified by centrifugation at 10.000 rpm by 3 min. The supernatant was applied on Protein Pack SP 5PW, 0.78 X 7.0 cm. The elution of samples was performed using a linear gradient of concentration from 0.05M to 1.0 M Ammonium bicarbonate.

The chromatographic run was monitored at 280 nm and constant elution flow of 2.0 ml/min.

Measurement of PLA₂ activity

The PLA₂ measurement was made following the method described by Cho and Kézdy, (1991) and Holzer and Mackessy (1996). In our experiments we used a PLA₂ solution of concentration at 1.0 mg/ml and used a 100 µg per test. The PLA₂ activity was measured during 20 min of incubation at 37° C, in 0.1M Tris- HCl buffer, pH 8.0 with 2.5 mM CaCl₂. After that the reaction was stopped by addition of Triton solution.

Anticoagulant activity

Anticoagulant activity was assayed by measuring the recalcification time of fresh, platelet-poor, rat plasma. The plasma (0.5 ml) was incubated with saline or isolated venom fraction (0.1ml), at 37° C for 3 min and the time for clotting after addition of 0.1ml of 0.25M CaCl₂ was measured (Alvarado and Gutiérrez, 1988; Selistre *et al.*, 1990).

Reduction and carboxy methylation of MP-III 4R

One milligram of the purified MP-III 4R toxin was dissolved in 6M guanidine chloride (Merck, Darmstadt, Germany) containing 0.4M Tris-HCl and 2 mM EDTA at final pH 8.15, reduced with DTT and carboxy-methylated with ¹⁴C-iodoacetic acid

(Marangoni *et al.*, 1995). Desalting was performed on a Sephadex G 25 column in 1M acetic acid at 25° C and the eluted RC-MP-III 4R was lyophilized.

Digestion of the MP-III 4R and purification of the resulting peptides

Reduced and carboxymethylated MP-III 4R (RC-MP-III 4R) was digested with *Staphylococcus aureus* protease V8 for 16 hr at 37° C, using a 1:30 enzyme to substrate molar ratio and stopping the reaction by lyophilization (Houmard and Drapeau, 1972). RC-MP-III 4R was also digested with clostripain for 8 h at 37° C and lyophilized (Cintra *et al.*, 1993).

Both protease V8 and clostripain digests were fractionated by reverse phase HPLC using a Waters PDA 991 system and a C18 μ Bondapak column under a concentration gradient of acetonitrile in 0.1% trifluoroacetic acid (v/v).

Sequence procedure

Direct sequencing from the N-terminal and also of the toxin peptides was carried out with the native protein followed by those of the isolated peptides from the RC-MP-III 4R, using an Applied Biosystem 477 A automatic sequencer. The phenylthiohydantoin (PTH) amino acid was identified according to their retention time compared to those of the 20 PTH amino acid standards.

Peptides containing (14C) CM-Cys were monitored by detecting the radioactive label using a liquid scintillation counter (Beckman, model L-250).

Light Microscopy

Swiss mice weighing 25-30 g were injected in the right thigh gastrocnemius muscle with either 0.1 ml of MP-III 4R (1.3 μ g/g), dissolved in Tyrode solution (n=3) or with 0.1 ml of Tyrode solution alone (controls) (n=3). After 3 hours, the animals were killed by cervical dislocation and samples of muscle were fixed for 10-12 h in Bouin's fixative. The gastrocnemius samples were then washed three times (10 min each) in distilled water, dehydrated in graded series of ethanol (70%, 95% and 100%) and embedded in Histoiresin JB-4 (LKB-Bromma, Sweden). Sections 2-3 μ m thick were cut using a Leica RM 2035 microtome (Leica, Wien, Austria) and stained with 0.5% Toluidine blue solution for examination by light microscopy. Photomicrographs were taken using a Zenalumar Zeiss light microscope (Carl Zeiss, Jena, Germany).

Results

MP-III 4R was isolated by HPLC methods as described above after three chromatographic steps. This fraction showed a moderate PLA₂ and anticoagulant activities and strong myotoxic activity. The first chromatographic step was made according to the method described by Toyama *et al.*, 1995, which used the RP HPLC for initial purification of the PLA₂.

The MP-III fraction (Fig. 1), obtained after RP HPLC, was repurified on a Protein Pack SP 5PW (Waters) (0.78 X 8 cm) ion exchanger HPLC column. Thirty mg of MP-III fraction were dissolved in 0.05M ammonium bicarbonate buffer, pH

7.8 and clarified before application to the column. The elution of fractions (MP-I, MP-II, MP-III, BpIV, BpV, BpVI) was made using a linear gradient of 1.0M ammonium bicarbonate, pH 7.8.

From the first chromatography were obtained six major fractions (Fig.1) among them a new myotoxic fraction MP-III with moderate PLA₂ and anticoagulant activities, that was repurified on ion exchange HPLC. After this chromatographic step, five major fraction were purified (Fig. 2). MP-III 4 isolated in this second chromatography demonstrated anticoagulant, PLA₂ and myotoxic activities. This fraction MP-III 4 was subjected to new RP HPLC for further purification (Fig. 3). The protein isolated from the MP-III 4 by ion exchange chromatography shows a single elution peak that was called as MP-III 4R. This protein has a molecular weight of around 14 kDa by SDS-PAGE and contained a basic protein.

An enzymatic assay was performed according to Cho and Kézdy, (1991) and Holzer and Mackessy (1996) and showed values of 102.31 (nmoles/min/mg) indicating a moderate PLA₂ activity. The anticoagulant activity using doses of 2.5 – 3.5 µg showed a recalcification time of up to 45 min, that was also considered moderate.

Fig. 4 shows the chromatographic profile of the *S. aureus* V8 protease digest of 500 µg of reduced and 14C alkylated MP-III 4R, which was purified using a RP-HPLC analytical µ-Bondapack C18 column. The chromatographic profile of the MP-III 4R digest with clostripain purified under the same conditions as of the purification of protease V8, is shown in Fig. 5.

The complete sequence of MP-III 4R is shown in Fig. 6. The sequence of the first 42 N-terminal amino acids was obtained by direct automated sequence from the RC-MP-III 4R. The rest of the sequence was determined from the amino acid sequencing of the peptides purified from the protease V8 and clostripain digest purification (Fig. 6). The effective important peptides that contributed for determination of the primary structure were CIt 5, CIt 18, CIt 12, SV8 2 and SV8 4.

The myotoxic activity of MP-III 4R D49 was assayed using direct histological examination of sections the injected skeletal muscle. 3 hours after injection, the predominant pattern of myonecrosis was characterized by densely clumped myofilaments alternating with both clear areas of sarcoplasm which appeared to be empty and hypercontract regions of cells (Fig. 8 B). Comparison with histological sections of control gastrocnemius (Fig. 8 A) revealed the marked disintegration of muscle cells of envenomated mice which lost striation, became swollen and had ruptured sarcolemma, indicating a powerful myotoxic activity of MP-III 4R D49. This toxin did not cause hemorrhage.

Discussion

In this paper, we described a new procedure for the isolation of PLA₂ from whole venom. Classically, bothropic and other PLA₂s were purified using molecular exclusion or ion exchange chromatography as the initial step, followed by ion exchange rechromatography and a final RP-HPLC step. *Bothrops* myotoxin isoforms described can not be resolved by common chromatographic method

based on size (such as molecular exclusion or SDS-PAGE) or net charge using ion exchange methods or chromatofocusing methods, which have failed to isolate the highly basic myotoxins. Recently, RP HPLC has been used for separation of these isoforms (Gutiérrez and Lomonte, 1995) and Toyama *et al.*, (1995) showed an alternative purification method of two myotoxins from *Bothrops pirajai* venom, which started with RP-HPLC. This method showed advantages in relation to the conventional chromatographic methods when there is a small amount of venom to be purified. In our experiments we used around 20 mg of whole venom which is considered a very low amount when compared to conventional chromatography that normally uses around 250 mg to 1.0g.

In this work, the MP-III 4R PLA₂ isolated from *Bothrops pirajai* whole venom showed three kinds of biological activities: a moderate PLA₂, anticoagulant activities and a strong myonecrotic-inducing effect.

Phospholipases A₂ are classified in four groups snake venom PLA₂ belonging to groups I and II. Type II PLA₂s can be subdivided into two subgroups: D-49 (Asp-49 PLA₂) which have an aspartic acid residue calcium binding site and high enzymatic activity and K-49 PLA₂-like (Lys-49 PLA₂-like), which have a lysine residue at position 49 and low enzymatic activity (Selistre de Araújo *et al.*, 1996). MP-III 4R exhibits a sequence homology degree of around 60% when compared to K-49 PLA₂ and up to 75% homology when compared to other D-49 PLA₂.

MP-III 4R, isolated from whole venom of *Bothrops pirajai* has a moderate PLA₂ activity if compared to others from Crotalic venoms. MP-III 4R has 70% homology with BthTX-II, a myotoxin isolated from *Bothrops jararacussu* venom (Fig. 7), which has low lipolytic activity (i.e. low PLA₂ activity) and its primary

structure was completely determined by Pereira *et al.*, (1998). According to these authors, the presence of Y28, G30, G32, D49, H48, Y52 and D99 are direct or indirectly involved in the catalysis. BthTX-II shows contain of mutation F5 → W5, which is considered a strategic position for enzymatic catalysis expression. The presence of F5 in the MP-III 4R (PLA₂ activity), shows that this residue contributes to the presence of hydrophobic inner in the N-terminal region that is highly conserved and foldis into a “hydrophobic wall” which is responsible for the access of phospholipids to the catalytic site. In the active PLA₂ there is a conserved triplet that contributes to form this hydrophobic wall: F5, A103 and F106 (Arni and Ward, 1996) which is conserved in MP-III 4R.

Kini and Iwanaga (1986) and Kini and Evans (1987, 1989) have proposed several models to explain the pharmacological and biological activities of PLA₂. In these models, the PLA₂ has two separate sites: one responsible for the catalytic activity and a pharmacological site responsible for the expression of the biological activity. The pharmacological site could be located at the surface of the PLA₂ molecule.

According to the model proposed by Kini and Evans (1987), the anticoagulant site should be located in the region between residues 53 and 76. This region is positively charged in the strong PLA₂ anticoagulant and negatively charged in the weak or non anticoagulant PLA₂. The anticoagulant region is placed in a distinct and separate region of the predicted neurotoxic and myotoxic sites.

Although, Corredano *et al.*, (1998) published crystallographic studies of two PLA₂s in which the presence of glutamic acid at position 53 is common in the

strong anticoagulant PLA₂ and may be important in the expression of anticoagulant activity.

MP-III 4R does not possess E53 that replaced by K53. This replacement is considered an important contributes to low anticoagulant activity.

MP-III 4R is a rare example of PLA₂ (class II) with phospholipase A₂, anticoagulant and myotoxic activities, which has determined its complete amino acid sequence determined.

MP-III 4R is a fraction that was capable of inducing necrosis of mouse skeletal muscle cells. It has detectable moderate PLA₂ activity, but strong myotoxicity. In this context, it induces a qualitatively similar myonecrotic picture greatly similar to those produced by other Bothrops venoms, independent of whether PLA₂ activity or not (see, Gitiérrez and Lomonte, 1986). Some of these myotoxins do not reveal histological alterations of blood vessels or nerves after intra-muscular injection, suggesting that muscular lesion occurs through a direct effect in the cell membrane (Gutiérrez *et al.*, 1984, 1991). The PLA₂ activity of MP-III 4R, though mild, probably contributes to membrane plasmatic destabilization resulting in a generalized increase in membrane permeability to macromolecules and ions. In skeletal muscle, the increase in calcium levels results in hypercontraction of the myofilaments, activation of calcium-dependent proteases and endogenous phospholipases which cause further cell damage (Gutiérrez *et al.*, 1984; Gutiérrez and Lomonte, 1995).

Probably, MP-III 4R PLA₂ plays important role in the *Bothrops pirajai* venom function because it has multi-functionality. It differ from PrTX-I and PrTX-II in that it acts only in the myonecrosis process. MP-III 4R probably has another mechanism

of action different from that of other PLA₂ described to *Bothrops pirajai* (Bahia's jararacussu) and *Bothrops jararacussu* (jararacussu).

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Legends of figures:

Figure 1. Chromatographic profile of purification of *Bothrops pirajai* whole venom by RP-HPLC on μ -Bondapak C18 semi-preparative column (7.8 X 300 mm). Purification of fraction were made using a linear gradient of acetonitrile (Solvent B).

Figure 2. Chromatographic profile of purification of MP-III 4R performed on Protein Pack, SP 5PW cation exchange column. The elution of samples was performed

Figure 7. The amino acid alignment of MP-III 4R with other PLA₂. PrTX-I (Toyama *et al.*, 1998), BthTX-I (Cintra *et al.*, 1993), Myotoxin II (Francis *et al.*, 1991), App K-49 (Maraganore and Heirikson, 1986), T.g PLA₂-V (Nakai *et al.*, 1995), BthTX-II (Pereira *et al.*, 1998), Myotoxin III (Kaiser *et al.*, 1990), VRV-PL-VIIIa (Gowda *et al.*, 1994), TFPLA₂P (Ogawa *et al.*, 1992) and Bp PLA₂ (Fleer *et al.*, 1978). The symbols ★ (L/F), ⊗ (E/D) and ⊕ (K/P).

Figure 8. Light micrograph of skeletal muscle from a mouse after i.m. injection of MP-III 4R (1.3 µg/g)

(A) Control gastrocnemius injected with Tyrode solution. Longitudinal section of fibers with normal cross striation, peripheral nuclei and regular diameters. (B) Right gastrocnemius showing extensive myonecrosis 3h after injection of toxin. Note the necrotic cells characterized by total disorganization of myofibrils; the cells have lost striated appearance, showed hypercontracted areas (h) and tortuous clump of condensed myofibrils (arrowhead) intercalated among empty areas (e). Bars = 50 µm.

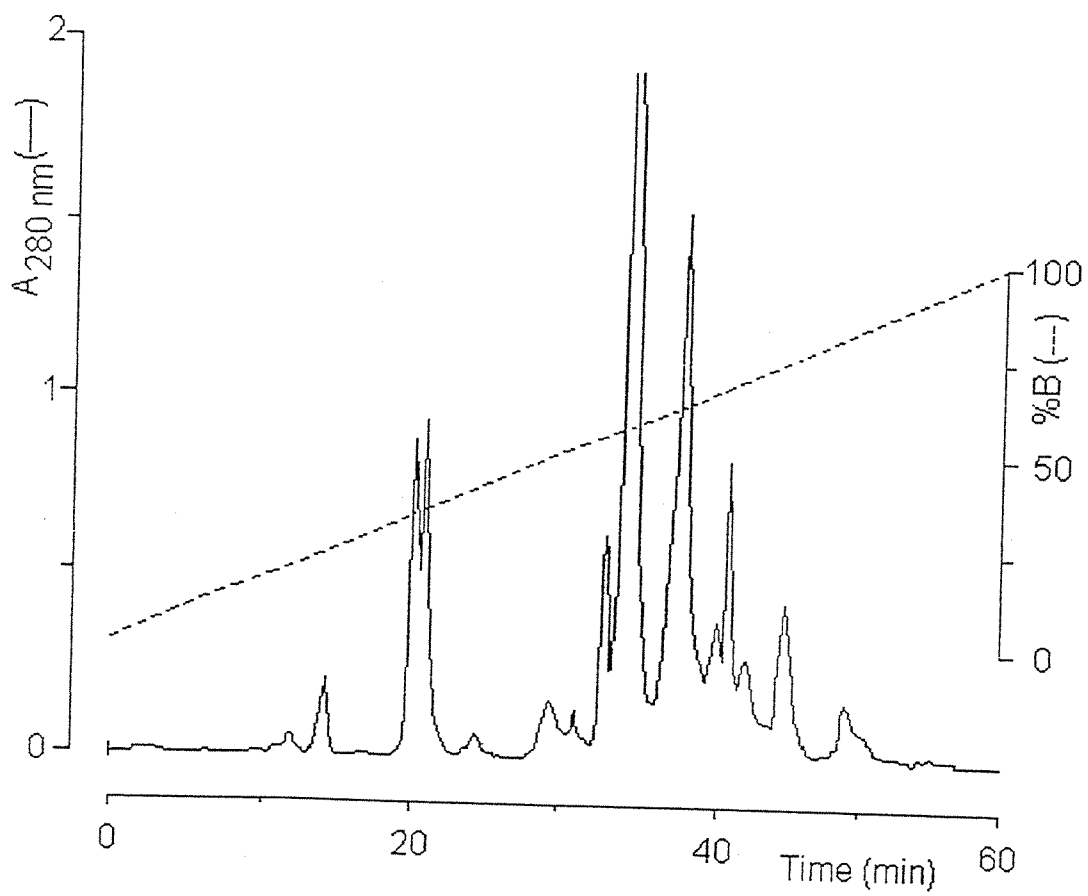
using a linear gradient of concentration from 0.05M to 1.0 M ammonium bicarbonate.

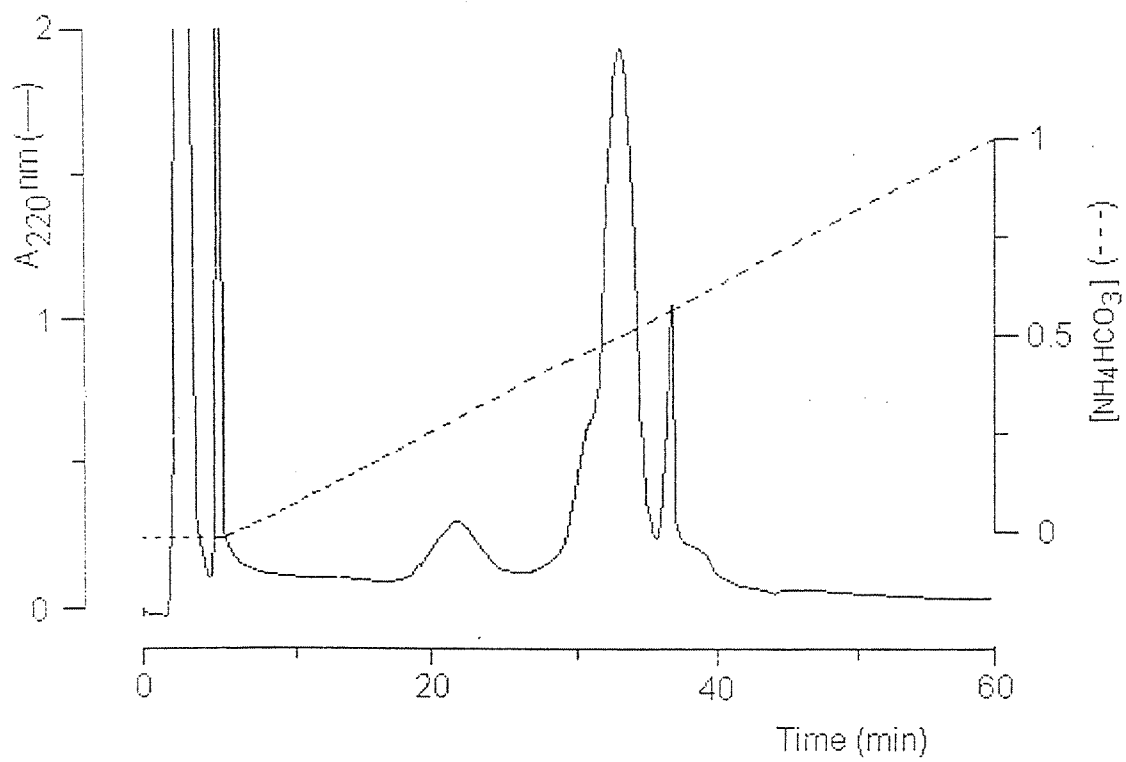
Figure 3. Repurification of MP-III 4R performed on analytical RP-HPLC column (0.39 X 300 mm). The repurification was made using a linear gradient of concentration of acetonitrile concentrations (Buffer B).

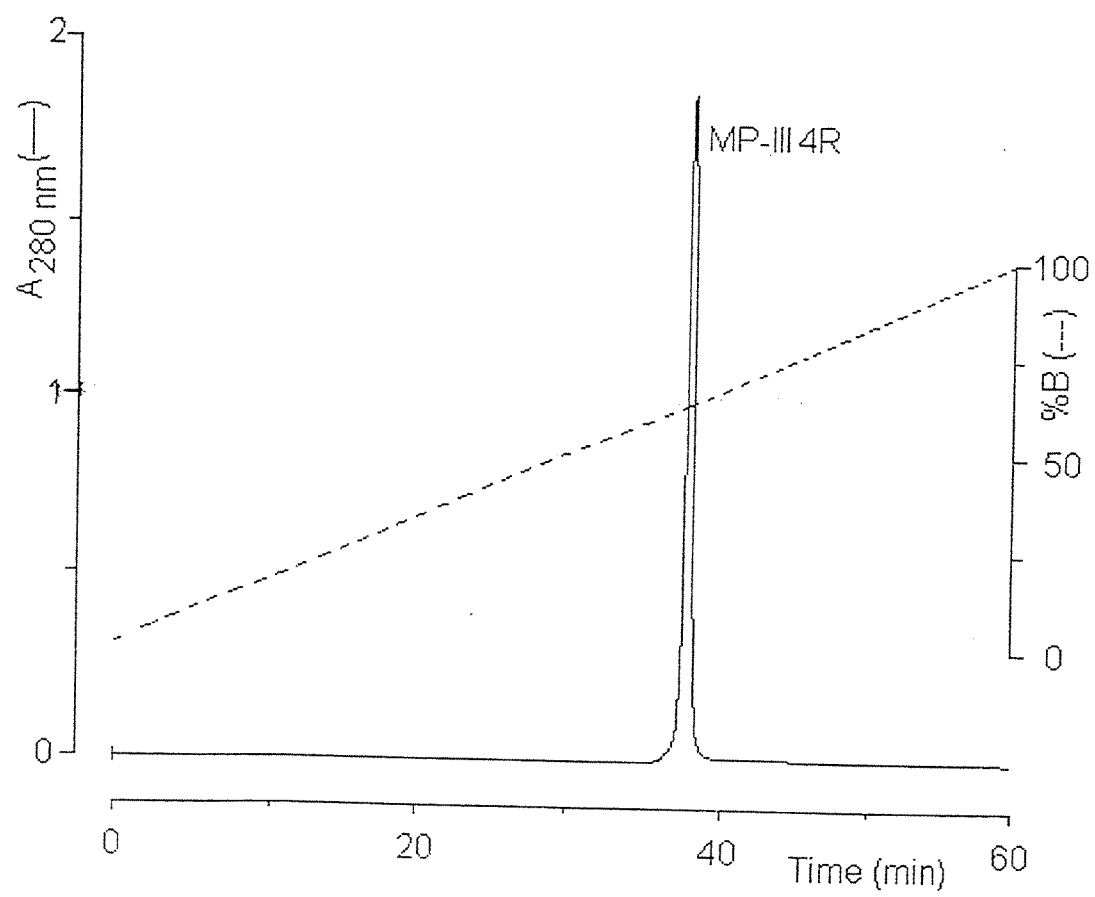
Figure 4. Chromatographic profile of purification of the peptides produced by digesting the RC-MP-III 4R protein with protease V8 from *Staphylococcus aureus* V8. The RP-HPLC carried out on a μ -Bondapak C18 (0.39 X 30 cm) column using a discontinuous acetonitrile gradient, at constant flow rate of 1.0 ml/ min with detection of peaks made using an Absorbance of 220 nm.

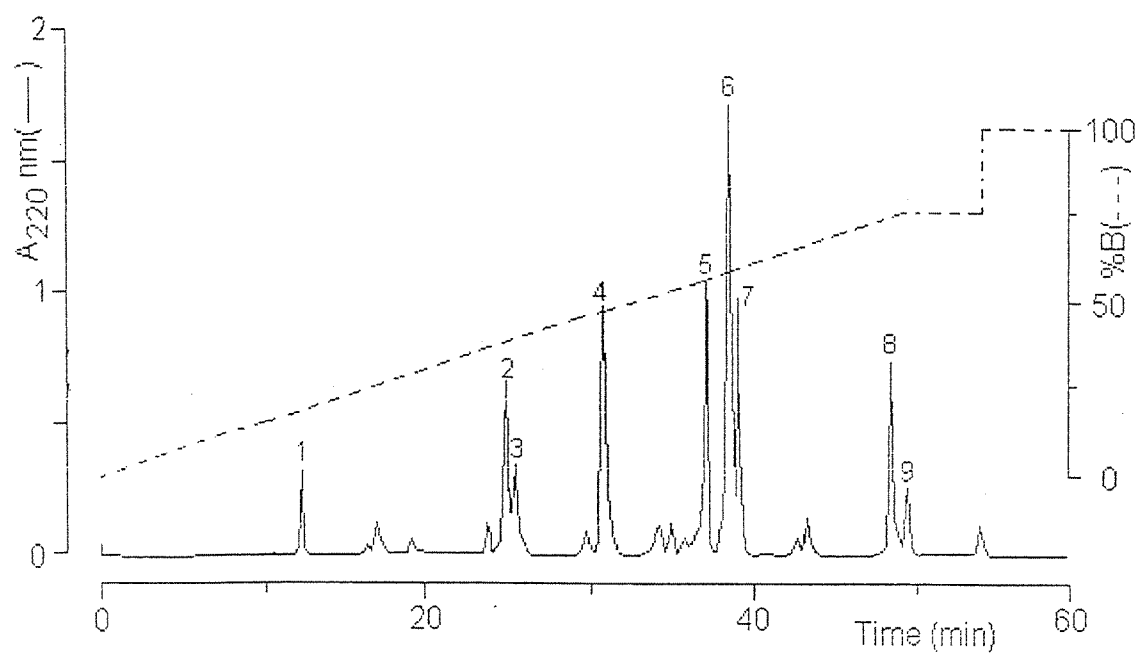
Figure 5. Purification of peptides produced by digesting the RC-MP-III 4R protein with clostripain.

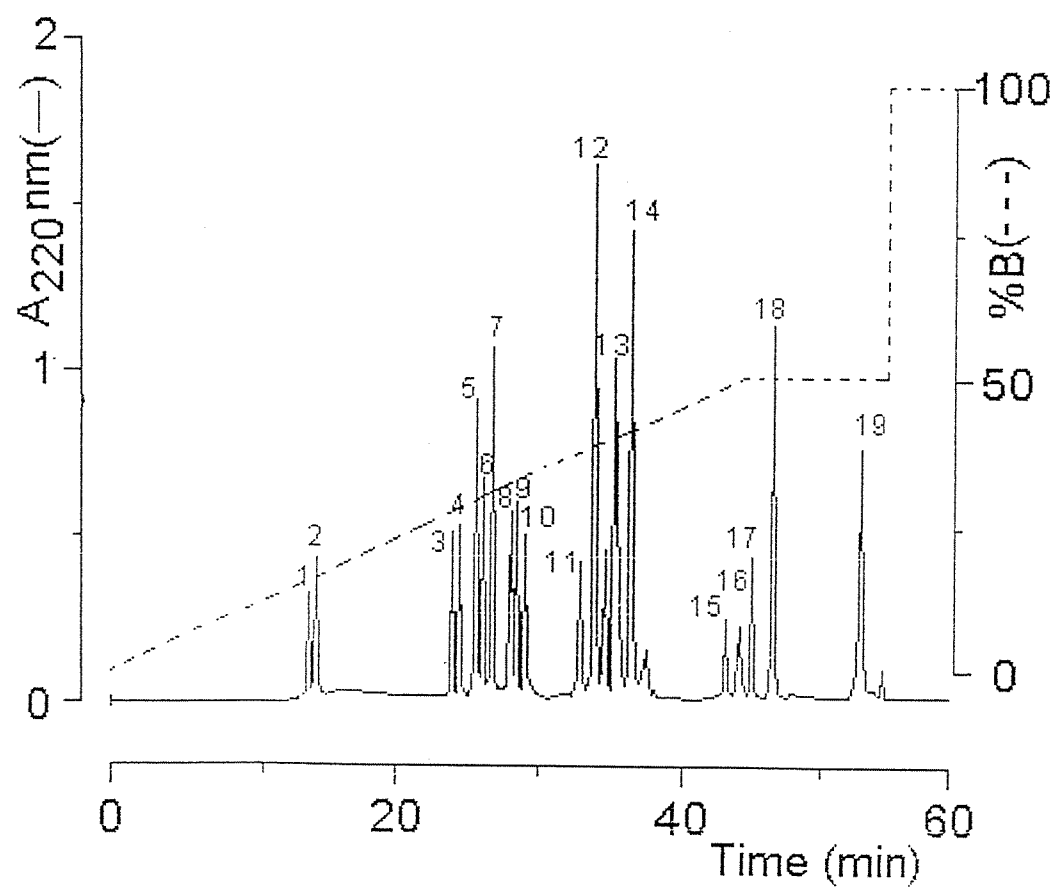
Figure 6. The primary structure of Lys-49 PLA₂-like MP-III 4R, showing the alignments of the peptides from the *Staphylococcus aureus* V8 protease (SV8) and from the clostripain (Clit) digest. The first 45 residues were directly sequenced.











1 10 20 30 40 50 60 70
DLWQFGQMILKETGKLPEFPYYTYGGCYCGVGRRGLGTDKTDRCYVHDCYKKLTGCPKTDDRYSSWL
|-----|
direct sequence

|-----|
Cl t 5

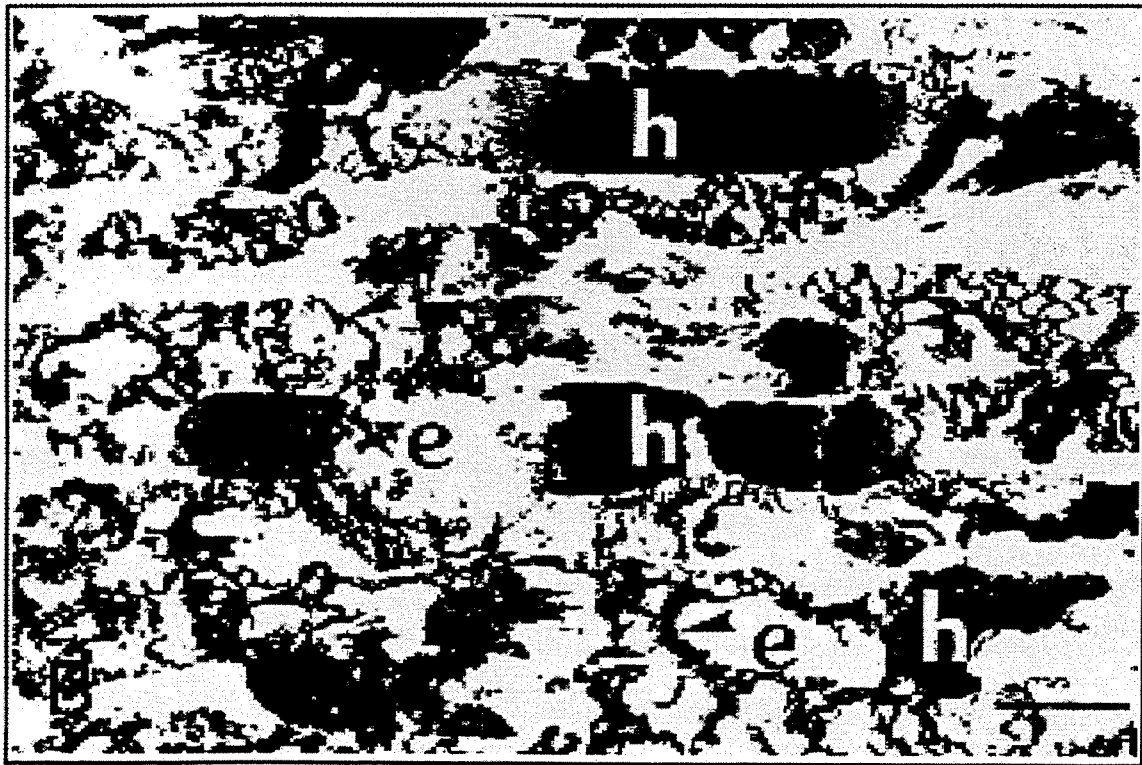
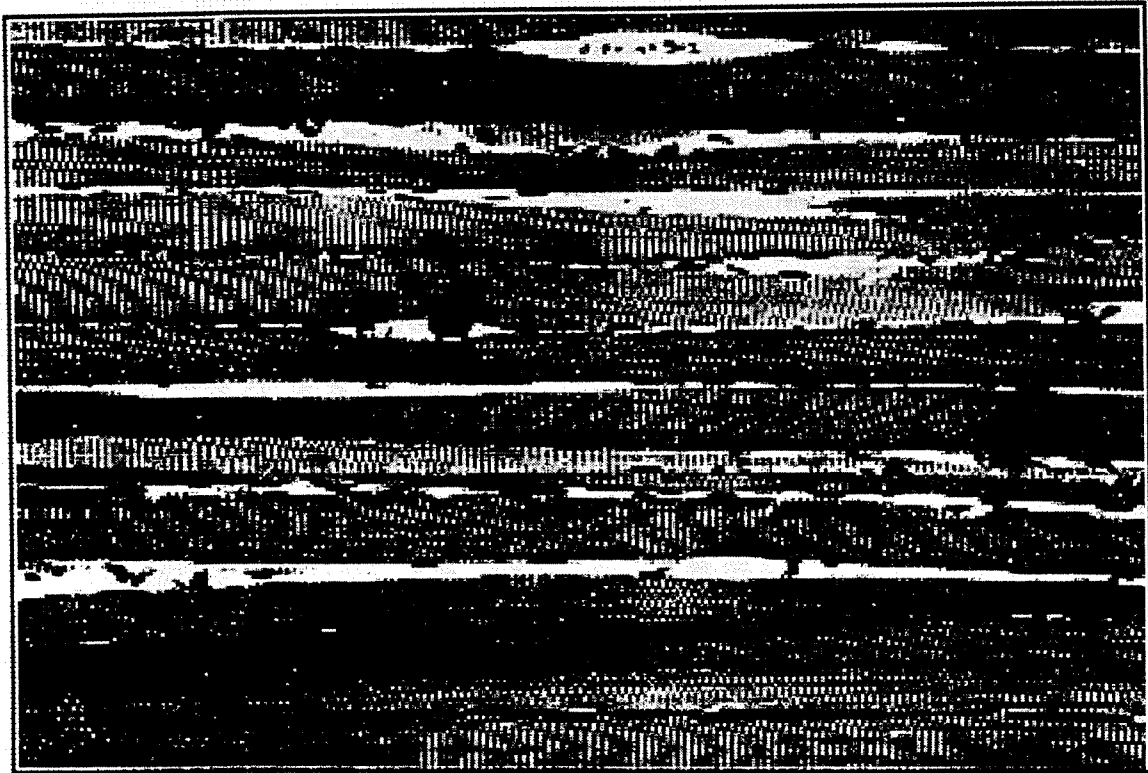
|-----|
Cl t 18

80 90 100 110 120
DLTIVCGEDDFCKELCECDKAIAVCFRENLGTYNKKYRYHLKPCKKADKPC

|-----|
SV8 2 SV8 4

|-----|
Cl t 12

|-----|
Cl t 18



4-Discussão

Purificação das PLA₂ miotóxicas de *Bothrops pirajai*

A purificação de miotoxinas de *Bothrops pirajai* através de cromatografia líquida convencional foi realizada usando-se dois passos subsequentes a cromatografia de exclusão molecular em Sephadex G75.

A etapa de purificação em SP Sephadex C25 foi realizada baseada no trabalho desenvolvido por Homsí-Brandeburgo et al., (1988), desenvolvido para a purificação das fosfolipases A₂ miotóxica de *Bothrops jararacussu*.

Algumas modificações em relação ao método desenvolvido por Homsí-Brandeburgo foram introduzidas ao longo do projeto de doutorado. Deste modo, ao invés do uso de acetato de amônio pH 5,0, que necessitava de uma molaridade alta ao redor de 3,0 M para eluição das amostras, principalmente as de caráter básico, como as miotoxinas PLA₂ "like". Foi usado o tampão acetato de amônio pH 6,0 que necessita de uma força iônica menor, ao redor de 1,5 M de acetato para eluição de todas as frações.

Esta mudança de pH das soluções tampões pode levar a diminuição da carga líquida positiva da proteína, deste modo diminuindo as interações da proteína com a fase estacionária.

A grande vantagem desta modificação sugerida acima foi a diminuição da concentração iônica para a eluição das amostras de fosfolipásica A₂ e das miotoxinas PLA₂ "like". Contudo a reprodução desta metodologia em sistema HPLC seria difícil, pelas próprias limitações técnicas do sistema, principalmente em relação ao sistema de bombas, da própria coluna e do sistema analítico de leitura. De acordo com os manuais de técnicos de HPLC Waters PDA 991 e de outros HPLC da família Waters e mesmo sistemas de HPLC preparativos para a purificação de proteínas como os LC/ Waters é expressamente proibitivo o uso de soluções salinas com uma molaridade acima de 1,0 M. Nestes sistemas cromatográficos recomenda-se o uso de soluções abaixo de 1,0 M.

Portanto, a mudança da fase estacionária também mostrou ser necessária para tentar adaptar as metodologias convencionais às metodologias em HPLC. Durante o desenvolvimento experimental de novas metodologias em cromatografia de troca iônica, concluímos que a mudança da fase estacionária e do tipo de tampão usados, mostraram ser mais eficientes para a purificação de miotoxinas PLA₂ "like", como mostrado segundo

trabalho desta tese (Soares et al., 1998). Basicamente a fase estacionária SP Sephadex C25, que é um trocador forte, foi substituída por uma CM Cellulose 52, mais fraca. O tampão de acetato de amônia foi substituído pelo Bicarbonato de amônia, o que resultou em um aumento do pH da fase móvel. Estas modificações permitiram a eluição das PLA₂ miotóxicas básicas com menor força iônica.

Este trabalho de tese propiciou o desenvolvimento de uma nova metodologia adaptada da cromatografia convencional. Nos utilizamos como uma alternativa a metodologia de cromatografia de troca iônica de baixa pressão, uma coluna de HPLC de troca iônica forte como a Protein Pack SP 5PW (Waters) e como fase móvel usamos o Bicarbonato de amônio. Através desta nova metodologia, conseguimos purificar com maior seletividade as proteínas de serpentes botrópicas, como a *Bothrops jararacussu*.

Conseguimos, desenvolvendo este método, purificar cerca de 40 a 50 mg de veneno bruto, a eluição de todas as amostras foi conseguida com uma concentração final de 0,45 a 0,6M de Bicarbonato de amônia, usando um gradiente total de 120 minutos com: 20 minutos de equilíbrio, 60 minutos de corrida, 10 minutos de limpeza da coluna e 30 minutos de reequilíbrio para a próxima injeção de amostra.

Neste trabalho também desenvolvemos outras metodologias para purificação e caracterização de venenos botrópicos através de sistemas de HPLC, principalmente o de HPLC de fase reversa.

Devido a alta capacidade resolução e seletividade dos picos oferecido pelo sistema de HPLC de fase reversa, duas miotoxinas PLA₂ "like" foram isoladas, primeiramente chamadas de MP-I e MP-II, que foram isoladas do veneno total de *Bothrops pirajai* em duas etapas cromatográficas. MP-I, que é a toxina majoritária deste veneno, foi obtido praticamente pura em uma única etapa.

A identidade entre MP-I e PrTX-I ou entre MP-II e PrTX-II foi evidenciado pelo perfil de eluição das respectivas toxinas, pela suas atividades biológicas e pelo seqüenciamento da N-terminal como descrita por Toyama et al., 1995, nesta tese.

Embora esta nova metodologia restrinja o volume de veneno a ser usado em cada etapa cromatográfica para aproximadamente 10 a 20 mg, a metodologia usando HPLC propiciou um rendimento de cerca de 35% para MP-I, quando comparado aos 19,7%

obtidos pelo método convencional. Isso representa uma estratégia de purificação vantajosa, quando grandes quantidades não são disponíveis.

Análises posteriores com outros venenos botrópicos bem como de suas PLA₂ miotóxicas principais isoladas em sistemas de HPLC de fase reversa e submetido a análises bioquímicas mostraram que as PLA₂ miotóxicas destas serpente possuem várias características em comum: possuem um grau de hidrofobicidade e basicidade semelhantes, massas moleculares similares, formam dímeros quando em solução, a atividade PLA₂ é ausente ou residual, possuem alta homologia seqüencial.

Determinação das estruturas primária das piratoxinas (PrTX-I, PrTX-II e PrTX-III) correlação entre estrutura e função.

O isolamento geográfico de algumas espécies de serpentes pertencentes a um único gênero pode levar a determinadas alterações que incluem modificações morfológicas e bioquímicas que tornam uma determinada espécie diferente da espécie original, tornando-a uma nova subespécie ou uma nova espécie distinta. Este é o caso da *Bothrops pirajai*, que é encontrada unicamente na região sudeste do Estado da Bahia (regiões de Ilhéus e Itabuna). O fracionamento do veneno foi previamente discutido por Mancuso et al., (1995), que mostrou similaridades com o veneno de *Bothrops jararacussu*. PrTX-I é a miotoxina de *Bothrops pirajai* que corresponde a Bothropstoxina I (BthTX-I), cuja seqüência foi determinado por Cintra et al., (1993).

Os resultados apresentado nesta tese mostram que PrTX-I possui uma alta homologia seqüencial (95%) com outras miotoxinas botropicas. Este valor cai para 80% quando comparadas com as miotoxinas de outras serpentes não botropicas e para cerca de 70 a 75% para as PLA₂ Asp 49.

Asp 49 é considerada vital, contudo não suficiente (Pereira et al., 1998) para atividade catalítica, que não é o caso da PrTX-I. Para as PLA₂ D49 existem, via de regra, três resíduos conservados na porção N-terminal que são Q(4), F(5), I(10), enquanto que nas variantes PLA₂ K49 possuem L(5) no lugar de F(5).

Estudos de homologia seqüencial mostra que nas PLA₂ D49 existem determinados resíduos altamente conservados ao longo da cadeia polipeptídica que são: Y(25), G(26),

C(27), Y(28), C(29), G(30), G(32), G(33), P(37), D(39), D(42), R(43), C(44), C(45), H(48), C(50), C(51) e Y(52). Dentro destes, o segmento 44 - 50 e 28 - 32 representam as regiões mais diretamente envolvidas com a função catalítica (Arni & Ward, 1996). Na PrTX-I nos temos N(28) para Y(28), L(32) para G(32) e K(49) para K(49). Esta última substituição foi decisiva para a perda da atividade PLA₂, sendo agravado por estas outras mudanças. Nas PLA₂ tem-se G(32) e G(33), esta seqüência não é encontrada nas PLA₂ K49, como foi o caso da PrTX-I.

O alinhamento das seqüências da PrTX-I e BthTX-II mostra que a presença de duas únicas mutações ao longo da cadeia peptídica, L(107) para K(107) e D(122) para P(122). A presença de L(107) e D(122) na PrTX-I substituídas por K(107) e P(122) da BthTX-I podem conferir a BthTX-I um caráter ligeiramente básico. Contudo esta diferença não é suficiente para produzir uma diferença de mobilidade eletroforética em PAGE sem SDS (Mancuso et al., 1995). PrTX-I e PrTX-II mostram pouca atividade anticoagulante, diferente do encontrado para PrTX-III, que possui uma atividade PLA₂ e uma atividade anticoagulante moderada, estes resultados podem estar relacionados a ausência da Lys 107. Nas PLA₂ um segmento peptídico de 20 resíduos C-terminal rico em Lys tem sido considerada a região responsável pela atividade anticoagulante das PLA₂ (Scott et al., 1990). Finalmente a presença de uma D(122) e P(123) no lugar de D(122) e D(123) poderia contribuir ainda mais para a perda da atividade anticoagulante, diminuindo o caráter catiônico da região C-terminal.

Contudo, em nossos trabalhos um outro segmento importante estaria entre os resíduos 53 e 76. Esta região é fortemente carregada positivamente na PLA₂ anticoagulantes fortes e é carregada negativamente ou moderadamente para as PLA₂ com atividade anticoagulante fracas. Nossas análises sugerem que uma mutação no resíduo E (53) poderia ser determinante para a perda da atividade anticoagulante.

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