UNIVERSIDADE ESTADUAL DE CAMPINAS

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Estudo das frações protéicas derivadas do veneno de serpentes "CROTÁLICAS" e "BOTHROPICAS" com atividade antibacteriana. Isolamento, purificação e caracterização bioquímica e biológica.

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"De tudo ficaram três coisas:
a certeza de que estava sempre começando,
a certeza que era preciso continuar
e a certeza de que seria interrompido antes de terminar.

Fazer da interrupção um caminho novo,
fazer da queda, um passo de dança,
do medo, uma escada,
do sonho, uma ponte,
da procura, um encontro."

Fernando Pessoa

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RESUMO

Nesta tese foram apresentados os resultados da purificação de várias proteínas biologicamente ativas do veneno de serpentes crotálicas (*Crotalus durissus terrificus*, *Crotalus durissus cascavella*, *Crotalus durissus ruruima* e *Crotalus durissus collilineatus*); botrópica (*Bothrops pirajai*) e coral (*Micrurus dumerilii carinicauda*). Muitas destas frações foram purificadas e caracterizadas do ponto de vista biológico, bioquímico e farmacológico. Frações como: crotamina, fosfolipase A_2 (PLA_2), crotapotinas e outras mostraram uma significativa atividade antibacteriana contra bactérias fitopatogênicas e patogênicas. As análises ultra-estruturais e outros dados bioquímicos indicam que estas frações exercem essa atividade de formas diferenciadas. Foram observados quatro mecanismos de ação: 1.interação das frações com a membrana bacteriana, induzindo a ruptura e desorganização da mesma com posterior lise celular, 2. desorganização citoplasmática através da entrada das frações por poros na membrana causando lise celular, 3. digestão da membrana com posterior extravasamento do conteúdo interno bacteriano com lise celular, 4.lise da membrana através dos radicais livres gerados pela reação enzimática do veneno com a membrana bacteriana.

Durante o desenvolvimento da tese foram publicados e submetidos à publicação os seguintes artigos:

1.Oliveira et al., 2002 apresentaram o fracionamento do veneno de *Crotalus durissus terrificus* em HPLC de fase reversa, com obtenção das frações: crotapotinas (F5 e F7) e PLA_2 (F15, F16 e F17). As frações apresentaram atividade antibacteriana contra *Xanthomonas axonopodis pv. passiflorae.* A fração F17 apresentou atividade anticoagulante e foi seqüenciada apresentando uma homologia estrutural com outras PLA_2 em torno de 60-90%. Sua massa molecular de 14,6kDa foi determinada através de tricina SDS-PAGE, eletroforese de duas dimensões e MALDI TOFF.

2.Toyama at al., 2003 verificaram a atividade enzimática, neurotóxica e antibacteriana da fração PLA_2 (F15) isolada do veneno de *Crotalus dirissus terrificus.*

3.Oliveira et al., 2003 isolaram, purificaram, seqüenciaram a fração crotapotina, um componente ácido da crotoxina do veneno de *Crotalus durissus cascavella*, e realizaram testes para verificar a atividade antibacteriana em bactérias fitopatogênicas Gram-positivas e Gram-negativas, além de testes inflamatórios e nefrotóxicos.

4.Havt et al., 2005 isolaram uma nova lectina tipo C (BPL) de *Bothrops pirajai* que foi caracterizada biológica e bioquimicamente. Esta fração também mostrou um efeito nefrotóxico em perfusão em rins isolados de ratos.

5.Toyama et al.,2004 isolaram, purificaram e caracterizaram bioquimicamente uma nova crotamina símile do veneno de *Crotalus durissus cascavella*. Foram identificados os peptídeos biologicamente ativos da crotamina, os quais foram responsáveis pela estimulação de secreção de insulina.

6.Gandhi et al., 2004 purificaram uma nova lectina tipo C (crotacetin), obtida do veneno de *Crotalus durissus cascavella*. Esta nova lectina induziu a agregação plaquetária, a aglutinação eritrocitária e também mostrou uma atividade antibacteriana.

7.Cavada et al., 2004 purificaram e caracterizaram uma nova lectina da semente de *Lonchocarpus sericeus,* com alta especificidade por N-acetilglicosamina.

8.Cháriston et al, 2004 isolaram e determinaram a estrutura primária de uma nova PLA_2 de *Micrurus dumerilli carinicauda*.

9.Toyama et al., 2004 isolaram, purificaram e caracterizaram uma nova isoforma da crotamina do veneno de *Crotalus durissus ruruima* e mostraram seu efeito antibacteriano.

10.Toyama et al., 2004 isolaram, purificaram e caracterizaram uma nova PLA_2 do veneno de *Crotalus durissus collilineatus*. Nestes trabalhos foram determinadas as regiões moleculares responsáveis pela atividade antibacteriana.

11. Toyama et al., 2004 purificaram e caracterizaram uma nova L-amino oxidase do veneno de *Crotalus durissus cascavella* (Casca LAO) através de exclusão molecular e HPLC de troca iônica. Casca LAO apresentou uma grande inibição bacteriana em bactérias Gram-negativas (*Xanthomonas axonopodis pv. passiflorae*) e Gram-positivas (*Streptococcus mutans*).

Os estudos destas frações apresentam perspectivas futuras na aplicação terapêutica, como agentes antibacterianos, de novos peptídeos sintéticos ou naturais.

ABSTRACT

In this dissertation we present the results of purification of several biologically active protein from the crotalic rattlesnake venom (*Crotalus durissus terrificus*, *Crotalus durissus cascavella*, *Crotalus durissus ruruima* e *Crotalus durissus collilineatus*); from bothropic (*Bothrops pirajai*) and coral (*Micrurus dumerilli carnicauda*). Many of these fractions were purified and biochemical, biologically and pharmacologically characterized, some were reported and other submitted for publication in specialized journals. Some fraction as crotamine, phospholipase A2 (PLA_2), crotapotins and other showed a significant antibacterial activity against phytopatogenical and pathogenic bacterium. Ultra structural and biochemical analyses suggest that this fraction exert this antibacterial activity by different ways, which are grouped according to the mode action into four different mechanisms: 1.interaction of some fractions with the bacterial membrane inducing cellular lyses by membrane rupture and disorganization. 2. Bacterial cell lyses by action of fraction from the venom that form pore on the membrane and consequently cytoplasmatic disorganization and membrane rupture. 3. Membrane cell digestion induced by protein from the venom and consequently lost of cytoplasmatic content and cellular lyses. 4. Membrane cell lyses induced by free radical generated by enzymatic action of protein form the venom.

During the development of this thesis we reported and submitted some articles listed below:

1. Oliveira et al., 2002 presents the purification of crotapotins (F5 and F7) and PLA_2 (F15, F16 and F17) from the *Crotalus durissus terrificus* venom by reverse phase HPLC. These fraction showed significant antibacterial activity against *Xanthomonas axonopodis pv. Passiflorae*. Fraction F17 also showed an anticoagulant activity and its amino acid sequence showed high amino acid sequence identity with 60-90% with other PLA_2 from different sources. Its molecular mass was 14,6kDa determined by Tricine PAGE-SDS, two dimensional electrophoresis and MALDI TOFF.

2. Toyama et al., 2003 showed the enzymatic, neurotoxic and antibacterial activities of PLA_2 isoform (F15) from the *Crotalus durissus terrificus* venom.

3. Oliveira et al., 2003 isolated, purified and determined the amino acid sequence of crotapotin fraction, an acid compound of crotoxin from the *Crotalus durissus cascavella*, and

determined its antibacterial activity against Gram-positive and Gram-negative phytopathogenic bacterium, inflammatory and nephrotoxicity.

4. Havt et al., 2005 isolated a new type C-lectin (BPL) from the *Bothrops pirajai* that was biologically and biochemical characterized. This fraction also showed a nephrotoxic effect on the isolated perfused kidney.

5. Toyama et al., 2004 isolated, purified and characterized biochemical a new crotamine like from *Crotalus durissus cascavella* venom. In this work we identified the biologically active peptide from this crotamine, which were responsible for increasing insulin secretion.

6. Gandhi et al., 2004 purified a new lectin type C (crotocetin) from the *Crotalus durissus cascavella* venom. This lectin induced platelet aggregation, erythrocyte agglutination and also showed an antibacterial activity.

7. Cavada et al., 2004 purified and characterized a new lectin from the *Lonchocarpus serieus* seed that showed high specific binding against N-acetyl glucosamine.

8. Chániston et al., 2004 isolated and determined the amino acid sequence of new PLA_2 from *Micrurus dumerilli carinicauda*.

9. Toyama et al., 2004 isolated, purified and characterized a new crotamine isoform from the *Crotalus durissus ruruima* venom and showed its antibacterial activity.

10. Toyama et al., 2004 isolated, purified and characterized a new PLA_2 from the *Crotalus durissus collilineatus* venom. In this work we determined the molecular region of the PLA_2 responsible for the antibacterial activity.

11. Toyama et al., 2004 purified and characterized a new L-amino acid oxidase from the *Crotalus durissus cascavella* whole venom (Casca LAAO) by molecular exclusion and cation exchange HPLC. Casca LAAO inhibited strongly the bacterial growth rate of Gram-negative (*Xanthomonas axonopodis pv passiflorae*) and Gram-positive (*Streptococcus mutans*).

These studies presented here showed a future perspective to therapeutic application of these peptides, synthetically or naturally obtained as potential antibacterial substances.

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. Quando seco apresenta entre 80-90% 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 A_2 (PLA_2); o magnésio e o zinco, que também são importantes íons para ação das principais metaloproteases do veneno como as "trombinas-likes" (Tu, 1977; Jia et al., 1996).

O citrato é encontrado em altas concentrações no veneno total de determinadas serpentes. Experimentalmente, foi demonstrado que o citrato atua como um inibidor de várias enzimas, como as nucleotidases, esterases, proteases e fosfolipases A_2 (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 "aminas biogênicas": a bradicinina, a histamina, a 4-hidroxitriptamina, a N-metil-5-hidroxitriptamina, a N'-N'-dimetil-5-hidroxitriptamina e a serotonina, que atuam como mediadores da dor provocada pelo veneno das serpentes (Ferreira et al., 1992).Classicamente estas proteínas podem ser agrupadas nos seguintes grupos:

1.1.1-Lectinas

As lectinas de serpente apresentam massa molecular de 14 kDa. São específicas para açúcares como -galactosídeos, mas requerem Ca^{2+} para sua atividade (Hirabayashi & Kasai, 1991). As lectinas de venenos são constituídas por cadeias polipeptídicas que correspondem à região relativa ao CRD (domínio de reconhecimento de carboidratos), ou seja, sua estrutura primária é homóloga à região que contém o CRD em outras proteínas ligantes de açúcar. São constituídas de aproximadamente 130 resíduos de aminoácidos e não possuem outros domínios acessórios. Elas são o próprio domínio de ligação a carboidratos observados em outras lectinas. Desta forma é vista como um CRD livre (Drickamer, 1993).

As lectinas de serpentes não são tóxicas para mamíferos, já que injeções intramusculares ou subcutâneas em coelhos de laboratório não causam reações adversas

(Ogilviem et al., 1986). Contudo estas lectinas estão intimamente relacionadas a vários eventos biológicos importantes, como indução da agregação plaquetária, atividade antibacteriana e apoptose.

1.1.2-Oxidoredutase

O veneno total de serpentes possui duas enzimas responsáveis por reações redox, as L-aminoácido oxidase (LAO) e a lactato desidrogenase. As LAOs convertem o aminoácido livre em um α-cetoácido. Muitos venenos de serpentes possuem LAO ativas, que são responsáveis pela cor amarela do veneno seco. Sua função biológica não é totalmente conhecida. Contudo já é conhecido que as LAO são capazes, em determinadas condições laboratoriais, de induzirem a agregação plaquetária (Li et al., 1994) e a apoptose em (determinadas células) (Suhr & Kini, 1996). Mclean et al. (1971) descreveram que o veneno de algumas serpentes catalisa a conversão do lactato em ácido pirúvico. A presença da lactato desidrogenase foi demonstrada em vários venenos, principalmente nos das serpentes do gênero *Naja* e *Dendroaspis*.

1.1.3-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 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 & Chua, 1966).

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

1.1.4-Glicosidases

A hialunonidase 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. Ela

teria a função de facilitar a difusão das toxinas do veneno para o interior dos tecidos das vítimas (Meyer et al., 1960). Também são encontradas duas outras enzimas, as α-amilases e a NAD nucleotidases.

1.1.5-Proteases

As proteases encontradas no veneno das serpentes podem ser reunidas em dois grupos: endopeptidases e 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, abundantes em venenos de serpentes *Crotalidae* e *Viperidae*. Um grande número de metaloproteases tem sido isolado de diferentes venenos e caracterizado como hemorraginas. As metaloproteases desses 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; Tu, 1982) e enzimas com ampla especificidade de substratos que não induzem hemorragia (Assakura et al., 1985; Sanches et al., 1987; Tu, 1982).

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

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

1.1.6-Neurotoxina de Canal de Sódio: Crotamina

É uma proteína neurotóxica de baixo peso molecular, de caráter básico, caracterizada pela primeira vez por Gonçalves & Vieira (1950). Esta toxina é encontrada no veneno de *Crotalus durisssus terrificus*, de certas regiões do Brasil, Argentina e Bolívia; possui ponto isoelétrico (pI) de 10,3 uma massa molecular de 4,8 kDa e três pontes disulfeto, (Gonçalves, 1956); apresenta um pequeno número de resíduos de aminoácidos quando comparados a outras neurotoxinas (Ryden et.al., 1973); é resistente ao calor de 700°C por até 18h, sem

perder sua propriedade tóxica. Toyama et al. (2000) identificaram duas isoformas de crotapotina denominadas F2 e F3. Estudos mostraram que, quando é injetadas intraperitonealmente em ratos, causa neles depressão respiratória, lacrimejamento, paralisia das patas posteriores, convulsão e morte. Recentemente Macin et al. (1997) mostraram que a crotamina em baixas concentrações é capaz de induzir um efeito anestésico local cujo mecanismo de ação é desconhecido.

1.1.7-Lípases

As fosfolipases e as acetilcolinesterases são enzimas mais facilmente encontradas nos venenos de serpentes. As PLA_2 são as enzimas mais estudadas, não somente pelas suas propriedades químicas, mas por sua importância biológica (Tu, 1977).

Além das enzimas hidrolíticas, o veneno total também possui outras moléculas de importância, das quais se destacam as desintegrinas. Estas, sem atividade enzimática possuem massas moleculares variadas e são proteínas que inibem a interação entre o fibrinogênio e o complexo glicoproteico I Ib - I Ia, na coagulação sangüínea. Possuem uma seqüência de três resíduos de aminoácidos conservados, que são responsáveis pela atividade biológica (Musial et al., 1990).

1.2-Proteínas e Peptídeos antimicrobianos

1.2.1-Peptídeos antimicrobianos

Podem ser reunidos em cinco grandes grupos, classificados de acordo com a estrutura tri-dimensional: 1. peptídeos lineares livres de cisteína com uma estrutura em α-hélice amfipática, 2. peptídeos com três pontes disulfeto com um estruturação básica em folhas β, 3. peptídeos sem organização definida, ricos em prolina, arginina, triptofano ou histidina, 4.bacteriocinas, 5. peptídeos básicos antimicrobianos.

Todos os grupos de peptídeos antibacterianos são capazes de alterar a estrutura da membrana e promover, a partir desta interação, a atividade antimicrobiana. Destacam-se, pela importância e perspectivas de aplicabilidade, três classes principais (Fig.1).

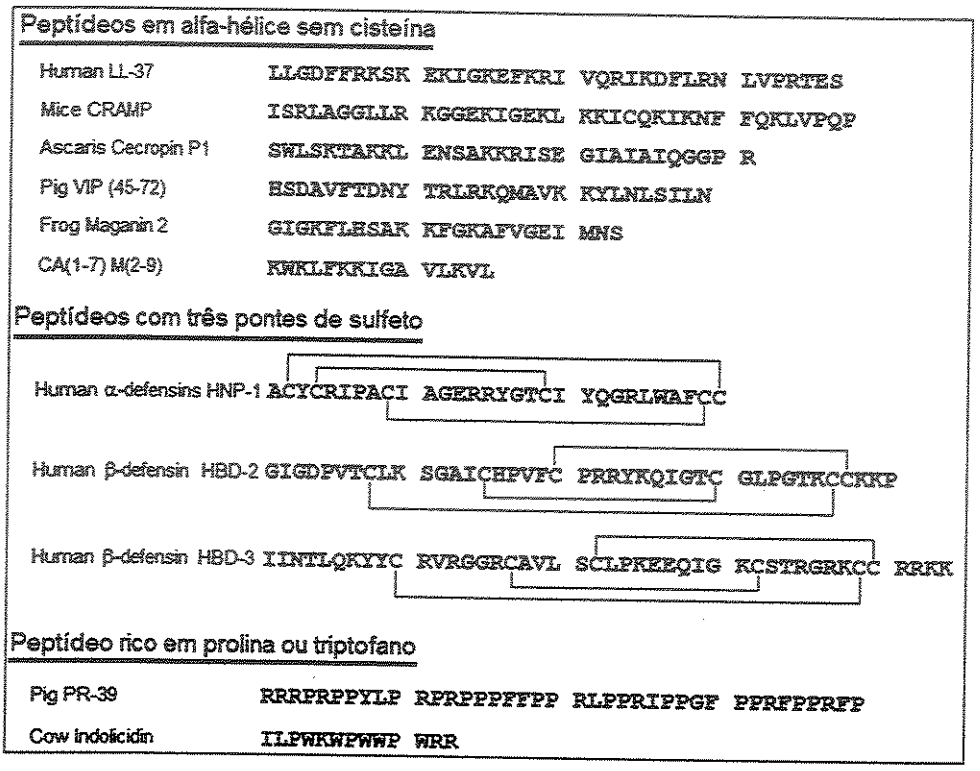

Figura 1. Sequência dos peptídeos antibacterianos pertencentes aos três maiores grupos (Boman, 2003).

Os peptídeos antibacterianos desempenham um papel importante na defesa inata de muitos organismos, como os dos ranídeos. São proteínas constituídas de 10 a 50 resíduos de aminoácidos que interagem com a bicamada lipídica de bactérias e alteram sua permeabilidade, com conseqüente lise bacteriana (Kourie & Shorthouse, 2000). Muitos destes peptídeos, pelos vários tipos de membranas, incluídas aquelas constituintes de protozoários, bactérias, leveduras, fungos e células de mamíferos, têm pouca ou quase nenhuma afinidade específica (Brogden et al., 2003). Estudos estruturais destes peptídeos mostram que eles apresentam estruturas secundárias em α-hélice, folhas β que podem ou não ser estabilizadas por 2 a 3 pontes de sulfeto. Estes peptídeos também possuem altos teores de aminoácidos hidrofóbicos como triptofano, prolina e histidina (Brahmachary et al., 2004), os quais lhe conferem um caráter anfipático que lhe proporciona, por membranas bilipídicas, grande afinidade. Muitos destes peptídeos podem produzir poros em membranas. Isto permite sua penetração que leva à ruptura celular e perda do seu conteúdo citoplasmático (Shai, 2002). Peptídeos com uma seqüência menor de aminoácidos mostraram uma atividade antibacteriana

mais eficiente do que a dos de maior massa molecular (Sitaram & Nagaraj, 1999). Apesar de serem diferentes em sua estrutura primária e terciária, todos estes peptídeos dependem da presença de resíduos básicos para desenvolverem um mecanismo comum de atividade antibacteriana (Sitaram & Nagaraj, 1999)(Fig.2).

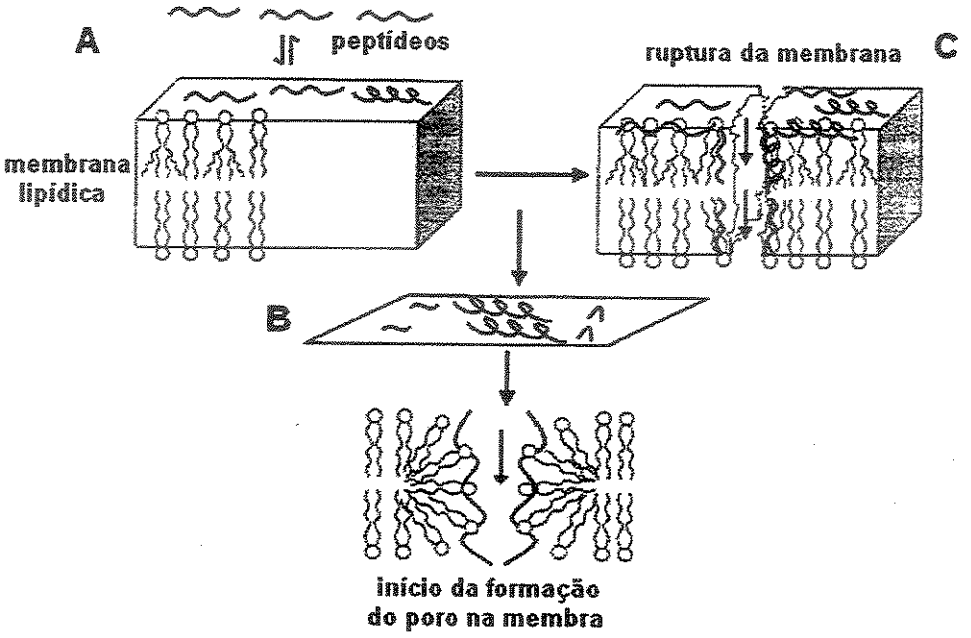

Figura 2. Modelo de ação dos peptídeos antibacterianos. A. Interação dos peptídeos com a membrana bacteriana. B. Início da formação do poro na membrana. C. Ruptura da membrana bacteriana (Sitaram & Nagaraj, 1999).

1.3-Veneno de serpentes como antimicrobianos

1.3.1-L-amino ácido oxidase (LAO)

Skarnes (1970) foi um dos primeiros a mostrar os efeitos antimicrobianos induzidos por LAO isolada do veneno total de *Crotalus adamanteus*. Stiles et al. (1991) também mostraram que duas LAOs isoladas do veneno total de *Pseudechis australis* mostraram uma forte atividade antimicrobiana tanto contra as bactérias Gram-positivas como Gram-negativas. Recentemente Tempone et al. (2001) mostraram que a LAO do veneno de *Bothrops moojeni* destruíram promastigotas de *Leishmania* spp "in vitro" e mostraram uma possível ação terapêutica contra a leishimaniose e outros parasitas. No trabalho realizado por Tempone et al. (2001) a atividade leishimanicida está associada à produção de água oxigenada (H_2O_2).

Experimentos posteriores mostraram que a catalase inibiu significativamente o efeito leishmanicida da LAO de *Bothrops moojeni*. Desta forma a atividade enzimática desta proteína pode ser extremante importante para a atividade antibacteriana.

1.3.2-Fosfolipase A_2 (PLA_2)

Buckland & Wilton (2000) fizeram uma correlação entre o aumento da expressão das PLA_2 secretórias do grupo II (presentes nos venenos de serpentes crotálicas e viperídicas e nas células humanas) com a progressão de infecções. Contudo a importância desta enzima no combate às infecções só foi claramente estabelecida com sua caracterização estrutural e molecular. Existem vários tipos de peptídeos antibacterianos produzidos por vertebrados. Estes têm, na membrana bacteriana, seu principal sítio de atividade (Ganz & Lehrer, 1998). Estes peptídeos têm preferência por interfaces aniônicas que interagem com os resíduos carregados positivamente como os resíduos de lisina e arginina (Grans & Lehrer, 1998). As PLA_2 secretórias classe II mostraram, devido à capacidade de hidrólise da membrana, habilidade de penetração de *Staphylococcus aureus*, o que foi confirmado por estudos de cinética enzimática realizados com *Micrococcus leteus* (Weirauch et al., 1996; Buckland et al., 2000). Estes também evidenciaram que essa atividade foi capaz de reduzir significativamente a sobrevivência das bactérias, pelos danos causados à integridade da membrana. Trabalhos realizados por Weinrauch et al. (1998) e Qu & Lehrer (1998), comprovaram que a atividade antibacteriana era claramente dependente da atividade enzimática das PLA_2. Experimentos realizados com quelantes de cálcio, como o ácido etilenoglicol-bis (β-aminoetileter)-N,N,N',N'-tetracético (EGTA) e o etilenodiamino tetracético (EDTA) ou agentes alquilantes, com o ρ-bromofenancil provocaram a inativação da atividade enzimática e preveniram a atividade antibacteriana das PLA_2. Estes estudos também mostraram a incapacidade de algumas PLA_2 em inibir ou lisar as bactérias, confirmaram que a atividade antibacteriana depende da capacidade das PLA_2 de penetrar em suas membranas cuja presença de cargas positivas mostraram-se cruciais para este evento (Fig.3). Experimentos realizados por PLA_2 secretória de coelhos mostraram que estas também foram capazes de interagir com bactérias agregadas em forma de grumos (Dominiecki et al., 1999).

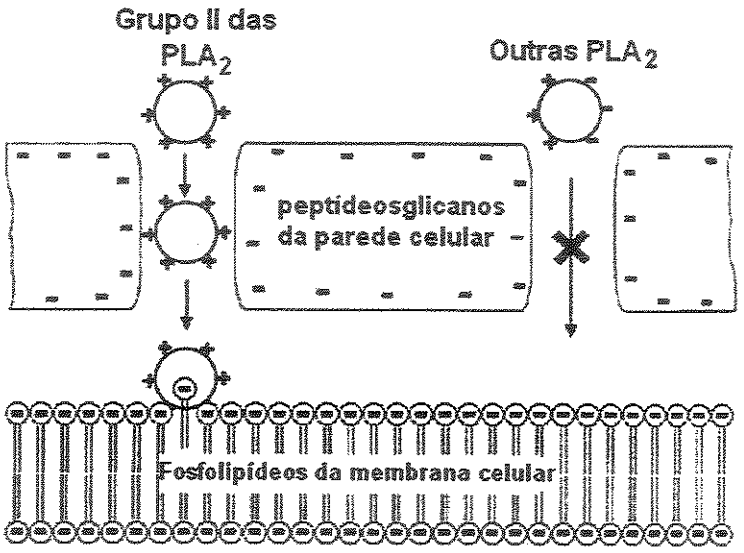

Figura 3. Mecanismo de ação da penetração das PLA_2 do Grupo II e de outras PLA_2 na membrana celular de bactérias Gram-positivas (Buckland & Wilton, 2000)

1.3.3-Crotaminas

As crotaminas, além de sua atividade miotóxica, também promovem a liberação de histamina em mastócitos, o que poderia explicar sua atividade analgésica. Foram recentemente descritos que seus efeitos estão relacionados à dose e ao tempo de exposição, podendo ser 30 vezes mais potente que a morfina (Mancin et al., 1997; Mancin et al., 1998).

A crotamina possui grande similaridade molecular com β-defensinas. Recentemente Siqueira et al. (2002) e Nicastro et al. (2003) mostraram que a crotamina possui estrutura secundária e terciária semelhante às β-defensinas e rede de membrana potencial positiva similar com presença de uma gama de aminoácidos básicos. A atração eletrostática e a interação hidrofóbica, incluindo hormônios e peptídeos citolíticos, são responsáveis por promover os estágios iniciais de adesão a polipeptídeos ativos de membrana incluindo hormônios e peptídeos citolíticos. Provavelmente o efeito antibacteriano da crotamina poderia estar relacionado à presença de cargas positivas, presentes em sua estrutura molecular. Vários trabalhos mostram que peptídeos catiônicos apresentam alta afinidade por lipopolissacarídeos (LPS), os quais são os principais componentes da membrana externa de bactérias Gram-negativas. Apesar da crotamina ser descrita como neurotoxina, recentemente Nicastro et al. (2003) mostraram que ela apresenta grandes similaridades estruturais com as β-defensinas, que são peptídeos antimicrobianos produzidos naturalmente por animais. Estes peptídeos

caracterizam-se pela presença de três pontes dissulfeto e de vários resíduos de aminoácidos básicos.

1.4-Bactérias como Modelo Biológico

1.4.1-Bactérias Fitopatogênicas

Bactérias são microorganismos unicelulares abundantes na natureza. Podem estar associadas à planta ou parte dela como residentes (relação temporária, sem causar doença), como epífitas (relação duradoura, sem causar doença), ou como patógenos (colonizando os tecidos e provocando doença). A grande maioria das doenças bacterianas de plantas é causada por várias bactérias, entre elas: *Agrobacterium*, *Clavibacter*, *Erwinia*, *Pseudomonas* e *Xanthomonas*. As bactérias são facilmente disseminadas através do ar, água, máquinas, insetos e animais. Normalmente penetram nos tecidos das plantas através de ferimentos ou aberturas naturais e necessitam para sua multiplicação de alta umidade para infecção no tecido e colonização na planta. São identificadas através de características de suas colônias em meios de cultura, por testes bioquímicos e tintoriais, por reação sorológica e pelo ciclo da planta hospedeira. Existem testes mais modernos de diagnose que utilizam técnicas de análise de DNA (Lopes & Santos, 1994).

A-Gênero *Xanthomonas*

A bactéria do gênero *Xanthomonas* é Gram-negativa, aeróbica obrigatória, com um único flagelo polar (raramente dois) e de coloração amarela pela presença do pigmento xantomonadina, característico do gênero. As colônias geralmente são amarelas brilhantes de 4-5 mm de diâmetro e viscosas (Bradbury, 1984). Deve ser armazenada liofilizada ou suspensa em solução de glicerina a 10% em freezer. É cultivada em vários meios, entre eles o Nutriente Agar. Seu período de incubação é de 72h a 28°C.

Xanthomonas axonopodis pv. passiflorae é patogênica ao maracujeiro, em diferentes regiões do Brasil. Essa bactéria apresenta ampla disseminação no território nacional onde esta fruta é cultivada comercialmente (Marques et al., 1994). Em condições naturais, ocorrem no maracujá amarelo, roxo e doce (Neto et al., 1984). Juntamente com outros patógenos, está envolvida com a anomalia denominada "morte precoce" do maracujazeiro, que reduz drasticamente seu período de exploração comercial e de vida. (Oliveira et al., 1986).

B-Gênero *Clavibacter*

A bactéria do gênero *Clavibacter* é Gram-positiva, aeróbica obrigatória, sem flagelo. As colônias são brancas devido à ausência de pigmento com 1-3 mm de diâmetro. Necessitam de meios ricos em nutrientes e 72h à 28ºC para crescerem (Bradbury, 1984).

Clavibacter michiganensis subsp. *michiganensis* causa principalmente nas regiões Sul, Sudeste e Centro-Oeste do Brasil o cancro-bacteriano, uma doença bastante freqüente em tomateiro estaqueado. Essa bactéria pode colonizar o tomateiro de forma sistêmica, quando atinge a região vascular, ou localizada, formando lesões superficiais. Um ataque severo pode provocar morte da planta (Lopes & Santos, 1994).

1.4.2-Bactérias Patogênicas

Os gêneros *Streptococcus* e *Enterococcus* englobam os cocos Gram-positivos, catalase negativos, de maior importância em medicina humana e animal. De modo geral, esses microorganismos são nutricionalmente exigentes, mas crescem bem em agar sangue e em caldo nutriente contendo gliose. São anaeróbicos facultativos. Alguns podem apresentar melhor crescimento em atmosfera rica em CO_2 (5%) ou em anaerobiose. O arranjo celular característico é em forma de cadeias, o que deu origem à denominação "estreptococo" Trabulsi & Alterthum (2004).

Streptococcus mutans

Streptococcus mutans pertence ao grupo viridans, que constitui um conjunto de microorganismos de caracterização pouco definida e padronizada que a dos demais estreptococos. Entre as suas principais características destaca-se a negatividade nos testes que auxiliam na identificação das outras categorias de estreptococos: não são beta-hemolíticos, não possuem antígenos dos grupos B ou D, não são solúveis em bile nem sensíveis à optoquina e não crescem em caldo contendo altas concentrações de sal; estão presentes na cavidade oral e devido à sua capacidade de sintetizar glicanas a partir de carboidratos têm um papel importante na formação da placa dental (Trabulsi & Alterthum, 2004).

2. OBJETIVOS

2.1 Isolar, purificar e caracterizar as proteínas e peptídeos da semente de *Lonchocarpus serieus* e do veneno de serpentes crotálicas (*Crotalus dirussus terrificus*, *Crotalus durissus cascavella*, *Crotalus durissus ruruima* e *Crotalus durissus collillineatus*), bothrópicas (*Bothrops pirajai*), corais (*Micrurus dumerilli carnicauda*);

2.2 Avaliar a atividade antibacteriana das frações purificadas em bactérias fitopatogênicas (*Xanthomonas axonopodis pv. passiflorae* e *Clavibacter michiganensis* subsp. *michiganensis*) e patogênica (*Streptococcus mutans*);

2.3 Caracterizar bioquimicamente, através do seqüenciamento do N-terminal e determinação da estrutura primária, as proteínas com atividade antibacteriana;

2.4 Avaliar através da análise ultra-estrutural das frações em contato com as bactérias, seus mecanismos de inibição;

3. TRABALHOS PUBLICADOS

Structural and Functional Characterization of Basic PLA_2 Isolated from *Crotalus durissus terrificus* Venom

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Structural and Functional Characterization of Basic PLA_2 Isolated from *Crotalus durissus terrificus* Venom

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The venom of *Crotalus durissus terrificus* was fractionated by reverse-phase HPLC to obtain crotapotins (F5 and F7) and PLA_2 (F15, F16, and F17) of high purity. The phospholipases A_2 (PLA_2s) and crotapotins showed antimicrobial activity against *Xanthomonas axonopodis pv. passiflorae*, although the unseparated crotoxin did not. The F17 of the PLA_2 also revealed significant anticoagulant activity, although for this to occur the presence of Glu 53 and Trp 61 is important. The F17 of the PLA_2 showed allosteric behavior in the presence of a synthetic substrate. The amino acid sequence of this PLA_2 isoform, determined by automatic sequencing, was HLLQFNKMLKFETRK NAVPFYAFGCYCGWGGQRRPKDATDRCCFVHDCCYEKVTKCNTKWDFYRYSLKSGY ITCGKGTWCKEQICECDRVAAECLRRSLSTYKNEYMFYPDSRCREPSETC. Analysis showed that the sequence of this PLA_2 isoform differed slightly from the amino acid sequence of the basic crotoxin subunit reported in the literature. The homology with other crotalid PLA_2 cited in the literature varied from 60% to 90%. The pL was estimated to be 8.15, and the calculated molecular weight was 14664.14 as determined by Tricine SDS–PAGE, two-dimensional electrophoresis, and MALDI-TOFF. These results also suggested that the enzymatic activity plays an important role in the bactericidal effect of the F17 PLA_2 as well as that of anticoagulation, although other regions of the molecule may also be involved in this biological activity.

KEY WORDS: Crotoxina; bacteria; enzyme; kinetics; sequencing; PLA_2.

1. INTRODUCTION

Crotoxin consists of a reversible protein complex composed of two nonidentical subunits, a basic phospholipase A_2 (PLA_2)[5] and an acidic nonenzymatic component known as crotapotin (Rubsamen *et al.*, 1971; Hendon and Fraenkel-Conrat, 1976). In isolation, both crotapotin and the PLA_2 are reported to be pharmacologically inactive, but crotapotin is thought to act as a chaperone protein for PLA_2 that increases the neurotoxic potency of this enzyme (Breithaupt, 1976; Habermann and Breithaupt, 1978).

Crotoxin, which is known for its presynaptic action, exists in various isoforms, which result from the random association of the PLA_2 with crotapotin (Faure *et al.*, 1991) after post-translational modification of a unique precursor of crotoxin or the expression of different mRNAs (Faure *et al.*, 1994). The principal role of the PLA_2 is that of digestion, but snake venom PLA_2 displays a wide range of other pharmacological activities, including neurotoxicity, myotoxicity, edema formation, hypotension, platelet aggregation, cardiotoxicity, and anticoagulant action (Lambeau *et al.*, 1996).

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[5] Abbreviations: Cdcasca, *Crotalus durissus cascavella*; Cdcolli, *Crotalus durissus collilineatus*; Cdt, *Crotalus durissus terrificus*; HPLC, high-performance liquid chromatography; PLA_2, phospholipase A_2.

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In the present research, various isoforms of crotapotin and PLA_2 from *Crotalus durissus terrificus* (Cdt) venom were purified, and their antimicrobial and anticoagulant activities were studied. The primary structure of these PLA_2 isoforms and several of their kinetic properties were then investigated.

2. MATERIALS AND METHODS

2.1. Isolation of PLA_2

Twenty milligrams of desiccated whole venom was dissolved in 750 μl of 0.1% (v/v) trifluoroacetic acid (solvent A). After clarification, the supernatant was applied onto a μ-Bondapack C18 column (0.78 cm × 30 cm) (Waters 991-PDA system). Fractions were eluted using a nonlinear gradient (0–66.5%, v/v) of acetonitrile (solvent B) at a constant flow rate of 2.0 ml/min. Chromatography of these fractions was monitored at 280 nm, and the resulting fractions were collected, lyophilized, and stored at −20°C.

2.2. Electrophoresis and MALDI-TOFF

Tricine–PAGE in a discontinuous gel and buffer system (Schagger and von Jagow, 1987) was used to estimate the molecular mass of the proteins. Two-dimensional electrophoresis was conducted as described by Anderson and Anderson (1991). MALDI-TOF mass spectrometry was performed using the highly purified proteins from HPLC; the F17 and PLA_2 were spotted onto a sample plate and introduced to the MALDI-TOFF mass spectrometer.

2.3. Reduction and Carboxymethylation of Protein and Digestion of F17

One milligram of purified PLA_2 was dissolved in 6 M guanidine chloride (Merck) containing 0.4 M Tris–HCl and 2 mM EDTA (pH 8.15); this was reduced with Dithiothreitol (DTT) and then carboxymethylated with ^{14}C-iodoacetic acid (Toyama *et al.*, 2000). Desalting was performed on a Sephadex G-25 column in 1 M acetic acid at 25°C, and the modified protein (RC-F17) was lyophilized. The reduced and carboxymethylated protein was digested with *Staphylococcus aureus* protease V8 for 16 h at 37°C using a 1:30 enzyme-to-substrate molar ratio; the reaction was stopped by lyophilization. The RC-F17 was also digested with clostripain for 8 hr at 37°C and then lyophilized again (Toyama *et al.*, 2000).

The digested products of this treatment were fractionated by reverse-phase HPLC using a Waters PDA 991 system and a C18 μ-Bondapack column. The elution of peptide peaks was made using a linear gradient consisting of 0–100% of acetonitrile in 0.1% trifluoroacetic acid (v/v).

2.4. Sequencing Procedure

The sequencing of the N-terminal was made using the reduced and carboxymethylated protein only, and the complete sequence was deduced using the peptides purified from the digested protein. The sequences were performed in the Procise f automatic sequencer. The phenylthyoidantoin amino acids were identified by comparing their retention times with those of the 20 phenylthyoidantoin amino acid standards. Peptides containing ^{14}C-CM-Cys were monitored by detecting radioactivity with the use of a liquid scintillation counter (Beckman model L-250).

2.5. Measurement of PLA2 Activity

PLA_2 activity was measured using the assay described by Cho and Kezdy (1991) and Holzer and Mackessy (1996), adapted for 96-well plates. The standard assay mixture contained 200 μl of buffer (10 mM Tris–HCl, 10 mM $CaCl_2$, 100 mM NaCl, pH 8.0), 20 μl of substrate, 20 μl of water, and 20 μl of PLA_2 in a final volume of 260 μl. After the addition of PLA_2 (20 μg), the mixture was incubated for up to 40 min at 37°C, with the absorbance being read at 10-min intervals. The enzyme activity, expressed as the initial velocity of the reaction (Vo), was calculated based on the increase in absorbance after 20 min. The inhibition of PLA_2 activity by crotapotin was determined by coincubating both proteins for 30 min at 37°C and then assaying the residual enzyme activity. The pH and temperature optima of the PLA_2 were determined by incubating the enzyme in six buffers of different pH (4–10) and in Tris–HCl buffer, pH 8.0, at 5° intervals from 15°C to 60°C. The optimum pH was determined using different buffer with a pH range from 4 to 10 and optimum temperature was determined using the Tris-HCl buffer; pH 8.0 assayed with temperature range from 15°C to 60°C. All assays were conducted in triplicate, and the absorbances at 425 nm were measured using a SpectraMax 340 multiwell plate reader (Molecular Devices).

2.6. 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 a venom fraction (0.1 ml) at 37°C for 3 min, and the clotting time after the addition of 0.1 ml of 0.25 M $CaCl_2$ was measured (Toyama *et al.*, 2000).

2.7. Bactericidal Effect

Xanthomonas axonopodis pv. passiflorae was harvested from fresh agar plates and suspended in distilled sterile water (A600 nm = 3×10^8 CFU/ml) solution. Aliquots of this suspension were diluted to 10^{-5} CFU/ml and were incubated with venom and toxins (250 μg/ml) for 60 min at 37°C, after which survival was determined with nutrient (Difco) plates (n = 5).

2.8. Statistical Analysis

The results are reported as the mean ± SEM of n experiments, as appropriate. The significance of differences between means was assessed by analysis of variance followed by Dunnett's test for comparison with the control group. The confidence limit for significance was set at 5%.

3. RESULTS

3.1. Purification of F17 of the PLA_2

Fractionation of Cdt venom by reverse-phase HPLC (Fig. 1a) showed the elution of seven main fractions: F2 and F3 were identified as crotamine isoforms, F5 and F7 showed crotapotin, and F15, F16, and F17 exhibited PLA_2 activity.

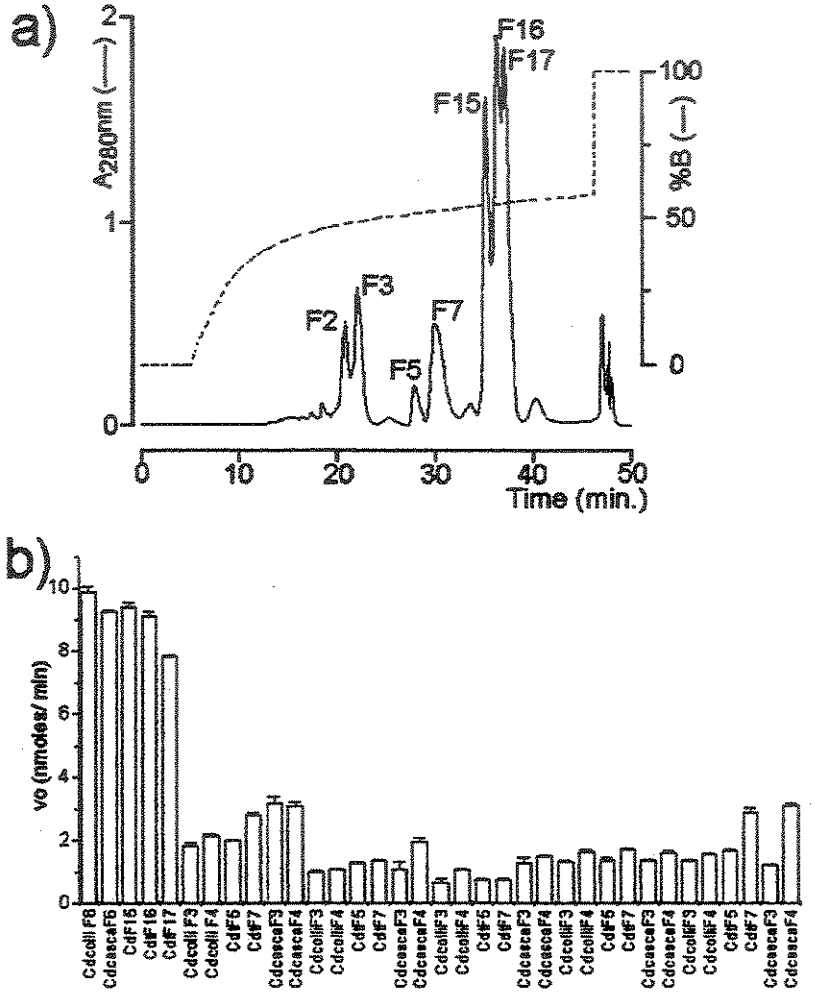

Fig. 1. (a) Elution profile of *Crotalus durissus terrificus* venom showing purification of the F17 phospholipase A_2 (PLA_2) isoform in a single step, using a μ-Bondapack C18 column. (b) Activity of PLA_2s isolated from venoms of *C. d. terrificus* (Cdt F15, F16, and F17), *C. d. collilineatus* (Cdcolli F6), and *C. d. cascavella* (Cdcasca F6) venoms. Each PLA_2 was incubated with various crotapotins for 30 min at 37°C, and the residual PLA_2 activity was then determined.

Together, F5, F7, F15, F16, and F17 represented approximately 50% of the whole venom, with F17 (PLA_2) being the main fraction. The F17 PLA_2 accounted for 35% of whole crotoxin, showing little enzymatic activity in comparison with the PLA_2 isolated from *Crotalus durissus collilineatus* (Cdcolli) and *Crotalus durissus cascavella* (Cdcasca). It appears as a single electrophoretic band with an estimated molecular mass in SDS–PAGE of approximately 15 kDa. The MALDI-TOFF spectrophotometric results showed that F17 has a molecular mass of 14.7 kDa. Its molecular homogeneity and basic composition were confirmed by two-dimensional electrophoresis that showed a single protein spot of 15 kDa with an estimated pL in 8.2.

All isolated crotapotins inhibited PLA_2 activity from different sources, but this activity was neither uniform nor constant, as it depended on the specific PLA_2 that was used. The PLA_2 from Cdcasca was the least sensitive to inhibition by the crotapotin isoforms isolated here, whereas PLA_2 from Cdcolli was the most sensitive. The most effective inhibition of PLA_2 activity was observed for the crotapotins F3 and F4 isolated from Cdcolli (Fig. 1b). The recalcification time for this PLA_2 was 30–40 min, with a protein concentration of 3–5 μg.

3.2. Enzymatic Composition

The PLA_2 activity of F17 was examined using the synthetic substrate 4-*nitro*-3-(octanoloxy)benzoic acid (Holzer and Mackessy, 1996). Under the conditions used, PLA_2 revealed sigmoidal behavior, mainly at low substrate concentrations and in the presence of crotapotin (Fig. 2a). However, in the presence of heparin, the activity of F17 increased and the *Km* decreased. The *Vmax*

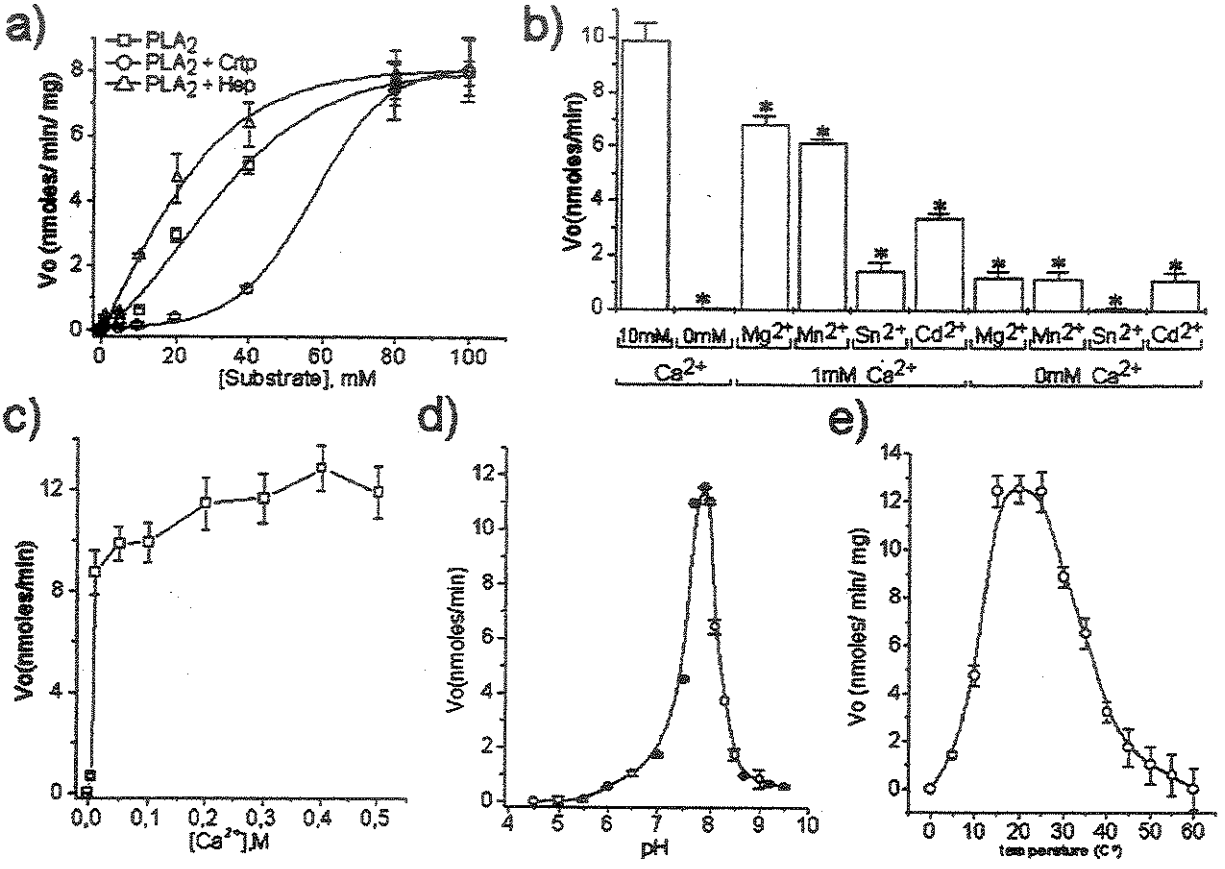

Fig. 2. (a) Effect of substrate concentration on the kinetics of F17 in the absence and presence of crotapotin (Crtp) and heparin (Hep). (b) and (c) pH and temperature optima for PLA_2 activity. (d) Influence of Ca^{2+} on PLA_2 activity. (e) Influence of ions on PLA_2 activity and crotapotin inhibition. The results are the mean ± SEM of three determinations.

was estimated to be 8.2 nmol/min/mg, and the K_m was 31.2 mM. Maximum enzymatic activity occurred at 10–30°C (Fig. 2b), and the optimum pH was 7.9 (Fig. 2c).

In the presence of 2 mM Ca^{2+}, the F17 PLA_2 isoform caused a significant increase in catalytic activity, which increased only slightly up to the maximum, 10–20 mM Ca^{2+}.

Although a certain activity is present with only 1 mM Ca^{2+} (Fig. 2d), this limited amount of Ca^{2+} is sufficient for the addition of Mn^{2+} or Mg^{2+} (10 mM) to be effective as an enzymatic cofactor.

The addition of Zn^{2+} and Cd^{2+} (10 mM), however, resulted in inhibition. The replacement of the 10 mM Ca^{2+} with 10 mM Mg^{2+} or Cu^{2+} reduced the activity to levels similar to those found in the absence of Ca^{2+} (Fig. 2e).

3.3. Amino Acid Sequence Determination

The F17 PLA_2 was reduced and carboxymethylated as determined by Toyama *et al.* (2000). It was then subjected to analytical reverse-phase HPLC, and the purified fraction. Different samples of the purified protein were separately digested with Sv8 and clostripain resulted in seven major peaks, each, in reverse-phase HPLC, whereas treatment with CNBr gave only two major peaks.

The F17 PLA_2 revealed a sequence of 122 amino acids, with approximately 90% homology with crotoxin B and Mojave toxin, although this similarity fell to around 60% compared with the PLA_2 of other snake toxins (Fig. 3).

The digestion products used to determine the amino acid sequence are shown in Fig. 4.

3.4. Antimicrobial Action of Crotoxin and PLA_2

Both crotoxin and the whole venom showed the same antimicrobial activity against *X. axonopodis pv. passiflorae*. Similar values were found for the F15 isoform, which inhibited approximately of the bacterial growth (CUF; Fig. 5). Both F16 and F17 are shown to be more efficient antimicrobial agents in this experiment, with each inhibiting approximately 65% of such growth.

4. DISCUSSION

4.1. Purification and Biological Properties of F17

The purification protocols used here were simple, quick, efficient, and reproducible, yielding pure crotapotin and PLA_2 isoforms from the venom of Cdt. The Cdt F17 PLA_2 does not reveal the classic Michaelis-Menten behavior described by Breithaupt *et al.* (1976), which is in agreement with the results of Beghni *et al.* (2000) for the PLA_2 of Cdcasca. Our results also suggest that this PLA_2 isoform of Cdt venom behaved allosterically, as can be seen in the presence of crotapotin and heparin.

This component revealed great activity at a weakly alkaline pH (7.5–8.0), and this activity was enhanced by the presence of a low Ca^{2+} concentration, which is in agreement with the results of Shiomi *et al.* (1998). The partial or total replacement of Ca^{2+} by other divalent cations resulted in lower or no PLA_2 activity of F17 isoform, showing dependence for Ca^{2+} like other D49 $PLAs_2$ described by Pieterson *et al.* (1974) and Verheij *et al.* (1980).

According to Selistre de Araújo *et al.* (1996), the action of D49 PLA_2 can be classified as only neurotoxic or as a combination of neurotoxic and myotoxic. Following this classification proposed by Selistre de Araújo *et al.* (1996), the crotoxin B and Mojave toxin could be classified in the latter. We expected the PLA_2 (F17) fraction investigated here to reveal similar results as those found for crotoxin B and Mojave toxin, because F17 reveals high sequencial identity of 85–90% with Mojave toxin and crotoxin B previously characterized by Aird and Kaiser (1985) and Aird *et al.* (1989). However, this was not the case; it did not exhibit any evident neurotoxicity or myotoxicity, although it showed a strong inflammatory activity.

The amino acid sequence of the F17 showed the absence of the lysine-rich region, which, according to some authors, is responsible for heparin binding (Lomonte *et al.*, 1994; Selistre de Araújo *et al.*, 1996). According to Gutierrez and Lomonte (1995), this region is the principal one involved in myotoxicity, which could explain the absence of such activity to F17 isoform.

The discrepancy between our results regarding neurotoxicity or myotoxicity and those previously reported in the literature may be related to the degree of purity of the PLA_2 obtained, although it is also possible that there are genetic differences between the rattlesnake species in Brazil.

Our enzymatic results showed that heparin enhanced the catalytic activity of F17, probably acting as a positive modulator, which stimulates PLA_2 activity, despite the lack of a C-terminal Lys-rich domain of this enzyme. This suggests that heparin is binding to the F17 PLA_2 in some other region of the enzyme.

Kini and Evans (1987, 1989) have shown that the regions between residues 54 and 77 in the PLA_2 may be

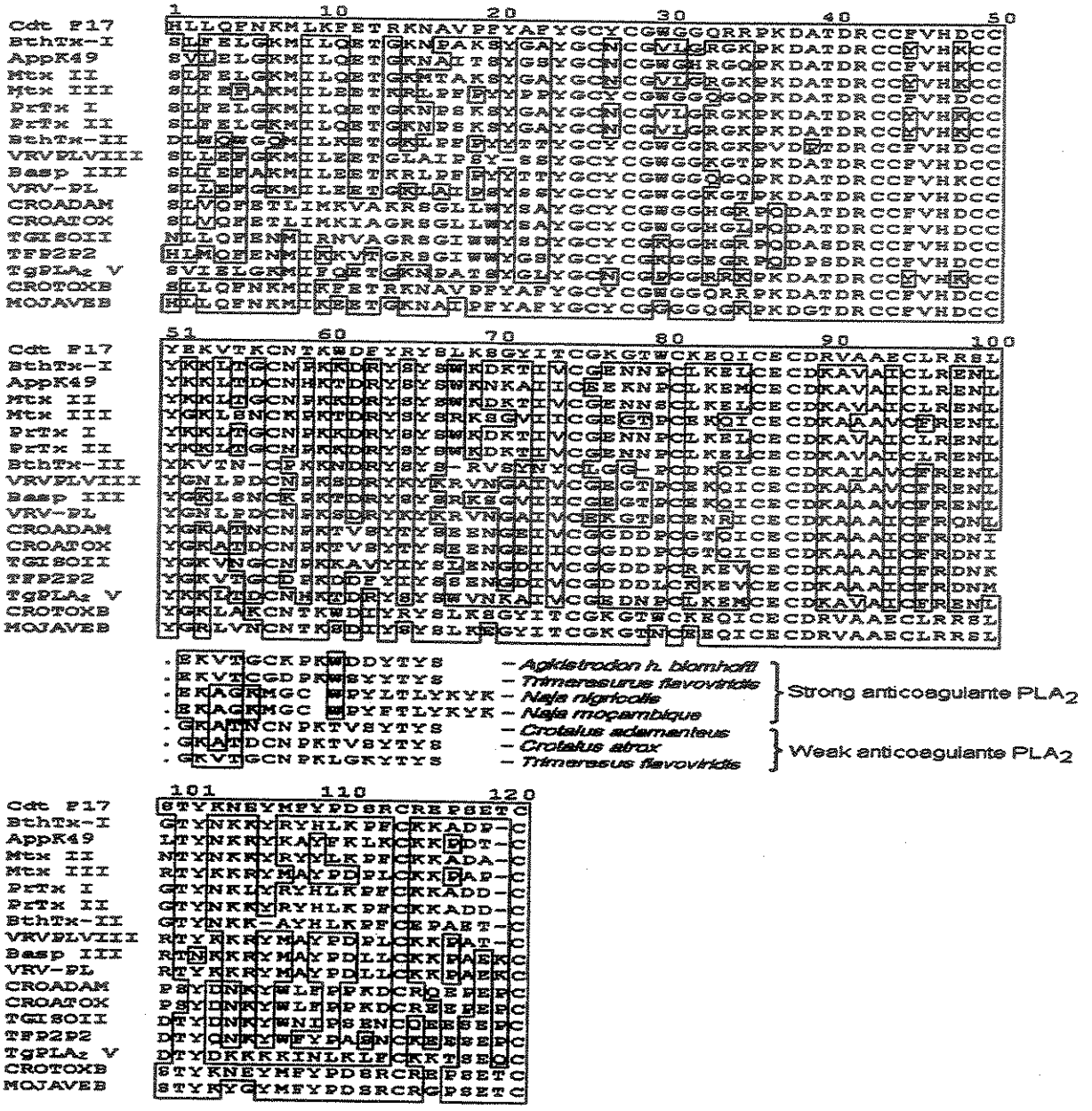

Fig. 3. The amino acid alignment of the F17 *phospholipase* A_2 (PLA_2) compared with other PLA_2s obtained from the BLAST protein databank (PubMed, MEDLINE): Cdt *(Crotalus durissus terrificus)*, BthTx-I and BthTx-II (botropsitoxin I and II from *Bothrops jararacussu*), App *(Agkistrodon piscivorus piscivorus)*, Mtx II and Mtx III (myotoxin I and II from *Bothrops asper*), PrTx I and PrTx II (piratoxin I and II from *Bothrops pirajai*), VRPV PLVIII (PLA_2 VIII from *Vipera russeli*), VRV (PLA_2 isoform from *Vipera russeli*), CROADAM (PLA_2 from *Crotalus adamanteus*), CROATROX (PLA_2 from *Crotalus atrox*), TGISOII (PLA_2 isoform II from *Trimeresurus gramineus*), TFP2P2 (PLA_2 from *Trimeresurus flavoviridis*), TgPLA2 V (PLA_2 isoform V from the *Trimeresurus gramineus*), CROTOXINB (basic unit of crotoxin from *Crotalus durissus*), and MOJAVEB (PLA_2 from the *Crotalus scutulatus scutulatus*).

the active site for anticoagulant activity and have proposed a model to explain this action by venom PLA_2. Although anticoagulant activity of the basic PLA_2 is not necessarily associated with the positive charges or overall molecular arrangement of these enzymes (Zhao *et al.*, 2000), some residues in the anticoagulant region, partic-

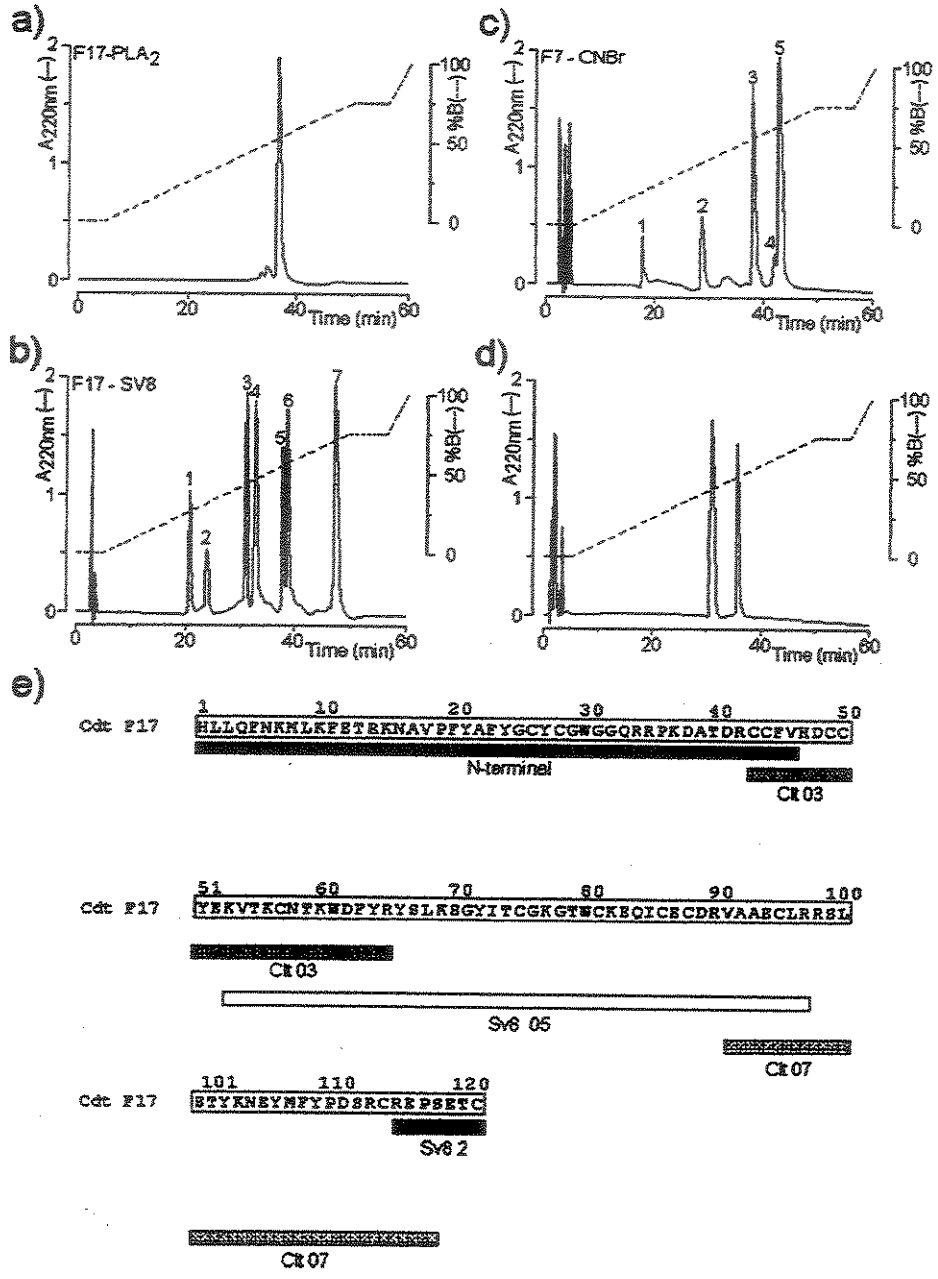

Fig. 4. (a) Elution profile of reduced and carboxymethylated F17. (b) Elution of the peptides produced by digestion of RC-F17 with protease V8 from *Staphylococcus aureus*. (c) Clostripain. (d) CNBr. Reverse-phase HPLC was done on a μ-Bondapack C18 colum. (0.39 cm × 30 cm) using a discontinuous acetonitrile gradient at a flow rate of 1.0 ml/min. (e) Digestion fragments used to determine the amino acid sequence of the F17 phospholipase A_2.

ularly Glu 53 and Trp 70, may be important for this activity as described by Carredano *et al.* (1998) and Paramo *et al.* (1998).

The crotoxin B chain was previously characterized as a strong anticoagulant PLA_2, but it does not have Glu 53 and Trp 70. However, the PLA_2 isoform purified here

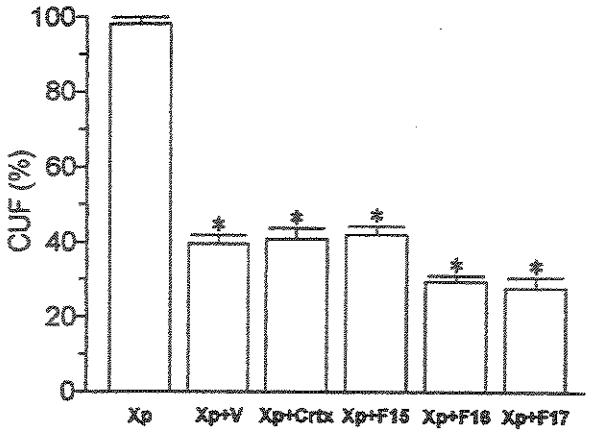

Fig. 5. Antibacterial activity of *Crotalus durissus terrificus* whole venom, crotoxin, F15, F16, and F17 phospholipases A_2, with a significantly reduced number of live bacteria, suggesting bacterial growth inhibition ($P < 0.05$).

(F17) did possess Glu 53 and, based on our sequence analysis, it seems possible that the Trp 61 in the case of F17 plays an important role in the anticoagulation activity showed by F17.

Crotapotin F7 inhibited the enzymatic and anticoagulant activities of F17. Zhao *et al.* (2000) showed that the strong anticoagulant action of certain PLA_2s is eliminated completely after treatment with p-bromophenacylbromide, although no significant changes in the arrangement of the PLA_2 molecule are revealed by crystallographic comparison with strongly anticoagulant PLA_2. Although the results suggest that the anticoagulant activity of F17 is not dependent on the presence of Glu 53 but should be dependent on Trp 61 as well as the catalytic fraction of the PLA_2 is required for full anticoagulant activity of this protein.

F17 alone exhibits a potent antibacterial activity compared with whole crotoxin, and the antibacterial activity of F17 was similar to that found by Paramo *et al.* (1998) and Soares *et al.* (2001). According to these authors, the PLA_2 from *Bothrops asper* venom, which is catalytically inactive, reveals potent antibacterial activity, an effect that is not dependent on enzymatic activity. The aromatic and basic amino acid residues located in the C-terminal region position 115–129 of this PLA_2 create this effect. However, in the case of F17, it is the catalytic activity that is important for antimicrobial activity, as shown when the PLA_2 is complexed with crotapotin.

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Structural, enzymatic and biological properties of a new PLA_2 isoform from *Crotalus durissus terrificus* venom

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Structural, enzymatic and biological properties of new PLA_2 isoform from *Crotalus durissus terrificus* venom

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Abstract

We isolated a new PLA_2 from the *Crotalus durissus terrificus* venom that designated F15, which showed allosteric behavior with a V_{max} of 8.5 nmol/min/mg and a K_m of 38.5 mM. The incubated heparin salt of this isolated F15 act a positive allosteric effector by increasing the V_{max} to 10.2 nmol/min/mg, with decreasing the V_{max} value to 20.5 mM. The crotapotin, on the other hand acts as a negative allosteric effector by increasing the V_{max} values to 58.4 mM. F15 also showed high calcium dependence for its catalysis similar to that found for other PLA_2 enzymes isolated from these snake venoms. The replacement of calcium by other divalent ions such Mg^{2+}, Mn^{2+}, Cd^{2+}, Sn^{2+} and Cu^{2+} resulted in lower enzymatic activity. The optimum pH and temperature for the enzyme was 8.5 and 18 °C, respectively.

F15 alone showed moderate neurotoxic activity in isolated mouse phrenic nerve diaphragm in comparison to other strong myotoxic PLA_2 such as bothropstoxin-I (BThtx-I), but this activity was highly neurotoxic in a chick biventrer cervis preparation, whereas BthTx-I did not reveal this high neurotoxicity. This new protein showed a high bactericidal effect against both Gram-negative and Gram-positive bacterial strains.

F15 contained 122 amino acid residues, with a primary structure of: HLLQFNKMIKFETRKNAVPFYAFYG-CYCGWGGQRRPKDATDRCCFVHDCCYGKLTKCNTKWDIYRYSLKSGYITCGKGTWCKEQICECDRVAAECLRRSL-STYKNEYMFYPKSRCRRPSETC. Its molecular mass and isoeletric point were 14.5 kDa and 8.85, both estimated by two dimensional electrophoresis. The amino acid sequence of the F15 revealed high sequence homology with F16 and F17. F15 and the other PLA_2s revealed highly conserved amino acid sequences principally for calcium binding loop and active site helix. F15 also showed a high homology with the lysine-rich region of myotoxic PLA_2.

Keywords: Bactericidal; *Crotalus durissus terrificus*; Neurotoxin; PLA_2; Rattlesnake venom

1. Introduction

Crotoxin, the main neurotoxin in the venom of the South American rattlesnake *Crotalus durissus terrificus*, accounts for approximately 50% of the dry weight of the venom and is responsible for most of the symptoms observed following envenomation. Crotoxin is a reversible protein complex consisting of a basic phospholipase A_2 (PLA_2) and an acidic, non-enzymatic component known as crotapotin (Habermann and Breithaupt, 1978). The crotapotin component, which is pharmacologically inactive, acts as a chaperon protein for PLA_2 and increases the biological activity of this enzyme (Breithaupt, 1976; Habermann and

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Breithaupt, 1978). Pharmacologically, crotoxin exerts pre- and post-synaptic actions, indicating the presence of various interactions with excitable cells (Bon et al., 1979). In the present study, a new PLA_2 isoform from *C. d. terrificus* venom was identified and its primary structure is determined as well as several of its kinetic properties. The neurotoxic and antimicrobial action was also investigated.

2. Methods, results and discussion

Snake venoms from the cities of Ribeirão Preto and Batatais (state of São Paulo) and city of Curitiba (state of Pararâ) was analyzed with reverse phase HPLC, using a μ-Bondapack C18 column ($0.3 \times 30\ cm^2$, Waters). The chromatographic analysis of these venoms showed various fractions differences in the venom composition, but in all of the venom examined, F15 appeared as a common fraction, representing around 12% of the whole venom. PLA_2 activity was measured using the assay method described by Holzer and Mackessy (1996), modified for 96-well plate. The standard assay mixture contained 200 μl of buffer (10 mM Tris–HCl, 10 mM $CaCl_2$, 100 mM and NaCl, pH 8.0), 20 μl of substrate, 20 μl of water and 20 μl of PLA_2 giving a final volume of 260 μl. The F15 showed allosteric behavior with a V_{max} of 8.5 nmol/min and a K_m of 38.5 mM.

The addition of heparin 500 U induced a significant increase of the catalysis of F15 by increasing the V_{max} and decreasing the K_m values to 10.2 nmol/min/mg and 20.5 mM, respectively. In the presence an equal molar of crotapotin V_{max} decreased to 8.2 nmol/min while the K_m increased to 58.4 mM (Fig. 1(a)). F15 enzymatic activity was completely dependent on Ca^{2+} and the complete replacement of this ion by other divalent ions did not show a significant enzymatic activity; in the presence of a lower calcium concentration (1 mM) however, the presence of Mg^{2+}, Mn^{2+} or Cd^{2+} resulted in lower activity (Fig. 1(b)). F15 showed maximum catalytic activity at a pH of 7.8–8.5 and a temperature of 18 °C (Fig. 1(d) and (e)).

The PLA_2 from *C. d. terrificus* venom is a typical PLA_2, since it hydrolyzes synthetic substrates at position 2 and preferentially attacks substrates in their micellar state

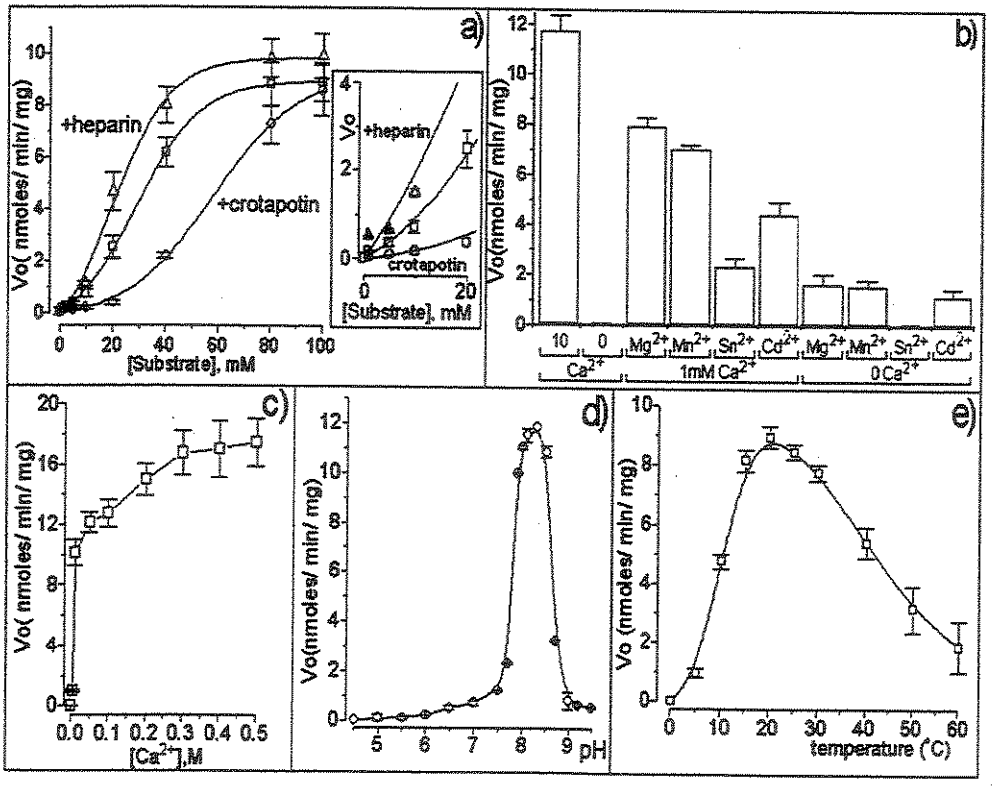


Fig. 1. (a) Effect of substrate concentration on the kinetics of F15 PLA_2 in the absence and presence of crotapotin (Crtp) and heparin (Hep), (b) influence of ions on PLA_2 activity and (c) influence of Calcium ion on PLA_2 activity, (d) and (e) show pH and temperature optima of the PLA_2 activity.

(Breithaupt, 1976; Holzer and Mackessy, 1996). Breithaupt (1976) has reported that this enzyme shows classic Michaelis–Menten behavior against micellar substrates. The PLA_2 activity of the F15 identified here was investigated using unmodified protein and showed allosteric behavior in the presence of with of synthetic substrates. This allosterism was more marked in the presence of heparin, which acted as a positive allosteric modulator, and crotapotin, which served as negative allosteric modulator. Similar allosterism has been noted in other PLA_2s (Oliveira et al., 2001). A strict requirement for Ca^{2+} is characteristic of many PLA_2 s (Pieterson et al., 1974), but the complete replacement of this ion by other ions such as Cu^{2+} and Zn^{2+} significantly reduces the enzymatic acitivity. Full enzymatic activity was restored when these ions were incubated in the presence of calcium (Breithaupt, 1976). Like other PLA_2, F15 was relatively resistant to heat, acid and urea, but was inactivated at pH >9; the highest activity occurred at pH 7–8 and 20–40 °C (Beghini et al., 2000; Oliveira et al., 2001).

Mouse phrenic nerve-diaphragm preparation was prepared as described by Bülbring (1946) and suspended in a 10 ml organ bath, maintained under a tension of 2 g in Tyrode

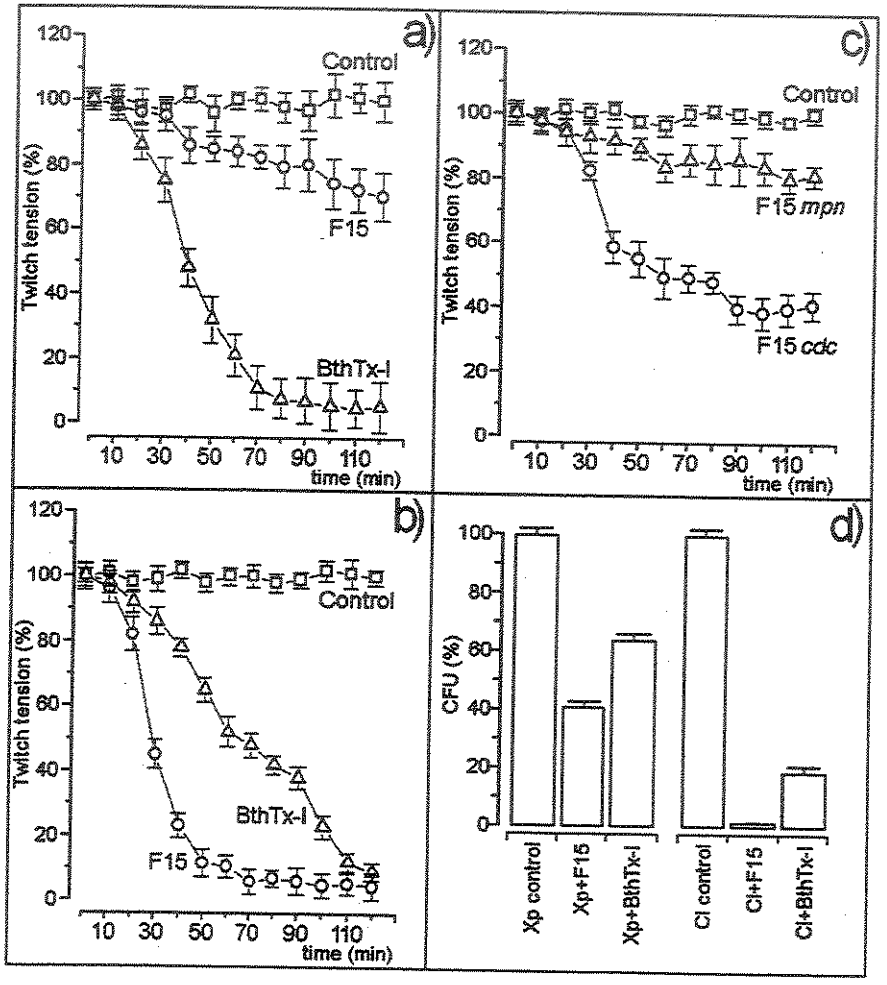
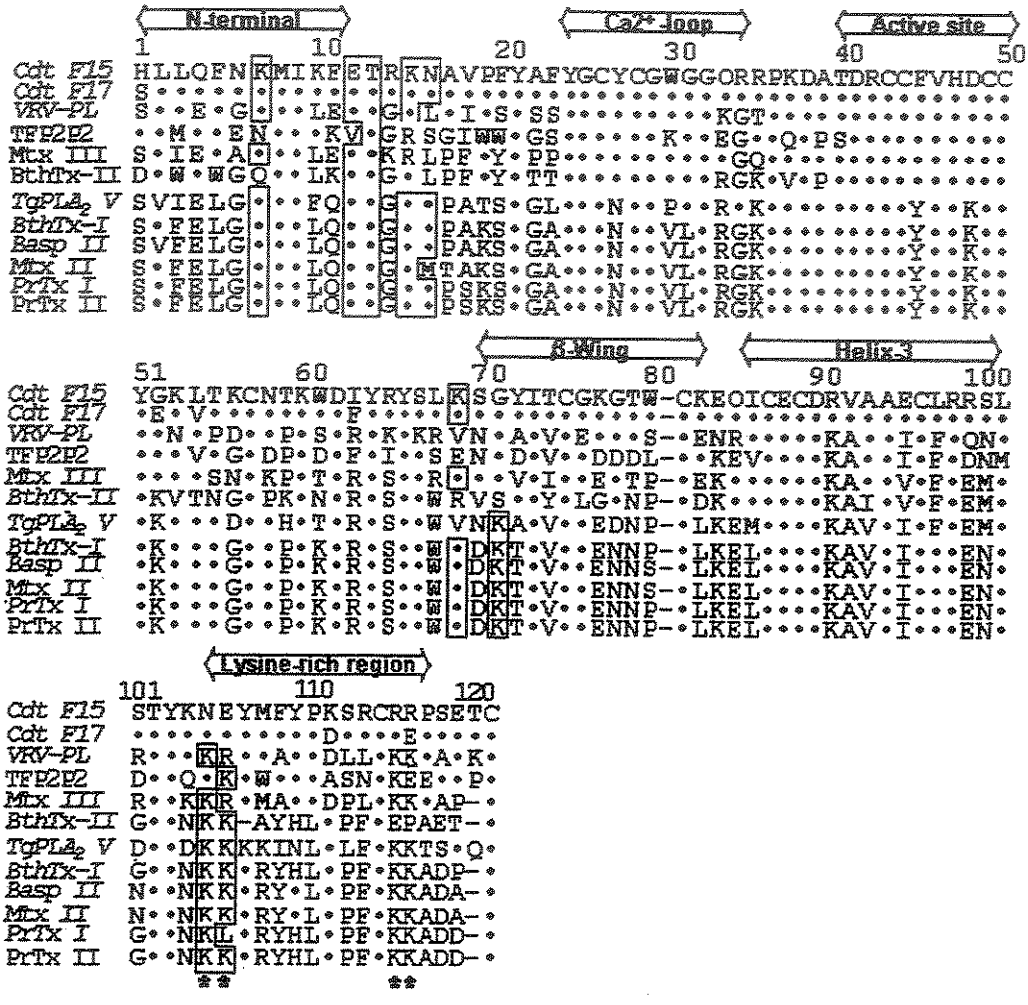


Fig. 2. (a) Neurotoxic activity of F15 and BthTx-I in mouse phrenic nerve-diaphragm preparations, which were allowed to stabilize for at least 20 min before the addition of a single dose (20 μg/ml) of each of the toxins followed by incubation for 120 min ($n = 4$). (b) Neurotoxic activity of F15 and Bthtx-I in the chick biventer cervicis preparation, which were allowed to stabilize for at least 20 min before the addition of a single dose (10 μg/ml) of each of the F15 or BthTx-I. (c) Neurotoxic effect of isolated F15 incubated with heparin on the mouse phrenic nerve-diaphragm preparation (F15 mpn) and in the chick biventer cervis (F15 cbc). (d) Antimicrobial activity of the isolated F15 and Bthtx-I on *Xanthomonas axonopodis psv passiblorae* (Xp) and *Clavibacter michiganensis michiganensis* (Cl).

Table 1
Amino acid sequence and molecular homology of F15 PLA_2 with other PLA_2



solution (pH 7.4, 37 °C). Stimuli delivered by a GRASS S4 electronic stimulator were applied through bipolar electrodes attached to the nerve (0.1 Hz, 0.2 ms, supramaximal stimuli, 4 × threshold), and the isometric muscle tension was recorded by a force displacement transducer coupled to a physiograph. The preparations were prepared and allowed to stabilize for at least 20 min before the addition of a single dose (20 μg/ml) of the venom fractions. In this treatment, the isolated F15 PLA_2 showed no significant blockage effect ($n = 4$, 23 ± 3.5 blockage in 120 min), whereas isolated myotoxin BthTx-I showed a strong neurotoxic effect ($n = 4$, 96.7 ± 3.2 blockage in 90 min) (Fig. 2(a)).

Chick biventer cervicis preparation was prepared, as described by Ginsborg and Warriner (1960); it was mounted under a tension of 1 g in a 4 ml organ bath containing Krebs solution (pH 7.5, 37 °C). Contractures in the presence of exogenously applied acetylcholine (Ach, 55–110 μM for 60 s) and KCl (50 mM for 120–130 s) was obtained in the absence of nerve stimulation prior to the addition of PLA_2s (F15 or BthTx-I) and end of the experiment, as a further test for the presence of myotoxic and neurotoxic activities. In this experiment the F15 (10 μg/ml) eliminated the twitches evoked by indirect electrical stimulation, after a lag period of 20 min. The times required to achieve 50 and 80%

paralysis for the F15 were 28.4 ± 4.3 min and 34.4 ± 7.4 min, respectively, whereas for the BthTx-I they were 56.5 ± 8.8 min and 98.4 ± 8,7 min (Fig. 2(b)). In all experiments the contractures produced by Ach but not by KCl, were inhibited after exposure to the venom. These results suggest that both PLA_2 exhibit a postsynaptic neurotoxin activity.

In both the mouse phrenic nerve-diaphragm preparation and chick biventer cervicis preparation, isolated PLA_2 treated with heparin resulted in a significant decrease of neutoxic activity (Fig. 2(c)). F15 exhibits specific neurotoxic activity (as other D49 neurotoxic PLA_2), in the presence of acid subunits as seen in the mouse phrenic nerve-diaphragm but also independent of this subunit as seen in the chick biventer cervis preparation. In the agreement with our results, we conclude that toxicity, neurotoxicity or myonecrosis of F15 is not dependent on the presence of other molecules (as crotapotin) and probably involve mechanisms similar to those observed for bothropic PLA_2 (Toyama et al., 2001); the C-terminal region could play an important role for these activities because treatments of this protein with heparin significantly decrease the neurotoxic effects, as is the case of bothrops venom (Toyama et al., 2001; Gambero et al., 2002).

The bactericidal activity of BthTx-I was less than that of isolated F15 PLA_2, with the latter reducing the bacterial growth rate of *Xanthomonas axonopodis psv passiflorae* (Gram negative) to around 58.2%, while the BthTx-I reduced only 28.3%. When used to treat *Claribacter michiganensis michiganensis* (Gram positive), F15 reduced bacterial growth at 98%, whereas BthTx-I reduced it only 78.8% (Fig. 2(d)). This bactericidal effect was greater than that of Lys 49 or native crotoxin (Fig. 2(d)), which suggests that the catalytic activity of this enzyme play an important role in bacterial inhibition. Other biological activities such as neurotoxicity, myonecrosis, and oedematogenic effects seem to be independent of the catalytic activity; however, other molecular regions may also play a role in the toxic effects exerted by this PLA_2.

N-terminal sequencing using the reduced and carboxymethylated protein was conducted using a Procise f automatic sequencer as well as that of peptides purified from the protease digests. Phenylthioydantoin (PTH) amino acids were identified by comparing their retention times with those of the 20 PTH amino acid standards. Peptides containing ^{14}C-CM-Cys were monitored for radioactivity by using a liquid scintillation counter (Beckman model L-250).

The complete amino acid sequence of the F15 is shown in Table 1. Clostripain digestion of F15 rises to multiple peptides, only two of which were uniquely informative. The sequences of these peptides were DATDRCCFVHDCCYGKLTKCNTKWDIYR (clostripain 6) and YGKLTK CNTKWDIYRYSLKSGYITCGKGTWCKEQICECDRVAAECLRRSLSTYK (clostripain 9). The endoproteinase Glu-C (SV8 protease) resulted in two peptides that were important for determining the primary structure of F15, namely, RCCFVHDCCYGKLTKCNTKWDIYRYSLKSGYITCGKGTWCKE (SV8 5) and CLR RSLSTYKNEYMFYPKSRCRRPSE (SV8 7). Cyanogen bromide cleavage resulted in three peptides (CNBr 01, CNBr 02 and CNBr 03), the most significant being CNBr 01, with the amino acid sequence FYPKSRCRRPSETC. F15 showed high sequence homology with F16, F17, crotoxin B, Mojave B and VRV-PL (95%); it also showed high strucutural homology with other PLA_2s from the snake venom in the Ca^{2+} binding loop and in the active site loops (Table 1).

Similar to other myotoxic PLA_2s from the bothropic venoms, F15 also had a positively charged and hydrophilic C-terminal region; however F17 exhibits two important modifications in this region. Lys (111) and Arg (111) were found in the F15 or other basic myotoxins, which were replaced by Asp (111) and Glu(116), respectively.This region seems to be important in accounting for the biological action and interaction of these PLA_2 with the cell membrane and the presence of basic amino acid residues play important role for this activities (Toyama et al., 2000; Soares et al., 2000; Lee et al., 2001; Oliveira et al., 2001).

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Structural and biological characterization of a crotapotin isoform isolated from *Crotalus durissus cascavella* venom

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Structural and biological characterization of a crotapotin isoform isolated from *Crotalus durissus cascavella* venom

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Abstract

Envenoming by *Crotalus durissus* subspecies leads to coagulation disorders, myotoxicity, neurotoxicity and acute renal failure. The most serious systemic alteration and primary cause of death after snakebite is acute renal failure. In this work, we isolated crotapotin, an acid component (Crtp) of crotoxin from *Crotalus durissus cascavella* venom and we investigated its bactericidal and pro-inflammatory activities as well as its renal effects in rat isolated perfused kidneys. Crtp was bactericidal to the Gram-negative species *Xanthomonas axonopodis pv. passiflorae*, but was less effective against the Gram-positive *Claribacteri* ssp, probably because of differences in the cell wall composition. Crtp showed a high amino acid sequence homology with other Crtps described in the literature (around of 90%) and its A and B chains had high conserved regions corresponding to the calcium-binding loop, catalytic site and helix 3 of PLA_2. The Crtp showed moderate pro-inflammatory activity and increased significantly the inflammation evoked by PLA_2 when co-injected or co-incubated with PLA_2. The renal parameters evaluated included the perfusion pressure (PP), renal vascular resistance (RVR), urinary flow (UF), glomerular filtration rate (GFR) and percent of sodium tubular transport (%TNa^+). Crotapotin (5 μg/ml) significantly increased the PP and RVR, whereas the GFR, UF and %TNa^+ were unaffected. These results suggest that crotoxin is the main venom component responsible for nephrotoxicity and crotapotin contributes little to this phenomenom. The biological and bactericidal actions of Crtp also suggest that this protein may have functions other than simply acting as a chaperone for PLA_2.

Keywords: *Crotalus durissus cascavella*; Crotapotin isoform; PLA_2; Venom; Rattlesnake; Neurotoxins

1. Introduction

PLA_2 are classified into three groups, based on their primary structure, group I includes mammalian pancreatic and Elapidae snake venom PLA_2, group II is represented by the mammalian non-pancreatic and Viperidae snake

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venom PLA_2 and group III consists of bee and lizard venom PLA_2 (Dennis, 1994). PLA_2 can also be classified as monomeric, homomultimeric and heteromultimeric. The last of these groups includes crotoxin, the principal neurotoxin of *Crotalus durissus terrificus* (South American rattlesnake) venom. Crotoxin consists of two non-identical subunits, a weakly toxic basic PLA_2 subunit and a non-enzymatic subunit (crotapotin). Crotapotin acts as a chaperon protein for PLA_2 to increase the neurotoxicity and decrease the catalytic activity of this enzyme (Soares et al., 2001). Thus, crotapotin is a natural inhibitor of crotalic and other PLA_2.

In addition to their normal digestive action, a wide range of pharmacological activities, such as neurotoxicity, myotoxicity, edema formation, platelet aggregation, cardiotoxicity and anticoagulant action have been attributed to PLA_2 (Soares et al., 2001). These enzymes also affect cell signaling, proliferation and migration and have an antimicrobial action (Lambeau and Lazdunski, 1999; Valentin and Lambeau, 2000). We reported that the venom of *Crotalus durissus cascavella*, a subspecies of *C.durissus* common in northeastern Brazil, causes renal lesions by a direct action on tubule and glomerular cells (Martins et al., 1998). In the present study, we investigated the effects of crotapotin, obtained from crotoxin isolated from *C.d. cascavella* venom on renal function in the isolated rat kidney. The antimicrobial, inflammatory and enzyme inhibiting activities of crotapotin were also evaluated.

2. Materials and methods

2.1. Venom, chemicals and reagents

C.d. cascavella venom was a gift from the Regional Snake Laboratory of Fortaleza (LAROF), Ceará. PLA_2 of *Crotalus durissus collilineatus* and *Crotalus durissus terrificus* PLA_2 were previously purified on the Protein Chemistry Laboratory (Department of Biochemistry, UNICAMP). All chemicals and reagents used in this work were of analytical or sequencing grade.

2.2. Reverse phase HPLC

Twenty milligrams of desiccated venom were dissolved in 750 μl of 0.1% (v/v) trifluoroacetic acid (solvent A). The resulting solution was clarified by centrifugation at 10,000 rpm for 3 min and the supernatant applied to a C18 μ-Bondapack column (0.78 cm × 30 cm) (Waters 991-PDA system). The column was eluted with a linear gradient (0–66.5%, v/v) of acetonitrile (solvent B) at a flow rate of 2 ml/min, and absorbances were monitored at 280 nm. The fractions were collected manually, lyophilized and stored at −20 °C.

2.3. PLA_2 activity measurement

PLA_2 activity was measured as described by Cho and Kezdy (1991) and Holzer and Mackessy (1995). Substrate 2.98 mM (4-nitro-3-(octanoyloxy)-benzoic acid) was incubated with 100 μl of enzyme solution (1 mg/ml) at 37 °C for 20 min. The increase in absorbance at 425 nm due to product formation was monitored during this period and the activity was expressed as nmols of product formed nmols/min/mg. The inhibitory effect of crotapotin on PLA_2 activity was investigated by incubating the PLA_2 with crotapotin at 37 °C for 30 min prior to assaying the enzyme activing.

2.4. Reduction and carboxymethylation of crotapotin

One milligram of purified crotapotin dissolved in 6 M guanidine chloride (Merck, Darmstadt, Germany) containing 0.4 M Tris–HCl and 2 mM EDTA (pH 8.15) was reduced with DTT and carboxymethylated with ^{14}C-iodoacetic acid (Toyama et al., 2000). Desalting was done on a Sephadex G-25 column in 1 M acetic acid at 25 °C and the modified crotapotin (RC-F17) was lyophilized. Reduced and carboxymethylated crotapotin was digested with *Staphylococcus aureus* protease V8 for 16 h at 37 °C, using an enzyme to substrate molar ratio of 1:30. The reaction was stopped by lyophilization. The products of digestion were separated by reverse phase HPLC using a Waters PDA 991 system and a C18 μ-Bondapack column. The peptide peaks were eluted with a linear gradient (0–100%) of acetonitrile in 0.1% trifluoroacetic acid (v/v).

2.5. Sequencing procedure

The N-terminal amino acid sequence reduced and carboxymethylated crotapotin and the sequences of the purified digestion products were determined using a Procise f automatic sequencer (Applied Biosystem). The phenylthiohydantoin (PTH) amino acids were identified by comparing their retention times with these of 20 PTH amino acid standards. Peptides containing ^{14}C-CM-Cys were monitored using a liquid scintillation counter (Beckman, model L-250).

2.6. Bactericidal effect

Xanthomonas axonopodis pv. passiflorae and *Clavibacter* ssp were harvested from fresh agar Plate and suspended in distilled sterilized water (A600 nm = absorbância, corresponding to 3×10^8 CFU/ml). Aliquots of this suspension were diluted to 10^3 CFU/ml and were incubated with venom or toxin (250 μg/ml) for 60 min at 37 °C, after which survival was determined on nutrient (Difco) Plate ($n = 5$).

2.7. Rat paw and skin edema

All experiments were carried in accordance with the guidelines for animals care of the State University of Campinas (UNICAMP). Male Wistar rats (120–150 g) were used. Hind paw edema was induced by a single subplantar injection of carrageenan alone (1 mg/paw) or coinjected with crotapotin (50, 100 and 250 μg/paw). Paw volume was measured immediately before injection of the inflammatory agents and at selected time intervals thereafter (1, 2, 3, 4 and 5 h) using a hydroplethysmometer (model 7150, Ugo Basile, Italy). Crotapotin was always coinjected with carrageenan in a final volume of 0.1 ml. All drugs were dissolved in sterile 0.9% saline. The increase in paw volume (ml) was calculated by subtracting the basal volume.

Skin edema was measured after a single injection of crotapotin or phospholipase A_2 alone (1 mg site^{-1}) or after coinjection of crotapotin and PLA_2 (250 ug/100 ul). Experiments of rat dorsal skin edema were performed in male Wistar rats (200–300 g). The animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and maintenance doses were administered when required. Local plasma protein extravasation was measured in the shaved dorsal rat skin, in response to intradermally injected crotapotin from *C.d. cascavella* and PLA_2s from *C.d. terrificus*, *C.d. collilineatus* e *C.d. cascavella* (0.5 μg/site in Tyrode solution), according to Brain and Williams (1985). Agents were injected in a random order, according to a balanced site pattern. Plasma protein extravasation was measured by the accumulation of intravenously injected (i.v.) ^{125}I-human serum albumin (^{125}I-HSA; 2.5 μCi rat^{-1}) with Evan's blue dye (25 mg kg^{-1}) to act as a visual marker. The injected sites were punched out and counted for radioactivity, with the plasma samples in a γ-counter. Plasma extravasation was expressed as the volume (μl) of plasma accumulated at each skin site compared to total counts in 1 ml of plasma.

2.8. Renal perfusion

Adult male Wistar rats (240–280 g) were fasted for 24 h before each experiment but had free access to water. The rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p). The perfusion fluid was a modified Krebs–Henseleit solution (MKHS) of the following composition in mmol l^{-1}: Na^+ 147, K^+ 5, Ca^{2+} 2.5, Mg^{2+} 2, Cl^- 110, HCO_3^- 2.5, SO_4^{2-}1, PO_4^{2-}1. 6% bovine serum albumin (BSA, fraction V), 0.075 g of urea, 0.075 g of inulin and 0.15 g of glucose in a final perfusate volume of 100 ml. The MKHS was dialyzed for 48 h at 4 °C against 1.5 L of Krebs solution with changes after 24 h (Hanson and Ballard, 1968; Greg et al., 1978), and the pH was adjusted to 7.4.

The perfusion method has been described by Fonteles et al. (1983) and was based on Bowman's technique (Bowman, 1970) modified by the inclusion of an artificial lung to improve oxygenation (Hamilton et al., 1974; Fonteles et al., 1998) and a 1.2 μm Millipore filter (Pegg, 1971). The flow calibration and resistance of the system were determined before each experiment. The perfusion pressure was determined at the tip of stainless steel cannulae using a mercury manometer. The right renal artery was cannulated through the upper mesenteric artery and the kidney was isolated (Balhlmann et al., 1967; Nishiitsutji-Uwo et al.,1967; Ross, 1978; Fonteles et al., 1983) to allow an uninterrupted perfusion. After an equilibration period of 15–20 min, the experiments were run for 120 min with crotapotin being added after 30 min of perfusion.

The perfusion pressure was measured at 5 min intervals. Samples of the perfusate were collected every 10 min for the determination of sodium, chloride, potassium and insulin levels, as well as osmolality. The urine flow was also measured at 10 min intervals. Sodium and potassium concentrations were determined by flame photometry (flame photometer Model 445) and insulin levels were determined (Walson et al., 1955; Fonteles et al., 1983). The osmolality of the samples was measured in an Advanced Instrument osmometer (WESCOR 5100c vapor pressure).

2.9. Statistical analysis

The results were expressed as the mean ± SEM. The data were analyzed by analysis of variance (ANOVA) followed by Bonferroni test. The level of significance was set at $p < 0.05$.

3. Results

3.1. Purification and biochemical characterization of crotapotin from C.d. cascavella venom

C.d. cascavella venom contained several Crtp isoforms (F2–F5) (Fig. 1a). Fraction F3 was the main isoform, with ~60% homology with the other crotapotins in the venom. This fraction was repurified and yielded a single peak (insert, Fig. 1a). Reduced and carboxymethylated F3 showed three major peaks identified as chains A, B and C (Fig. 1b).

The primary structures of chains A, B and C were deduced by N-terminal sequencing following digestion with protease V8 (Fig. 2a). All chains showed a high degree of homology with other crotapotins described in the literature. Chains A and B also showed high homology with some segments of several PLA_2, especially in the calcium binding site, the catalytic site and helix 3. Other conserved regions in chain B compared to PLA_2 included the β-wing and helix 3. Chain C showed slight homology with the C-terminal of PLA_2 (Fig. 2b).

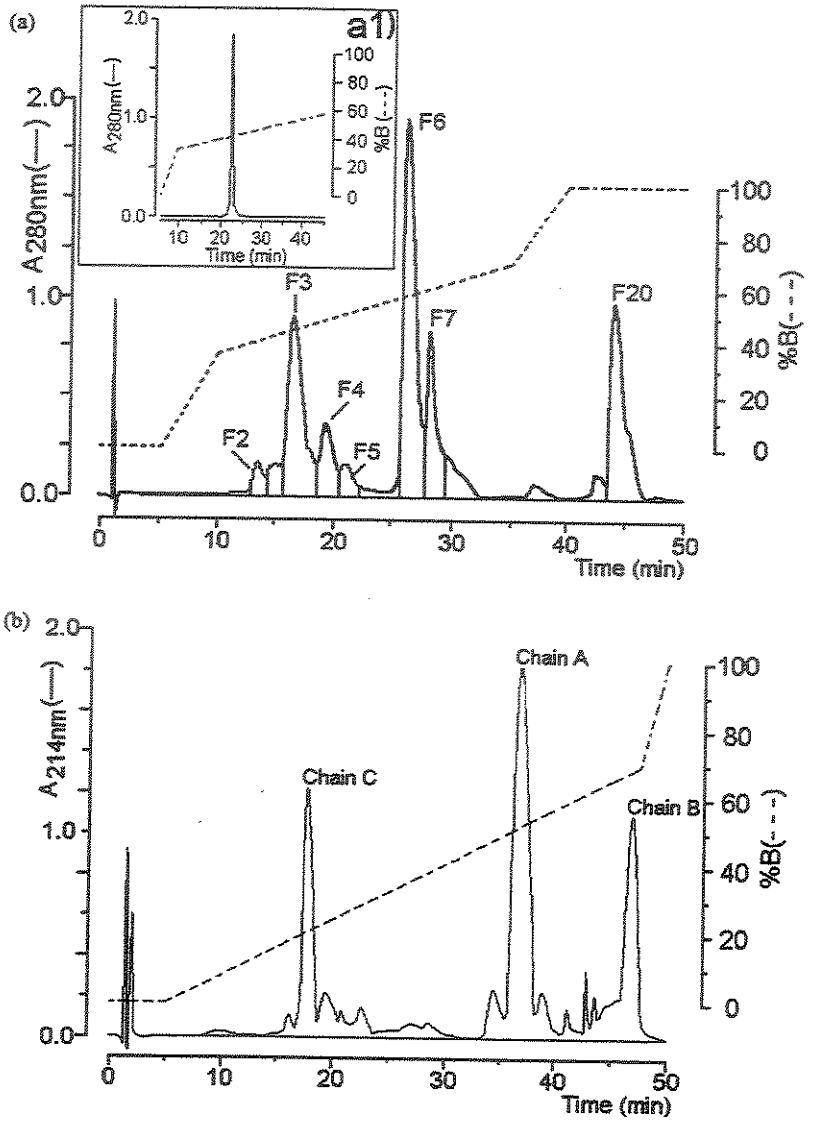

Fig. 1. The elution profile of crotoxin from *C.d. cascavella* venom in reverse phase HPLC (panel a). Fractions F2–F5 were crotapotin isoforms. Fractions F6 and F7 were PLA_2 isoforms. The chromatographic run was monitored at 280 nm. The insert (a1), shows the rechromatography of crotapotin (F3) in reverse phase HPLC. After purification, crotapotin was reduced and carboxymethylated and chains A, B and C were isolated by reverse phase HPLC using an analytical column (panel b).

3.2. Effect of crotapotin on the rat hind paw and skin edema

The edema induced by carrageenan was potentiated by crotapotin in the rat hind paw, with maximum activity seen 2 h after the injections (Fig. 3a). Crotapotin (Cdcasca F3) significantly increased the plasma extravasation in rat skin. Crotapotin did not inhibit the plasma extravasation induced by PLA_2. Indeed, the edema caused by PLA_2 from *C. d. terrificus* venom was potentiated by crotapotin (Fig. 3b).

3.3. Bactericidal effect of crotapotin isolated of C.d. cascavella venom

Crotapotin showed a bactericidal effect principally in the Gram-Negative bacteria in the presence of *Xanthomonas*

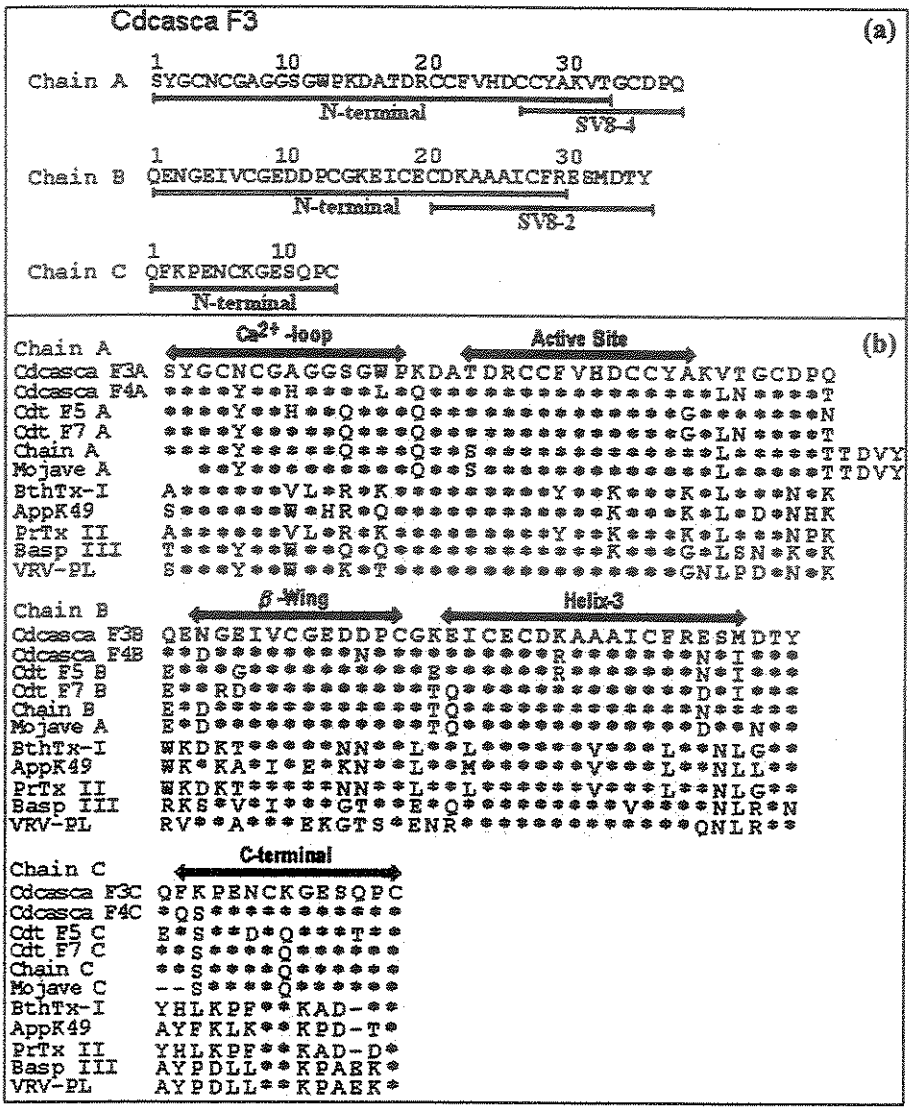

Fig. 2. The primary structure of chains A, B and C crotapotin (panel a). Panel b shows the amino acid alignment of chains A, B and C with other crotapotins and some PLA_2 segments. Cdcasca F3, Crotapotin from *C.d. cascavella* venom, Cdt F5 e F7, Crotapotin isoforms from *Crotalus durissus terrificus* venom, BthTx, PLA_2 from *Bothrops jararacussu* (Bothropstoxin I), AppK49, Asp49 PLA_2 from *Agkistrodon piscivorus piscivorus* venom. PrTx II, PLA_2 from *Bothrops pirajai* (piratoxin II), Basp III, PLA_2 from *Bothrops asper*, VRV-PL, PLA_2 from *Vipera russelli* venom.

axonopodis pv. passiflorae that was five fold higher than to *Clavibacter michaganensis michiganensis* (Gram-Positive) in the higher dose (Fig. 4). The antimicrobial activities of *C.d. cascavella* venom and crotoxin were higher in Gram-positive than in Gram-negative bacteria. PLA_2 showed a similar bactericidal effect in both types of bacteria, whereas crotapotin was more effective in Gram-negative bacteria (Fig. 5b).

3.4. Effect of crotapotin from C.d. cascavella venom on the enzymatic activity of PLA_2

Crotapotin F3 significantly inhibited the catalytic activity of PLA_2 from *C. d. cascavella* and *C. d. terrificus* venoms, but had little effect on PLA_2 from *C.d. collilineatus* venom (Fig. 5a).

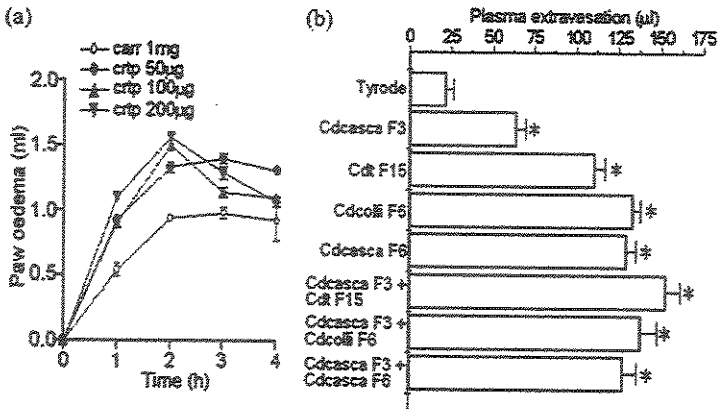

Fig. 3. (a) Inhibitory effect of crotapotin at different doses of 50, 100 and 200 µg on the paw edema induced by carrageenan (carr). Results for paw oedema are expressed as volume (ml) and each point represent the mean ± SEM of four animals. (b) Effect of crotapotin (0.5 µg/ site) from the *C.d. cascavella* venom (Cdcasca F3) incubated or not with different phospholipase A_2 (0.5 µg/ site) on the skin plasma protein extravasation. Results are expressed as µl plasma extravasated per site and each point represents the mean ± S.E.M of 3 animals. $^*p < 0.05$ (compared to Tyrode control. Cdcasca F6 = PLA_2 from the *C.d. cascavella*; Cdcolli F6 = PLA_2 from the *Crotalus durissus collilineatus*; Cdt F15 = PLA_2 from *Crotalus durussus terrificus*.

3.5. Renal perfusion

Under the experimental conditions used, the perfused kidneys were functionally stable for over 120 min (Fig. 6 and Table 1). Crotapotin (5 µg/ml) significantly ($p < 0.05$) increased the perfusion pressure and renal vascular resistance compared to control kidneys (Fig. 6a and b), but did not affect the urinary flow, glomerular filtration rate and percent of sodium tubular transport (Table 1).

4. Discussion

Crotoxin is composed of two non-covalently associated subunits; a basic and weakly toxic phospholipase A_2, subunit B (PLA_2) and an acidic protein, subunit A (crotapotin), which is non toxic and devoid of enzymatic activity. However, crotapotin, separately from the crotoxin complex has shown many biological activities (Landucci et al., 2000).

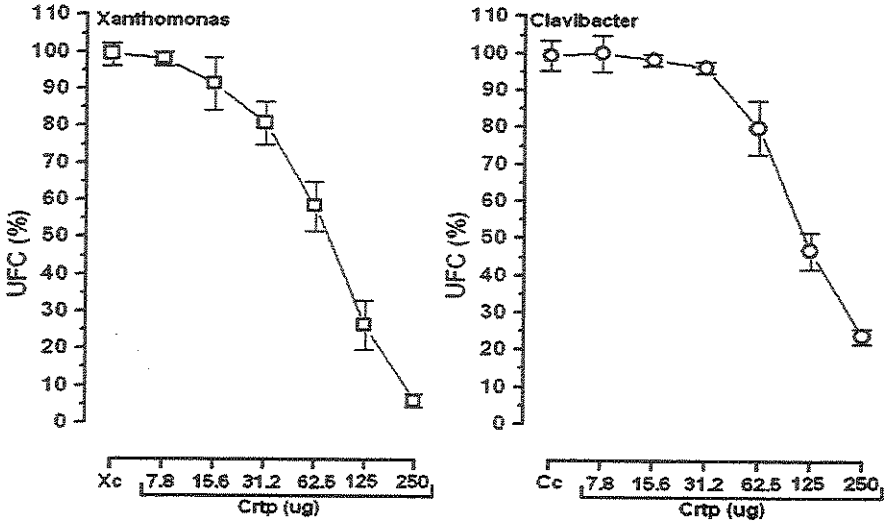

Fig. 4. The bactericidal effect of crotapotin (crpt) against two bacteria strain at different doses. Xc (*Xanthomonas axonopodis pv. Passiflorae*) and Cc (*Claribacter michaganensis michiganensis*).

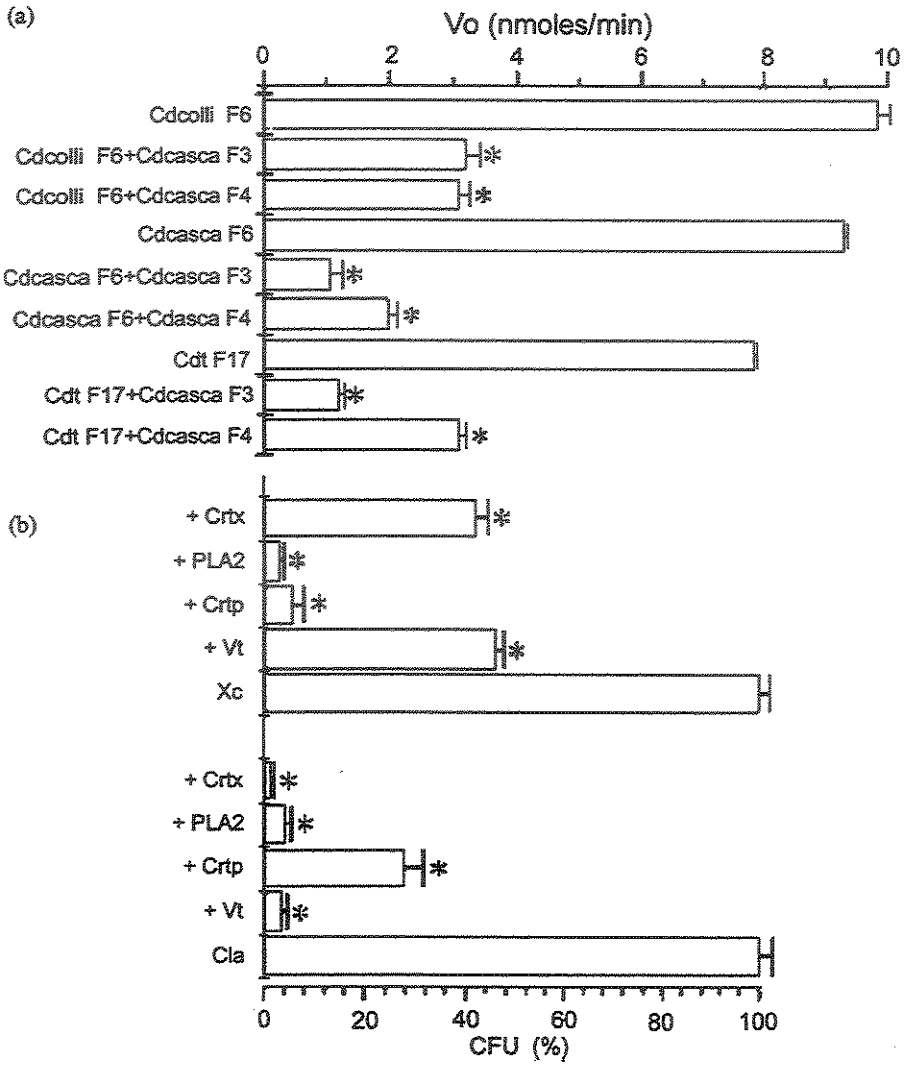

Fig. 5. (a) Inhibitory effect of crotapotins isoforms (50 μg) from the *C.d. cascavella* venom on the enzymatic activity of PLA_2 from *C. d. cascavella* (Cdcasca F6, 50 μg), *C.d. collilineatus* (Cdcolli F6, 50 μg) and *C.d. terrificus* (Cdt F17, 50 μg). Crotapotins were incubated for 20 min (w/w) before the measurement of enzymatic activity. Results are expressed as nmoles/min and represent the mean ± S.E.M of eight experiments. (b) Bactericidal effect of crotapotin (Crtp, 250 μg), phospholipase A_2 (PLA_2, 250 μg), Crotoxin (Crtx, 250 μg) and whole venom (vt, 250 μg) from the *C.d. cascavella*. The bacteria and toxins were incubated for 20 min and results were expressed as percent of CFU and each point represent the mean ± SEM of four experiment. $^*p < 0.05$ compared to bacteria in saline (control).

The edematogenic activity induced by PLA_2 was not decreased or inhibited by the addition of crotapotin from the *C.d. cascavella*, which may be associated to the crucial amino acid changes principally in the chain C by replacing of S(3) and Q(8) in the *Crotalus durissus terrificus* for K(3) and K (3) in the *C.d. cascavella*. In chain B the replacement of T(16) and Q(17) (*C.d terrificus*) for K(16) and E(17) (*C.d. cascavella*). Other important replacements in chain A were observed as Y(5), Q(11), Q(15) for N(5), S(11) and K(15). These structural changes probably induce critical modification in the function properties of the crotapotin from the *C.d. cascavella*.

Despite its ability to decrease the enzymatic activity of PLA_2 and to increase the neurotoxicity of PLA_2, crotapotin

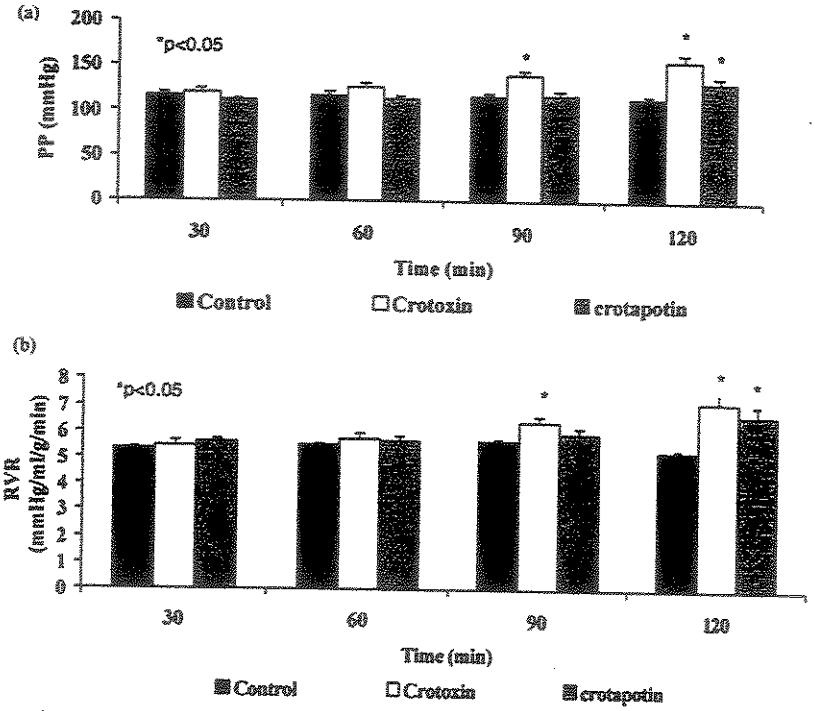

Fig. 6. Effects of crotoxin and crotapotin (a) on the renal perfusion pressure (PP) and (b) renal vascular resistance (RVR) in rat isolated perfused kidneys. Each toxin was tested at a concentration of 5 μg/ml. The columns and bars represent the mean ± SEM for six rats. The results were compared to the corresponding control group. *$p < 0.05$.

increased the inflammatory response to carrageenan in the rat hind paw edema and the response to PLA_2 from *C.d. terrificus* venom in rat skin. The primary structure of chains A, B and C of crotapotin showed that chain A had a conserved calcium-binding loop and contained the active site whereas chain B showed a conserved β-wing and helix-3 which are found in snake venom PLA_2. However, crotapotin from *C.d. terrificus* venom inhibits

Table 1
Effects of crotoxin and crotapotin (5 μg/ml each) on renal parameters

Variables	30 min	60 min	90 min	120 min
UF (ml g^{-1} min^{-1})				
Control	0.13 ± 0.01	0.15 ± 0.01	0.16 ± 0.01	0.15 ± 0.01
Crotoxin	0.12 ± 0.01	0.16 ± 0.02	0.27 ± 0.03*	0.42 ± 0.07*
Crotapotin	0.15 ± 0.01	0.14 ± 0.01	0.13 ± 0.01	0.13 ± 0.01
GFR (ml g^{-1} min^{-1})				
Control	0.60 ± 0.04	0.65 ± 0.03	0.66 ± 0.01	0.65 ± 0.01
Crotoxin	0.60 ± 0.03	1.01 ± 0.12*	0.71 ± 1.32	1.57 ± 0.33*
Crotapotin	0.69 ± 0.04	0.63 ± 0.04	0.60 ± 0.02	0.65 ± 0.05
% TNa^+				
Control	81.8 ± 0.20	81.9 ± 0.14	81.2 ± 0.20	81.2 ± 0.30
Crotoxin	81.9 ± 0.91	79.2 ± 1.32	75.1 ± 2.10*	71.9 ± 2.50*
Crotapotin	81.6 ± 1.21	81.2 ± 1.30	78.3 ± 0.81	77.7 ± 1.91

The results are expressed as the mean ± SEM of six rats for each experimental group. The toxins were added 30 min after the begging of each perfusion. UF, urinary flow; GFR, glomerular filtration rate; %TNa^+, percent of sodium tubular transport. *$p < 0.05$ compared to the corresponding control.

the inflammatory response induced by carrageenan in the rat hind paw (Landucci et al., 1995).

Crotoxin isolated from the *C.d. cascavella venom* was six fold less active in the Gram-negative than in Gram-positive bacteria. The PLA_2 from this venom showed the same bactericidal effect in both types of bacteria. Crotoxin probably exerted its bactericidal effect by a mechanism different from that induced used by PLA_2 or crotapotin.

The bactericidal effect of PLA_2 was probably dependent on the positively charge in the PLA_2 and mainly of the enzymatic activity as shown by Soares et al. (2001) and Oliveira et al. (2002). Crotapotin did inhibit the bacteria grow rate. Crotapotin showed a bactericidal effect, principally against the Gram-negative *Xanthomonas axonopodis pv. passiflorae* which was more susceptible than *Clavibacter michaganensis michiganensis* (Gram-positive). Thus the bactericidal effect of crotapotin may be dependent on the composition of the bacterial cell wall. The structural and biological activities suggest that crotapotin acts similarly to some antimicrobial peptides isolated from Brazilian frog Phyllomedusa distincta (Batista et al., 1999) or other synthetic peptides (Bennik et al., 1999). Basically, the combination of some positively charge amino acid in the molecule would be interacting with negatively charged groups of membrane and the combination of the hydrophobic amino acid residues may be affecting the membrane permeability or facilitating the membrane disruption.

According to Valentin and Lambeau (2000), PLA_2 produced their actions via specific receptors known as M- and N-type receptors. Lambeau and Lazdunski (1999) suggested that mutations in Gly30, Leu31 and Asp49 strongly decreased the binding of PLA_2 to the membrane receptors. These amino acid residues were also found in chain A of crotapotin. These results suggest that bacteria may have similar M-type and N-type receptors in their membranes, although the interaction of crotapotin and PLA_2 with these receptors is apparently dependent on the cell wall composition.

The most serious complication following envenomation by *C.d. subspecies* is acute renal failure (ARF) (Ribeiro et al., 1998). Rhabdomyolysis may contribute to renal lesions (Azevedo-Marques et al., 1985), but the pathogenesis is not well understood. Martins et al. (1998) demonstrated that *C. d. cascavella* venom altered renal functional parameters in the isolated perfused rat kidney method, probably through a direct action at both vascular and glomerular sites. *C.d. terrificus* venom and crotoxin, its major component also cause acute nephrotoxicity in the rat isolated perfused kidney (Monteiro et al., 2001).

Crotapotin increased the perfusion pressure and renal vascular resistance, but did not change the urinary flow, glomerular filtration rate and the fractional sodium transport in isolated kidneys.

The renal response to crotapotin was different from the effects caused by crotoxin. Whereas crotoxin increased the urinary flow and glomerular filtration rate (Martins et al., 2002), but crotapotin produced no such changes. These results suggest that crotoxin is the main venom component responsible for nephrotoxicity seen following envenomation by *C.d. cascavella* and that crotapotin contributes little to this phenomenon.

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A new C-type animal lectin isolated from *Bothrops pirajai* is responsible for the snake venom major effects in the isolated kidney

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Abstract

We investigated the biochemical and biological effects of a new C-type galactoside specific lectin termed BPL that was isolated from the snake venom of *Bothrops pirajai*. This lectin was purified using size exclusion HPLC followed by an immobilized lactose affinity column. The purified BPL was homogeneous by reverse phase HPLC and SDS-PAGE. We evaluated the nephrotoxicity of the whole venom of *B. pirajai* and its lectin. The whole venom of *B. pirajai* (10 μg/mL) showed similar results as those observed for BPL (3, 10 and 30 μg/mL) evaluated by the perfused rat kidney method. They caused reductions in perfusion pressure ($Control_{120} = 110.28 \pm 3.69$; $BP_{120} = 70.70 \pm 2.40$*; $BPL3_{120} = 113.20 \pm 4.40$; $BPL10_{120} = 67.80 \pm 3.00$*; $BPL30_{120} = 64.90 \pm 3.50$* mmHg; *: $P < 0.05$), renal vascular resistance, urinary flow, glomerular filtration rate ($Control_{90} = 0.695 \pm 0.074$; $BP_{90} = 0.142 \pm 0.032$*; $BPL3_{90} = 0.314 \pm 0.064$; $BPL10_{90} = 0.250 \pm 0.038$*; $BPL30_{90} = 0.088 \pm 0.021$* mL g^{-1} min^{-1}; *: $P < 0.05$) and sodium ($Control_{120} = 81.28 \pm 0.26$; $BP_{120} = 55.71 \pm 5.72$*; $BPL3_{120} = 80.94 \pm 0.93$; $BPL10_{120} = 65.23 \pm 1.47$*; $BPL30_{120} = 76.03 \pm 1.70$*%; *: $P < 0.05$), potassium and chloride tubular transport. Neither whole venom nor purified BPL induced direct vasoactive effects in perfused arteriolar mesenteric bed, and BPL did not potentiate bradykinin contraction in the ileum. We postulate that both *B. pirajai* and BPL promoted the same renal effects probably caused by the release of inflammatory mediators.

Keywords: *Bothrops pirajai*; Lectin; Biological activity; Biochemical characterization

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1. Introduction

Lectins are found in a diverse array of organisms. They consist of a large group of proteins with the ability of binding specifically, reversibly, and non-covalently to carbohydrate. Some of these molecules may also contain other sites that interact with a noncarbohydrate ligand. These proteins are more common in plants, including trees, particularly in seeds. So far, they have been reported in bacteria, roe, snails, vertebrates and mushrooms (Kilpatrick, 2002).

The animal lectins can be classified into four major groups: (i) C-type or calcium dependent lectins, (ii) S-type or the galactose binding galactins, (iii) the I-type lectins, and (iv) the luminal proteins of the endoplasmatic reticulum that interacts transiently with glycoproteins (Drickamer, 1995).

Lectin-like proteins have been found in snakes belonging to the families Elapidae, Viperidae and Crotalidae (Aragón-Ortiz, Brenes-Brenes, & Gubensek, 1990). They have been shown to be composed of two identical subunits with invariant amino acid residues, which are also found in the carbohydrate recognition domain of the C-type lectins (Weis, Khan, Fourme, Drickamer, & Hendrickson, 1991). They also have similarity with other snake venom proteins like echicetin, a glycoprotein Ib-binding protein (Polgár et al., 1997) and bothrocetin, a von Willibrand factor-binding protein (Usami et al., 1993).

Many biological effects of snake venom lectins have been reported, such as erythrocyte agglutination in vitro, mitogenic activity to lymphocytes, platelet aggregation, oedema induction, calcium release from internal stores and inhibition of cell proliferation (Marcinkiewicz et al., 2000).

Snake venom proteins of the C-type lectin family are characterized by very distinct biological activities despite their highly conserved primary structure, which is homologous to the carbohydrate recognition domain of true C-type lectins (Drickamer, 1999). Basically, there are two kinds of snake venom C-type lectins. The first has disulfide linked αβ heterodimer made of two homologous polypeptides of about 14 kDa, with erythrocytes agglutinating and carbohydrate biding activities and a lot of different pathological effects, similar to the ones found in Bothrops species (Castro, Lemos, Bon, & Zingali, 2003; Lee, Du, Lu, Clemetson, & Zhang, 2003). The second type has a high molecular weight (50–100 kDa). They correspond to disulfide-linked multimers that are associated by two to four αβ heterodimers, as found for Convulxin, which showed other important biological activities as platelet aggregation, but did not have a high erythrocyte agglutinating activity (Toyama et al., 2001).

The pathogenesis of renal alterations following envenomation by *Bothrops* species is not well defined and appears to be multifactorial (Nancy, Ahlstrom, Luginbuhl, & Tisher, 1991). The symptoms are due to the additive or synergistic effects of the different toxins and enzymes present in the venoms (Ferreira et al., 1992). To further evaluate the renal effects of *Bothrops* envenomation we isolated a new C-type lectin from the *Bothrops pirajai* snake venom and studied its role in the nephrotoxicity caused by the crude venom, using the isolated perfused rat kidney model. This snake is a species geographically located only in the south region of the Bahia State, in Brazil (Toyama et al., 1995).

2. Material and methods

The chemical and reagents used in this work were purchased from Promega (Madison, WI, USA), Sigma and Aldrich (Sigma Chemical Co., St. Louis, MO, USA), Merck (Merck Chemical Co, Germany), Perkin Elmer Applied Biosystems (Perkin Elmer, USA), and Bio Rad (USA).

2.1. Purification of a lectin-like material from B. pirajai

The *B. pirajai* venom was a gift from CEPLAC, CEPEC (Secretary of Agriculture, Itabuna, Bahia, Brazil). The venom (45 mg) was dissolved in 0.2 M ammonium bicarbonate, pH 8.0 and clarified by ultracentrifugation at 4500 × *g* for 2 min. The supernatant was applied and fractionated on a column (1 cm × 60 cm) of Superdex 75 (Pharmacia) pre-equilibrated with ammonium bicarbonate buffer (0.2 M, pH 8.0). The flow rate was 0.2 ml/min and the elution profile was monitored at 280 nm. Purification and the lectin activity were monitored for erythrocyte agglutinating activity. The lectin active peak from HPLC molecular

exclusion was followed by affinity chromatography on absorbed lactose column (0.8 cm × 5 cm), previously equilibrated with CTBS (Tris, 20 mM, NaCl, 150 mM, $CaCl_2$ 5 mM, pH 7.5). BPL was eluted using a gradient of 0.2–0.3 M lactose in CTBS. The lectin-like fraction was pooled and extensively dialyzed against ammonium bicarbonate buffer (0.1 M, pH 8.0) and lyophilized. The molecular mass and homogeneity of the eluted protein was evaluated by reverse phase HPLC (0.1 cm × 30 cm column of μ-Bondapack C18, Waters) using a linear gradient 0–100% of acetonitrile in 0.1% trifluoroacetic acid (v/v). The purified protein eluted at 66% acetonitrile. SDS-PAGE using 12% acrylamide Tricine gels confirmed the molecular homogeneity of this fraction.

2.2. Structural characterization of lectin

Two milligrams of the purified protein was dissolved in 200 μl of 6 M guanidine chloride in 0.4 M Tris–HCl and 2 mM EDTA, pH of 8.15. Nitrogen was blown over the top of the protein solution for 15 min, and the protein was then reduced with DTT (6 M, 200 μL) and carboxymethylated with ^{14}C-iodoacetic acid. Nitrogen was blown again over the surface of the solution prior to sealing the tube. The reaction mixture was incubated in the dark at 37 °C for 1 h and then desalted using Sephadex G25 column using 1 M acetic acid, followed by lyophilization. The amount of 600 μg of reduced and *S*-carboxymethyled-BPL (RC-BPL) was digested with *Staphylococcus aureus* protease V8 for 16 h at 37 °C, using an enzyme to substrate molar ratio of 1:30, and then lyophilized. Other aliquots of RC-BPL (600 μg) were digested with trypsin or clostripain for 8 h at 37 °C and lyophilized (Toyama et al., 2000).

The protease V8, clostripain and trypsin digests were fractionated by reverse phase HPLC using a Waters PDA 991 system and a C18 μ-Bondapack column. The elution of the peptide peaks was done using a linear gradient 0–100% of acetonitrile in 0.1% trifluoroacetic acid (v/v). RC-BPL (500 μg) was cleaved with a 150-fold molar excess of cyanogen bromide (CNBr) over methionine residues of RC-BPL in 70% formic acid (4 ml) under nitrogen for 24 h at room temperature. The reaction mixture was diluted with 40 ml of water and lyophilized. The excess reagents were removed by gel filtration on a Sephadex G-25 column (1.0 cm × 20 cm) equilibrated with 10% acetic acid. The CNBr peptide fragments were separated by reverse-phase HPLC. N-terminal sequencing using the reduced and carboxymethylated protein, and the sequencing of peptides purified from the protease digests were made using an automatic sequencer (Applied Biosystems). The phenylthiohydantoin (PTH) amino acids were identified by comparing their retention times with the 20 PTH amino acid standards separated by 140 C Microgradient System (Applied Biosystems).

2.3. Hemagglutination activity

Hemagglutination was assayed using serial dilutions of BPL incubated with ox, pig, horse, sheep and human (types A, B and O) erythrocytes, as previously described (De Carvalho, Marangoni, & Novello, 2002). Trypsinized erythrocytes were prepared by incubating the cells with 0.025% trypsin for 1 h at 37 °C. Inhibition of hemagglutination activity by several carbohydrates was performed using D-lactose, D-galactose, D-raphinose, D-glucose, D-sucrose, D-maltose, D-mannose, D-fructose, D-threalose and methyl-manopyrasonide.

2.4. Perfused rat kidney assay

Adult male Wistar rats weighing 250–300 g were fasted 24 h with water ad libitum before each experiment and anaesthetized with sodium pentobarbital (50 mg/kg body weight) following ethical guidelines approved by the local committee. The perfusate was a modified Krebs-Henseleit solution (MKHS) containing in mmol/L: 118 NaCl, 1.2 KCl, 1.18 KH_2PO_4, 1.18 $MgSO_4 \cdot 7H_2O$, 2.5 $CaCl_2 \cdot 2H_2O$, and 25 $NaHCO_3$ and 6 g% of bovine serum albumin (BSA, fraction V). MHKS was then dialyzed for 48 h at 4 °C. Immediately before the beginning of each perfusion we added 50 mg of urea, 50 mg of inulin and 100 mg of glucose to a final perfusate volume of 100 mL. The pH was then adjusted to 7.4 and the solution placed into the perfusion system.

The perfusion model followed the method described by Fonteles, Cohen, Black, and Wertheim (1983). The rate of perfusion was maintained between 25 and 35 mL/min per kidney. The perfusion pressure was measured at the tip of the stainless steel cannula and

was allowed to fluctuate under experimental conditions, but was carefully kept at 120–140 mmHg during the 30 min internal control period. In each experiment, the recirculating perfusion system employed 100 mL of MKHS and lasted 120 min. After an equilibration period of 15–20 min the experiments were started. Perfusion pressure was measured at 5 min intervals. Every 10 min, samples of urine, obtained by the cannulated right ureter and perfusate were collected for further analysis of sodium, potassium, inulin and osmolality. Clearance measurements were made according to Martinez-Maldonado, and Opava-Stitzer (1978) and Pitts (1971). Sodium and potassium were measured by flame photometry; inulin was determined by direct hydrolysis as described by Fonteles et al. (1983) and Walser, Davidson, and Orloff (1955). Osmolality was measured in a vapor pressure osmometer (WESCOR 5100c vapor pressure).

The whole venom of *B. pirajai* (10 μg/mL) and its lectin BPL (3, 10 and 30 μg/mL) were always added to the system 30 min after the beginning of each experiment ($n = 6$). Data were measured at 10 min intervals and averaged every 30 min at 30, 60, 90 and 120 min. Statistical evaluation was determined by analysis of variance (ANOVA) and corrected by Bonferroni test, used to compare data with control group, where kidneys were perfused only with MKHS, and four treated groups. Statistical significance was set at 5%.

2.5. Isolated perfused rat mesenteric blood vessels method

The perfusion was done as described (Mcgregor, 1965). Briefly, Wistar rats weighing 280–350 g were anesthetized with sodium pentobarbitone (50 mg/kg, body weight). After opening the abdomen, pancreatic-duodenal, ileum-colic and colic branches of the superior mesenteric artery were tied. Then, the superior mesenteric artery was cleaned of surrounding tissue and cannulated by a polyethylene tube (PE20). The intestine was separated from the mesenteric bed by cutting close to the intestinal border of mesentery. The mesenteric bed was perfused with Krebs-Henseleit solution containing: 114.0 mM of NaCl; 4.96 mM of KCl; 1.24 mM of $KH_2PO_4 \cdot H_2O$; 0.5 mM of $MgSO_4 \cdot 7H_2O$; 24.99 mM of $NaHCO_3$; 2.10 mM of $CaCl_2 \cdot 2H_2O$; and 3.60 mM of glucose. The perfusion solution was kept warmed at 37 °C and the mesenteric bed was perfused by a constant flow (4 mL/min), while the variable perfusion pressure was measured by means of a P23 Stratham pressure transducer (Gould, Oxnard, CA, USA) and recorded continuously on a DMP-4B Narco Bio-Systems (Houston, TX, USA) physiograph. We examined the direct vascular effects of the *B. pirajai* venom and its lectin (10 μg/mL/min; $n = 4$) and also evaluated its effects on mesenteric beds previously pre-contracted with phenylephrine (1–5 μM; to achieve ≈40–60% maximal response).

2.6. Guinea-pig Ileum

Male albino guinea-pigs weighing from 200 to 300 g were sacrificed by cervical displacement followed by exsanguination. After rapid excision of the proximal ileum, segments (3 cm) were suspended in 10 ml organ baths, with a resting tension of 1 g, in Tyrode solution (mM): NaCl 137, KCl 5, $CaCl_2$ 3.6, $NaHPO_4$ 0.4, $NaHCO_3$ 15 and glucose 5.5 kept at 37 °C, pH 7.4 and gassed continuously with air bubbling. The mechanical activity was recorded on smoked drums by means of an isotonic lever (6-fold magnification). After a resting period of 1 h, with 15 min washout interval, the tissues were exposed, for 1 min, to bradykinin (BK; single doses of 2.5, 5, 10 and 20 ng/mL) as an internal standard control. The concentration-response curve to BK was repeated until two similar curves were established. Thereafter, the contractile response of the guinea-pig ileum to 2.5 ng/mL BK was compared in the absence and presence of 1, 2 or 4 μg/mL of the lectin; 4 μg/mL of the crude venom of *B. pirajai* or 2 μg/mL captopril to test BK potentiation.

3. Results

Size exclusion HPLC of *B. pirajai* whole venom performed on Superdex 75 showed five major peaks designated Bp I, Bp II, Bp III, Bp IV and Bp V (lectin fraction) (Fig. 1). The pooled Bp V fraction was then lyophilized and 50 mg of this fraction was subjected to a purification step on affinity chromatographic method using immobilized lactose. The purified lectin peak was eluted by increasing the lactose concentration up to 200 mM. The yield was then identified as BPL (Fig. 1b). After dialysis against ammonium bicarbon-

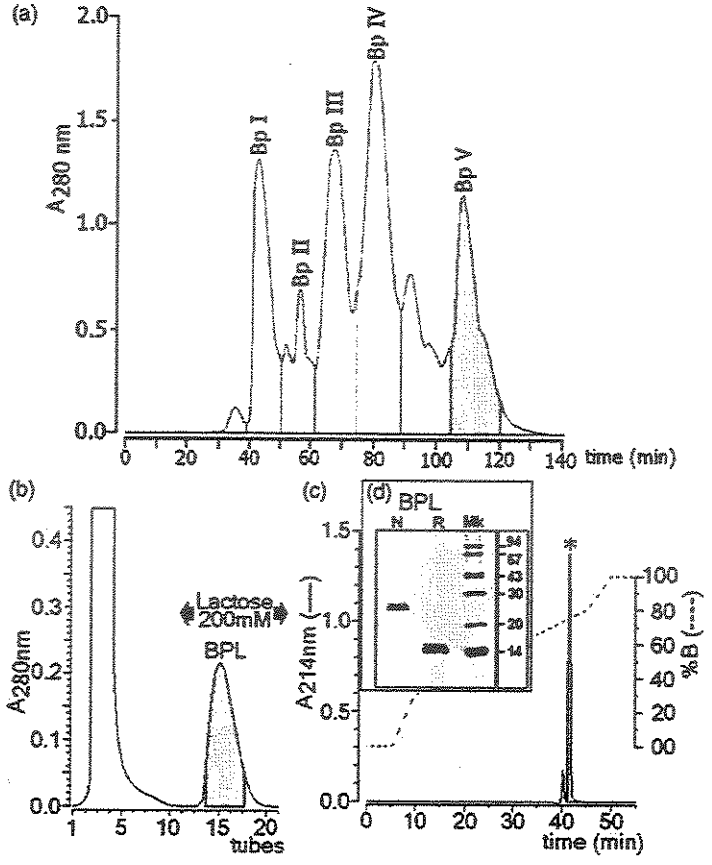

Fig. 1. Purification of BPL by HPLC size exclusion chromatography on Superdex 75 (a) and lactose affinity chromatography (b). The molecular homogeneity of the purified BLP was determined by reverse phase HPLC (c) and Tricine SDS-PAGE (d).

ate, the homogeneity of this fraction was verified by SDS-PAGE, which showed one major band of 28 and 15 kDa molecular mass in the presence or absence of 1 M of DTT, respectively (Fig. 1d). The purified lectin fraction obtained from the HPLC molecular exclusion and affinity chromatography showed one major peak (Fig. 1c).

The complete amino acid sequence of BPL was obtained following cyanogen bromide, trypsin, V8 protease and Clostripain digestion. The sequence of BPL was compared with other venom snake lectins as BjcuL (lectin from *Bothrops jararacussu*), BJL (lectin from *Bothrops jararaca*), PAL (lectin from *Bitis arietans*), BML (lectin from *Lachesis muta*), RSL (lectin from *Crotalus atrox*), and trombolectin or TL (lectin from *Bothrops atrox*) (Fig. 4). The sequences were arranged to maximize mutual homologies. BPL showed 90% of sequence homology to Bjcul, PAL, BJL and BML (Fig. 2).

BPL showed high hemagglutination activity in the presence of human erythrocytes, especially on trypsinized erythrocytes, which were agglutinated at concentrations of 2.5–0.35 μg/ml (Table 1). In addition, like other lectins, hemagglutination by BPL was inhibited by D-lactose (1.75 mM), D-galactose (0.75 mM) and D-raphinose (3.5 mM), showing similarities with other snake lectins (Table 2).

The lectin from *B. pirajai* caused the same renal effects described for the whole venom. Perfusion pressure (Fig. 3) and renal vascular resistance (Fig. 4) were decreased after the administration of the venom (10 μg/mL) and BPL (10 and 30 μg/mL). The lectin in the dose of 3 μg/mL did not alter those parameters. All doses of BPL and *B. pirajai* venom diminished

```
          1                                                  50
BPL       -NNCPGDWLPMNGLCYKIFNELKAWEDAEMFCRKYKPGCHLASIHIYG-E
BjcuL     -....Q.............D.....K.....................L..-.
BJL       -....Q.............D.....K.....................F.L..-.
PAL       -....P.............D.........R.............F.Q..-.
BML       -....Q.............D.Q.......................F.R..-.
RSL       -....L.............Q..T.....................F.R..-.
TL        --D...G.SSYE.N...F.QQKMN.A...R..SEQAKGG..V..K..SK.

         51                                                  100
BPL       SLEIAEYISDYHKGQAE-VWIGLWDKKKDFSWEWTDRSC---TDYLSWDK
BjcuL     .P..............-.....Q.....................---.....
BJL       .P..............-.........................---....T...
PAL       ................-.........................---.....
BML       ................-.........................---....T...
RSL       ..............-.N.....R.....................---....T...
TL        KDFVGDLVTKNIQSSDLYA....RVEN.E-------KQ.SSEWSDGSSVS

        101                                                144
BPL       NQPDHYENKEFCVELVSLTGYRLWEDQVCESKNAFLCQCKF
BjcuL     ......Q.....................................
BJL       .......G.................N.................
PAL       ......Q.................N....G.............
BML       .......G.................N.................
RSL       ......Q.................N.......D.........
TL        YENVVERTVKK.FALEKDLGFVL.INLY.AQK.P.V--C.SPPP
```

Fig. 2. Comparison of amino acid sequence of snake venom lectins and BPL. Gaps (-) and dots (...) were introduced to conserve homology. Bjcul (lectin from *Bothrops jaracussu*), BJL (lectin from *Bothrops jararaca* isoform I), PAL (lectin from puff adder), BML (lectin from *Lachesis muta muta*), RSL (lectin from the *Crotalus atrox*), TL (lectin from the *Bothrops atrox*).

urinary flow and glomerular filtration rate in the periods of 90 and 120 min. However, BPL in the dose of 10 μg/mL was not different from control values in the period of 120 min for both parameters (Figs. 5 and 6).

Sodium, potassium and chloride tubular transport were decreased after administration of *B. pirajai* venom and its lectin. Sodium tubular transport was affected especially in the periods of 90 and 120 min, but BPL dose of 3 μg/mL did not alter this parameter (Fig. 7). Potassium tubular transport was diminished in the periods of 90 and 120 min by the whole venom

Table 1
Hemagglutination activity of isolated *B. pirajai* lectin on erythrocytes of different species

Erythrocytes	MHC	
	Non-trypsinized (μg)	Trypsinized (μg)
Ox	25.5	3.5
Pig	3.5	1.5
Horse	Nd	Nd
Sheep	Nd	Nd
Human type A	2.5	0.35
Human type B	2.5	0.65
Human type O	1.5	0.55

MHC—minimum hemagglutination concentration able to agglutinate a sample of 100 μl of erythrocytes (2%).

Table 2
Carbohydrate inhibition on *B. pirajai* lectin hamagglutination activity

Carbohydrate	MIC (mM)
D-Lactose	1.75
D-Galactose	0.75
D-Raphinose	3.5
D-Glucose	>50
D-Sucrose	>50
D-Maltose	>150
D-Mannose	>200
D-Fructose	>200
D-Threalose	>200
Methyl-manopyrosonide	>200

MIC—minimum inhibitory concentration able to block *B. pirajai* lectin hemagglutination of a sample of 100 μl of type O human erythrocytes (2%).

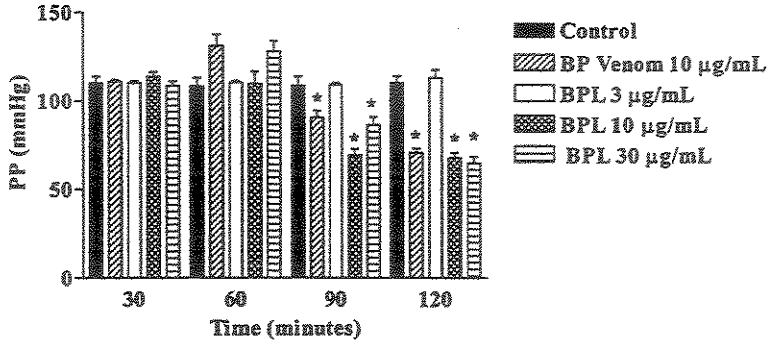

Fig. 3. The effects of *B. pirajai* (10 µg/mL) whole venom (BP venom) and purified BPL at three different concentrations (3, 10 and 30 µg/mL) on renal perfusion pressure (PP). Data are expressed as means ± S.E.M. from six different animals for each group. Statistical analysis was done by ANOVA, comparing the four treated groups with a control group, where kidneys were perfused with only MKHS with *: $P < 0.05$.

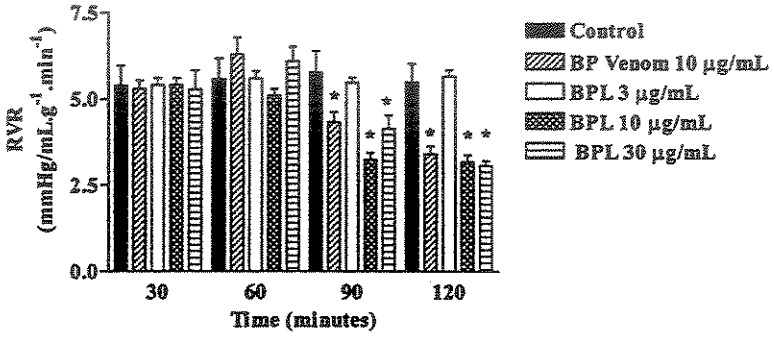

Fig. 4. The effects of *B. pirajai* (10 µg/mL) whole venom (BP venom) and purified BPL at three different concentrations (3, 10 and 30 µg/mL) on renal vascular resistance (RVR). Data are expressed as means ± S.E.M. from six different animals for each group. Statistical analysis was done by ANOVA, comparing the four treated groups with a control group, where kidneys were perfused with only MKHS with * $p < 0.05$.

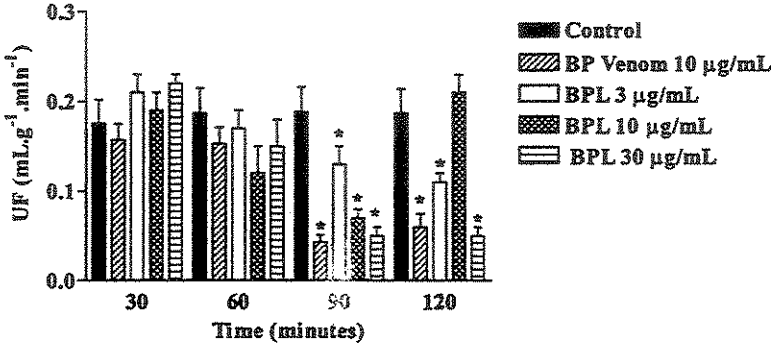

Fig. 5. The effects of *B. pirajai* (10 µg/mL) whole venom (BP venom) and purified BPL at three different concentrations (3, 10 and 30 µg/mL) on urinary flow (UF). Data are expressed as means ± S.E.M. from six different animals for each group. Statistical analysis was done by ANOVA, comparing the four treated groups with a control group, where kidneys were perfused with only MKHS with *: $P < 0.05$.

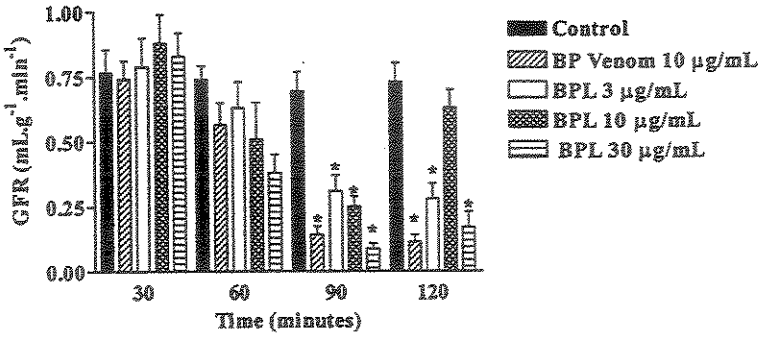

Fig. 6. The effects of *B. pirajai* (10 µg/mL) whole venom (BP venom) and purified BPL at three different concentrations (3, 10 and 30 µg/mL) on glomerular filtration rate (GFR). Data are expressed as means ± S.E.M. from six different animals for each group. Statistical analysis was done by ANOVA, comparing the four treated groups with a control group, where kidneys were perfused with only MKHS with *: $P < 0.05$.

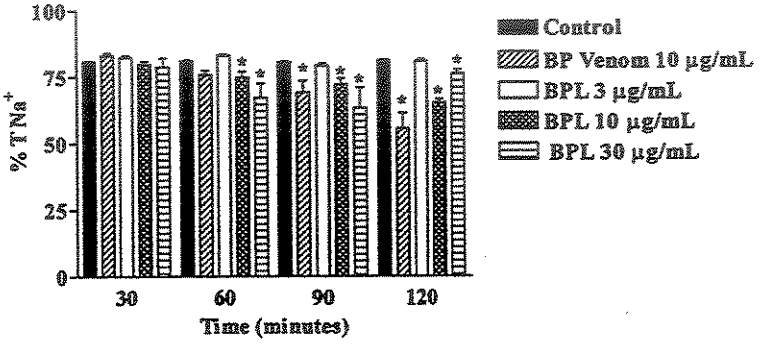

Fig. 7. The effects of *B. pirajai* (10 µg/mL) whole venom (BP venom) and purified BPL at three different concentrations (3, 10 and 30 µg/mL) on the percentage of sodium tubular transport (% TNa^+). Data are expressed as means ± S.E.M. from six different animals for each group. Statistical analysis was done by ANOVA, comparing the four treated groups with a control group, where kidneys were perfused with only MKHS with *: $P < 0.05$.

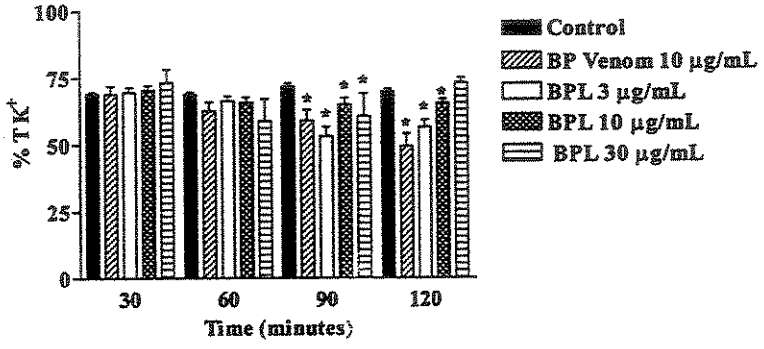

Fig. 8. The effects of *B. pirajai* (10 µg/mL) whole venom (BP venom) and purified BPL at three different concentrations (3, 10 and 30 µg/mL) on percentage of potassium tubular transport (% TK^+). Data are expressed as means ± S.E.M. from six different animals for each group. Statistical analysis was done by ANOVA, comparing the four treated groups with a control group, where kidneys were perfused with only MKHS with *: $P < 0.05$.

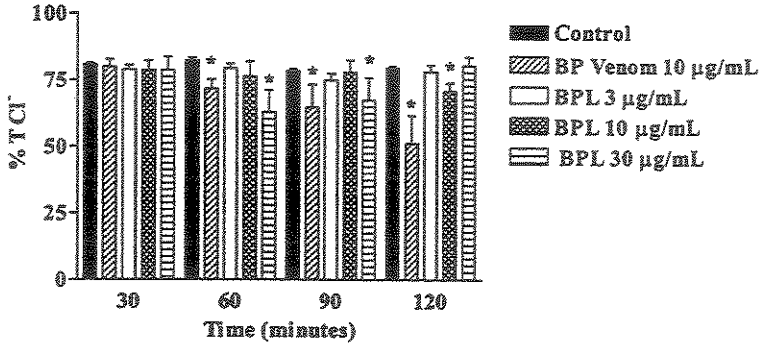

Fig. 9. The effects of *B. pirajai* (10 µg/mL) whole venom (BP venom) and purified BPL at three different concentrations (3, 10 and 30 µg/mL) on percentage of chloride tubular transport (% TCl^-). Data are expressed as means ± S.E.M. from six different animals for each group. Statistical analysis was done by ANOVA, comparing the four treated groups with a control group, where kidneys were perfused with only MKHS with *: $P < 0.05$.

and all doses of BPL (Fig. 8). *B. pirajai* venom reduced significantly chloride tubular transport in the periods of 60, 90 and 120 min. However, the BPL (10 µg/mL) affected this parameter only in the period of 120 min, while BPL (30 µg/mL) altered this ion tubular transport in the periods of 60 and 90 min (Fig. 9).

Neither BPL nor *B. pirajai* venom affected the basal perfusion pressure or the perfusion pressure of phenylephrine pre-contracted mesenteric vascular beds after 60 min of constant infusion (10 µg/mL/min) (Fig. 10). Similarly, neither BPL (1, 2 and 4 ng/mL) nor the crude venom potentiate bradykinin-induced contractions in the guinea-pig ileum (Fig. 11).

4. Discussion

The most common method to purify a lectin generally uses a single affinity chromatographic step, but in this work we performed an additional size exclusion HPLC method using Superdex 75. This procedure has the advantage to recover other important components present in the venom. Besides, this matrix is based on dextran and agarose polymers which have some affinity for lectins in the venom. The affinity step then yielded a highly purified lectin with less possibility of glycoprotein contamination.

Tricine SDS-PAGE showed the BLP subunit to be about 15 kDa, in good agreement with the 16 kDa molecular weight calculated from the amino acid

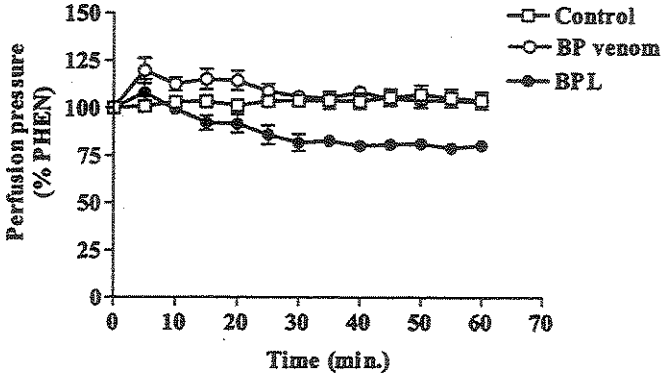

Fig. 10. Effects of *B pirajai* (BP venom; 10 µg/mL/min) and purified BPL (10 µg/mL/min) in the phenylephrine pre-contracted mesenteric beds. Data are expressed as mean ± S.E.M of three different experiments. Statistical analysis was done by ANOVA and Bonferroni test with $P < 0.05$. PHEN = phenylephrine.

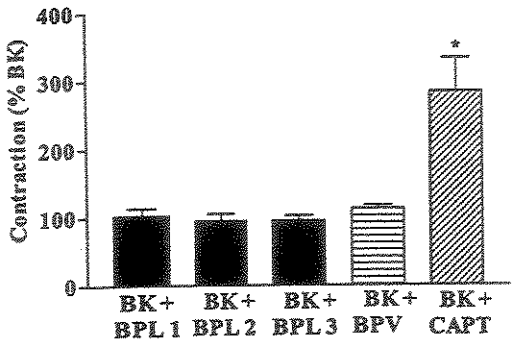

Fig. 11. Comparative effects of *B. pirajai* crude venom (4 μg/mL), BPL (1, 2 and 4 μg/mL) or captopril (CAPT; 2 μg/mL) in bradykinin ileum contraction (BK; 2.5 ng/mL). Data are expressed as means of relative bradykinin contraction ± S.E.M of four different experiments. Statistical analysis was done by Student *t*-test with *: $P < 0.05$. BPL1—1 μg/mL; BPL2—2 μg/mL; BPL3—4 μg/mL and BPV = *B. pirajai* crude venom.

sequence, similar to other lectins (Drickamer, 1988; Fonteles et al., 1983). BLP is highly hydrophilic, with high contents of charged and polar amino acid residues. The theoretical pI of *B. pirajai* lectin was found to be near 6.0, similar to the lectin isolated from *L. muta stenophyrs* (Aragón-Ortiz et al., 1996).

The primary structure of the snake venom lectins presents an amino acid homology around 90%, with conserved positions of cysteines that are involved in four intrasubunit disulfide bridges: Cys3–Cys4, Cys31–Cys133, Cys39–Cys135, and Cys107–Cys 125, while Cys 87 is involved in an intersubunit disulfide bridge. The N- and C-terminal were practically identical to other venom lectins. BPL also has homology for CDR motif, specific for N-acetylgalactosamine and galactose (Drickamer, 1988; Spies, 1990). C-type CDRs are characterized by a sequence motif of 32 conserved amino acids at fixed intervals, including 2 disulfide bonds. This conserved residue appears to form a general calcium dependent carbohydrate-binding framework. However, the amino acid alignment results suggested that this new lectin has similar structure organization as other lectins.

The hemagglutination activity of isolated BPL on the human erythrocytes and its inhibition by lactose and galactose suggested that galactose residues of erythrocytes are the recognition site for BPL. True lectins from other venoms agglutinate intact erythrocytes at concentrations of 10–400 ng/ml (Komori, Nikai, Tohkai, & Sugihara, 1999).

B. pirajai venom caused similar renal effects in vitro as other *Bothrops* venoms evaluated previously by our group (Barbosa et al., 2002; Havt, Fonteles, & Monteiro, 2001; Monteiro & Fonteles, 1999). We observed significant reductions in perfusion pressure (PP) and renal vascular resistance (RVR). We usually hypothesized that *Bothrops* snake venoms promoted renal vascular resistance drop by the presence of bradykinin-potentiating peptides, which where isolated from many other Bothrops species (Bourguignon et al., 2000; Ferreira, Bartelt, & Greene, 1970; Ferreira et al., 1992, 1998). We tested if these peptides could also be present in *B. pirajai* venom and would be causing the reductions seen for PP and RVR. However, neither BPL nor the crude venom potentiated BK-induced contractions in the guinea-pig ileum, contrasting with captopril, a known BK potentiator. Moreover, none of them affected the basal perfusion pressure of the arteriolar mesenteric bed or the perfusion pressure of phenylephrine pre-contracted arteriolar mesenteric beds. Hence, these results do not support our previous hypothesis that the crude venom or BPL would induce reductions in PP and RVR of perfused kidneys by a BK potentiation mechanism.

On the other hand, the results with BPL were similar to the *B. pirajai* venom and possibly, BPL is the major fraction responsible for this renal effect. But it is unlikely that a protein could interfere with these renal vascular parameters by itself. Probably these results are due to mediators released by mesangial or endothelial cells elicited by the venom or lectin. Recent reports demonstrated that plant and animal lectins (Alencar et al., 1999; Assreuy et al., 2002) could promote inflammatory mediators release. In addition, Barraviera, Lamonte, Tarkowski, Hanson, and Meira, 1995 reported that snake-poisoned patients could release prostaglandins, cytokines, bradykinin, complement fractions and platelet activating factor (PAF). All those substances were also shown to be released by kidney cells (Havt, Fonteles, & Monteiro, 2001; Koeppen & Stanton, 1997).

In this regard, renal prostaglandins could be also interfering in sodium, potassium and chloride tubular transportation, promoted by *B. pirajai* venom and its lectin. The renal eicosanoids play important autoregulatory roles in kidney function by modifying renal hemodynamics and glomerular and tubular physiology (Foegh, Hecker, & Ramwell, 1998).

Both *B. pirajai* venom and its lectin reduced urinary flow (UF) and glomerular filtration rate (GFR), perhaps by a primary vascular relaxation and consequently interfering in those parameters. However, after testing BPL in its smaller dose (3 μg/mL) we observed that UF and GFR were significantly reduced, without any change in PP and RVR decrease. These results suggest that the glomerular filtration rate reduction effect is independent of vascular resistance drop.

In conclusion, both *B. pirajai* crude venom and BPL, a new C-type galactoside specific lectin isolated from this venom, promoted the same in vitro renal alterations observed in the isolated perfused rat kidney. At the moment a probe for eicosanoid or PAF mediation is underway.

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Structural-Function relationship of a new crotamine isoform from the *Crotalus durissus cascavella*

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Diana Hardy
Editorial Assistant

Structure-Function relationship of new crotamine isoform from the *Crotalus durissus cascavella*.

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Abstract

In this work we isolated a novel crotamine like protein from the *Crotalus durissus cascavella* venom by combination of molecular exclusion and analytical reverse phase HPLC. Its primary structure was: YKRCHKKGGHCFPKEKICLPPSSDLGKMDCRWKRK-CCKKGSGK. This protein showed a molecular mass of 4892.89 Da that was determined by MALDI TOFF mass spectrometry. The approximately pI value of this protein was determined in 9.9 by two dimensional electrophoresis. This crotamine like protein isolated here and that named as Cro 2 produced skeletal muscle spasm and spastic paralysis in mice similarly to other crotamines like proteins. Cro 2 did not modify the insulin secretion at low glucose concentration (2.8 and 5.6mM), but at high glucose concentration (16.7mM) we observed an insulin secretion increasing of 2.7 - 3.0 fold than to control. The Na^+ channel antagonist tetrodoxin (6mM) decreased glucose and Cro 2 induced insulin secretion. These results suggested that Na^+ channel are involved in the insulin secretion. In this article we also purified some peptide fragment from the treatment of reduced and carboxymethiled Cro 2 (RC-Cro 2) with cyanogem bromide and protease V8 from *Staphylococcus aureus*. The isolated pancreatic β-cells were then treated with peptides only at high glucose concentration (16.7mM), in this condition only two peptides induced insulin secretion. The amino acid sequence homology analysis of the whole crotamine as well as the biologically active peptide allowed determining the consensus region of the biologically active crotamine responsible for insulin secretion was KGGHCFPKE and DCRWKWKCCKKGSG.

Key word: crotamine, small basic myotoxin, Crotalus, pancreatic β-cells, insulin secretion.

Abbreviations:

HPLC, High Performance Liquid Chromatography; PLA_2, Phospholipase A_2; MALDI TOF, Matrix Assisted Laser Desorption Ionization Time-of-flight; Tris, Tris (Hydroxymethyl) amino methane; EDTA, Ethylenedinitro Tetraacetic Acid; DTT, dithiothreitol; PICO-TAG, Water amino acid analyzer work station, Lys, Lysine; TTX, tetrodoxin.

1. Introduction

Crotamine is one of the main toxins in the venom of the South American rattlesnake (Rádis-Baptista, G., 1999) and is responsible for myonecrosis on snake envenomation (Bieber and Nedelkov, 1997). Crotamine belong to a family of small basic rattlesnake venom myotoxins that include myotoxin a (Cameron and Tu, 1978), peptide C (Fox et al., 1979), myotoxin I and II (Bieber et al., 1987) and the CAM toxin (Samejima et al., 1991). This toxin exhibits high primary sequence identity and similar motifs and this identity suggest that crotamine may represent a canonical structure of that protein family (Nicastro et al., 2003). This protein has been known for a long time to be able to induce membrane depolarization dependent muscle contractions by increasing the Na^+ permeability of skeletal muscle membrane and its mode action resemble of scorpion α-toxins (Nicastro et al., 2003). The crotamine structure also showed high homology with other to α-, β-defensins and insect defensins (Green and Loewenstein, 1988 and Dimarcq et al., 1998). It is a 42 amino acid-long cationic polypeptide, containing high content of basic residues (lysines and arginines), and six cysteines, involved in 3 disulfide bonds. Molecular biology studies have demonstrated that crotamine represents a family of proteins composed of a variety of isoforms characterized two crotamine isoforms (F2 and F3), using a simplified chromatographic procedure involving reverse phase HPLC (Toyama et al., 2003). Toyama et al., (2000) isolated two active crotamines from the *Crotalus durissus terrificus* venom, although both isoforms produced the characteristic spastic paralysis in mice, they differed in their ability to stimulate insulin secretion. Toyama et al., Toyama et al., 2000, 2001a, b and 2003 showed that pancreatic isolated β-cells is a useful model for neurotoxic assay and this models was able to show slight difference in the mode action of several protein such as crotamine. Crotamine has been characterized a rich molecule with high content of basic amino acid residues arginine and liysine, which are involved in the interaction with proteic receptor on the membrane. In case of PLA_2, several studies showed that chemical modification of these amino acid residues decrease significantly the myonecrotic and other biological activities of basic PLA_2 (Soares et al., 2004). Among of the some chemical used, anidrous acetic acid has been commonly used for the modification of lysine amino acid residue (Soares and Giglio, 2003). In case of crotamine, the chemical modification or correlation between the structures and neurotoxic, myotoxic or other biological activities is poor documented. Peptides rich in basic arginine and lysine residues can be internalized by cells both in vitro (Derossi et al., 1994, Fawell et al., 1994 and Vivés et al., 1997) and in vivo (Derossi et al., 1998, Schwarze et al., 1999 and Caron et al., 2001) and thus have been used for the intracellular delivery of genes, therapeutic agents, and diagnostic probes (Dilber, et al., 1999, Hawiger, 1999, Schwarze et al., 2000, Morris et al., 2000 and Futaki et al., 2001). The number of

known natural cell-penetrating peptides (CPPs) is limited, and they differ in primary sequence and penetrating capacity. The best-studied are Antp43-58 from the *Antennapedia homeodomain* (Derossi et al., 1994), Tat48-60 from the transcription-activating factor of HIV Green and Loewenstein (1988) and Frankel and Pabo (1988), and VP22 from the structural protein of Herpes simplex virus type I (HSV-1; Elliott and O'Hare (1997). Antp43-58 is internalized by cells in culture and is conveyed to the cell nucleus, but its use as a transduction molecule is limited because it transports only small peptides (Ford et al., 2000). This report showed that positively charged amino acid has crucial role for interaction with cell. In this work, we isolated a new crotamine protein from the *Crotalus durissus cascavella* venom and determined two important amino acid segments involved in the neurotoxic and physiological activities of this protein and we evaluated the action of anidrous acetic acid on the structure and function of this crotamine.

2. Material and Methods.

2.1. Reagents and Venom

The venom was obtained from the Instituto Butantan and all chemical used here was analytical, HPLC and sequence grade from Sigma and Aldrich chemical, Waters, Applied Biosystens, Pierce and Bio Rad.

2.2. Isolation, Electrophoresis and MALDI TOF spectrometry of the isolated Cro 2

Molecular exclusion chromatography: Approximatelly 35 mg of whole venom from the *Crotalus durissus cascavella* venom was dissolved in 400µl ammonium bicarbonate buffer (0.2M; pH 8.0) and homogenized at still complete dissolution, followed by clarification with high speed centrifugation (4500xg for 2min). The supernatant was recovered and injected on a molecular exclusion HPLC column (Superdex 75, 1x60cm, Pharmacia), previously equilibrated with same buffer used for dissolving the whole venom. The flow rate used for elution of the fraction was 0.2ml/ml; the chromatography was monitored at 280nm and the fractions isolated was immediately lyophilized and stored at -40°C. The crotamine from the *Crotalus durissus cascavella* venom was purified by reverse phase HPLC according to method described by Toyama et al. (2000). Briefly, three milligrams of whole crotamine was dissolved in 250µl of buffer A and centrifuged at 4500xg for 2 minutes and the supernatant was then applied on the analytical reverse phase HPLC, previously equilibrated with buffer A (0.1% trifluoroacetic acetic acid (TFA) for 15 minutes. The elution of the protein was then conducted using a

linear gradient of buffer B (66.6% Acetonitrile in buffer A) and the chromatographic run was monitored at 214nm of absorbance. After elution the fraction was lyophilized and stored at -40°C. The purity degree of crotamine was assayed using two dimensional (2D) electrophoresis and MALDI TOF mass spectrometry. 2D electrophoresis was conducted as described by Anderson (1991). The spectrometry used proteins purified by HPLC, with the protein was spotted on a sample plate and introduced into the MALDI TOF mass spectrometer.

2.3. Reduction and carboxymethylation; digestion and amino acid sequence determination of Cro 2

Ten milligrams of purified protein were dissolved in 200 µl of 6M guanidine chloride (Merck, Darmstadt, Germany) containing 0.4mM Tris-HCl and 2 mM EDTA (final pH 8.15). Nitrogen was flushed over the top of the protein solution for 15 min, which was then reduced with DTT (6M, 200 µl) and carboxymethylated with ^{14}C–iodoacetic acid and cold iodoacetic acid. Nitrogen was again flushed over the surface of the solution and the reaction tube sealed. This solution was incubated in the dark at 37°C for 1 h and desalting was done on a Sephadex G 25 column (0.7 x 12 cm) in 1mM acetic acid buffer. The eluted reduced and carboxymethylated (RC) protein was then lyophilized and stored at -20°C. One sample of this RC-protein (4.5 mg) was then digested by *Staphylococcus aureus* protease V8 for 16 h, 37°C and pH 7.4, using an enzyme to substrate ratio of 1:30. The Cro 2 peptide fragments obtained after the treatment of RC protein with protease V8 were separated by reverse phase HPLC, using analytical µ-Bondapack C18 column (0.39 x 30cm; Waters), with 0.1% TFA as solvent A and acetonitrile containing 30% of solvent A (solvent B). The elution profile was monitored at 214nm and peptides were lyophilized. Other aliquot of RC-protein (4.5mg) was digested with a 15-fold molar excess of cyanogens bromide (CNBr) over methionine residues in 70% formic acid (4ml) under nitrogen for 24 h at room temperature, after which the reaction mixture was diluted with 40 ml of water and lyophilized. Excess reagents were removed by gel filtration on a Sephadex G-25 column (1 x 20 cm) equilibrated with 10% acetic acid. The CNBr peptide fragments were separated by reverse phase HPLC, using analytical µ-Bondapack C18 column (0.39 x 30cm; Waters), with 0.1% TFA as solvent A and acetonitrile containing 30% of solvent A (solvent B). The elution profile was monitored at 214nm and peptides were lyophilized. Analysis of the amino acid sequence of the RC-protein was well as that of the enzymatically or chemically digested fragments, were performed with an Applied Biosystems model Procise f gas-liquid protein sequencer. The phenylthiohydantoin (PTH) derivatives of the amino acids were identified with an Applied Biosystems model 450 microgradient PTH-analyser.

2.4. Amino acid analysis

Approximately one nmol do the purified protein was treated hydrolyzed with 6N HCl (200µl) in presence of 10µl of phenol. The amino acid hydrolysis was performed at 106°C for 24 hour and after this time the excess of HCl was removed and hydrolyzed amino acid were redried with aqueous solution of (ethanol: water: triethylamine; 2:2:1 by vol). The pós-column derivatization was done with aqueous solution of phenylisothiocyanate (ethanol: water: triethylamine: phenylisothiocyanate; 7:1:1:1 by volume). Either sample or amino acid standard were derivatized using a PICO-TAG amino acid analyzer system. The analysis of the PTH-amino acid was made using a PICO-TAG amino acid analyzer (waters).

2.5. Chemical modification of Lys of Cro 2

The chemical modification of Lys amino acid residue by anhydrous acetic acid (Acetylation): Modification of lysine residues was performed at a protein: reagent molar ratio of 1:50. Protein (3mg) was dissolved in 1.5ml dos 0.2M Tris-HCl buffer, pH 8.0, and 10µl of anhydrous acetic acid (Sigma, USA) was added. The pH was adjusted again to 8.0 with NaOH (1M) after the addition of acetic anhydride. After 1hr of incubation at 25°C, protein was separated from the from the reagents by preparative reverse phase HPLC using a delta-pack C4 column (Waters), which was previously equilibrated with TFA 0.1% and the purification the modified Cro 2 was carried out by discontinuous acetonitrile (Acetonitrile 66.6% in TFA 0.1%) gradient.

2.6. Insulin secretion assay for Cro 2

To study the effect of Cro 2 and peptide fragment from the treatment of RC protein with protease V8 or CNBr on the isolated cells, we used the β-cells as model. These rat islets were isolated by collagenase (EC 3.4.24.3) digestion. Briefly, the pancreas was inflated with Hanks' balanced salt solution containing 0.7 mg collagenase/ml, excised and then maintained at 37 °C for 20 min. The digested tissue was washed four times and the islets separated by handpicking using a siliconized stretched Pasteur pipette. Groups of five islets were incubated for 30 min at 37 °C in 0.75 ml of Krebs-bicarbonate buffer of the following composition (mmol/ l): 115 NaCl; 5 KCl; 2.56 $CaCl_2$; 1 $MgCl_2$; 24 $NaHCO_3$; and 5.6 glucose; supplemented with 3 mg Bovine Serum Albumin (BSA) /ml, and aerated with a mixture of O_2-CO_2 (95: 5, v/v; pH 7.4). After incubation, the medium was replaced with a fresh buffer solution, and the islets incubated for 1 h in the presence of 2.8, 5.6 or 16.7 mmol glucose/l

(controls) and in presence of Cro 2, its peptide fragments or the chemically modified Cro 2. The insulin content of the incubation medium was measured by radioimmunoassay using rat insulin as the standard.

2.7. Statistical analyze

The results were expressed as the mean ± SEM. The data were analyzed by analysis of variance (ANOVA) followed by a Bonferroni test. The level of significance was set at $p< 0.05$.

3. Results

3.1 Purification of Cro 2

Crotalus durissus cascavella was the most important snake found in the Northeast of Brazil and responsible for several snakebite notifications. In this article we examine three differents *Crotalus durissus cascavella* venom extracted from the rattlesnakes in three different places in the Northeast of Brazil (São Luiz, Fortaleza and Salvador). All venoms were subjected to reverse phase HPLC and we determined the account of crotoxin, PLA_2, crotapotin and crotamine. According the Fig. 1a we showed that *Crotalus durissus cascavella* venom extracted from the Fortaleza was the only that showed presence of crotamine fraction, which are absence in the São Luiz and Salvador rattlesnakes. The Cro 2 fraction isolated here was purified from the venom collected from the rattlesnake specimens from Fortaleza. Crotamine like protein (Cro 2) was purified from the whole venom of *Crotalus durissus cascavella* by combination of two chromatographic procedures, molecular exclusion and other a reverse phase HPLC. Crotamine appears as single fraction eluted after crotoxin peak and accounting for approximately 12% of this venom (Fig. 2a). After this first purification, this fraction was then purified on the reverse phase HPLC. The reverse phase chromatogram allowed the purification of two major crotamine like isoforms Cro 1 and Cro 2, that showed the most neurotoxic and myonecrotic protein of whole crotamine fraction eluted in the molecular exclusion step. Cro 2 accounts for approximately 38% of whole crotamine fraction presented in this venom (Fig. 2b). After two chromatographic steps, Cro 2 was purified with high molecular homogeneity as shown in the Fig. 3a, its molecular mass was estimated by Tricine PAGE-SDS in presence of DTT (1M) in 5kDa. This protein also showed a pI value of 9.5 that was determined by two dimensional electrophoresis (Fig. 3b) and its purity degree was corroborated by MALDI-TOFF mass spectrometry that showed a molecular mass for crotamin of 4.98 kDa (Fig. 3c).

3.2. Amino acid sequencing of Cro 2

After the protein carboxymethylation and reduction, one aliquot of this sample was treated with cyanogens bromide that cleavage specifically Met-X bonds, after the incubation time, the resulting peptides were separated by reverse phase HPLC column. The chemical cleavage of Cro 2 give two major peaks (Fig. 4a), which are named as CNBr-1 and CNBr-2. The amino acid sequences of these peptides were YKRCHKKGGHCFPKEKICLPPSSDLGKM and DCRWKRKCCKKGSGK. The amino acid analysis of these fragments corroborated the sequencing results of CNBr-1 and CNBr-2 (table I). Other aliquot was treated with protease V8 from the *Staphylococcus aureus* that cleavage specifically Glu-X bounds, in this condition we recovered on the reverse phase HPLC three major fraction designated as SV8 -1, SV8-2 and SV8-3, which were determined as YKRCHKKGGHCFPKE (SV8-1), LGKMDCRWKRKCCKKGSGK (SV8-2) and KICLPPSSD (SV8-3) (Fig 4b). The amino acid composition of peptides corroborates the sequence data (table I). The primary structure of the Cro 2 was determined by direct sequencing of the RC Cro 2 and the peptides from the treatment of RC Cro 2 with cyanogen bromide and protease V8. The most important peptide fragments were SV8-2 and CNBr-1 (Fig. 5a). Cro 2 showed presence of 43 amino acid residues and showed a high amino acid sequence degree with other crotamines, small basic myotoxins and peptide C proteins (Fig.5b). In this article we used amino acid sequence of several myotoxins and crotamines deposited in the Swiss protein data bank and PubMed (Blast search). The sequence used were: crotamine isoform precursors from *C. durissus terrificus* (this work); MYX1_CRODU, MYX2_CRODU, MYX3_CRODU and MYX4_CRODU crotamine sequences from *C. durissus terrificus* (Smith and Schmidt, 1990); MYXC_CRODU - crotamine from *C. durissus terrificus* (Laure, 1975); CRO_Ile-19 - crotamine Ile-19 from *C. durissus ruruima* (Dos Santos et al., 1993); MYX1_CROVV - myotoxin-a from *C. viridis virids* (Fox et al., 1979); MYX_CROAD CAM- toxin from *C. adamanteus* (Samejima et al., 1987); MYXC_CROVH - peptide C from *C. v. helleri* (Maeda et al., 1978); MYX1_CROVC and MYX2_CROVC - myotoxin I and II from *C. viridis concolor* (Bieber et al., 1987). Cro 2 showed high homology with crotamine from the *Crotalus durissus terrificus* (MYXC_CRODU) and small basic myotoxin-a from *C. viridis virids* (MYX1_CROVV). Other small basic myotoxins and crotamines showed a amino acid similarities around 88%.

3.3. Insulin secretion

Crot 2 obtained from the repurification of crotamine on the reverse phase HPLC dose-dependently potentiated the insulin secretion only at high glucose concentration (16.7mM glucose/ l)

(Fig. 6a). Thus, in the presence of 1.0, 5.0, 10.0 and 15.0μg of Cro 2/ ml, insulin secretion was showed a dose dependent insulin secretion rate; at dose of 15μg/ml we have an insulin secretion 3.2 – 3.5 times higher than control ($p<0.001$). The previous incubation of pancreatic β-cell with tetrodoxin (6μM/ml) decreased the glucose (16.7mM) and Cro 2 (15.0μg/ ml) induced insulin secretion (Fig. 6b).

According these results we choice that 5.0 μg/ml (1.0 nmol/ ml of Cro 2) was the adequate for pancreatic β-cell stimulation. In this protocols we tested all peptide fragment obtained after treatment of RC-Cro 2 with cyanogens bromide and protease V8, which was previously showed in section 3.2. In this experimental condition we used same molar concentration of Cro 2 for evaluating of activity of CNBr-1, CNBr-2, SV8-1, SV8-2 and SV8-3. These entire fractions were adjusted at 1.0 nmol/ml and then applied to isolated pancreatic β-cell for insulin secretion performed at 16.7mM of glucose/ l. We showed that Cro 2 induced 1.5 – 1.7 times higher than control, CNBr-1 (1- 28) at same molar concentration of Cro 2 did not induced significant insulin secretion, but CNBr-2 (29-43) that correspond to the C-terminal region of Cro 2 induced a 1.4 – 1.7 times higher than to control (Fig. 7). The peptide SV8-1 (1-15) was able induce a 0.7 – 1.0 times than to control, whereas SV8-3 (16-24) did not induced any modification on the insulin secretion. The peptide SV8-2 (25-43) induces a 1.3 – 1.5 times higher than to control. All these results were summarized in the Fig. 7 and the analysis of these results showed that short N-terminal as well as the short C-terminal segments was involved in this effect. The amino acid alignment of the Cro 2 and its respective peptides fragments with high myotoxic and neurotoxic active fragments isolated from the myotoxin a of *Crotalus viridis viridis*(Baker et al., 1991). Amino acid analysis of the Cro 2 peptide fragments and myotixin a fragment allowed determining the consensus region involved in the biological activity of Cro 2 (Fig. 8).

4. Discussion

The *Crotalus durissus cascavella* venom from the species found in the Fortaleza was the only that showed the presence of crotamine. As this small basic myotoxin or crotamine are highly neurotoxic and myotoxic. Thus, this protein should be contributed for the high toxic effect of this venom if compared to other *Crotalus durissus cascavella* venom. The crotamine fraction has been described as homogeneous fraction but in this article we showed two different crotamine isoforms Cro 1 and Cro 2, but only Cro 2 showed neurotoxic and myotoxic activity. The analysis of Cro 1 and Cro 2 by MS were similar as well as pI values. Thus these results suggest that Cor 1 and Cro 2 were structurally very

similar, but these proteins have some amino acid replacements that were involved in the biological differences. Toyama et al., 2000, 2001a, 2001b showed that pancreatic β-cells is a good model for examine the interaction of drugs with excitable cells. The plasma membrane of pancreatic β cells contains selective channels for K^+ and Ca^{2+} which are essential for the initial steps of glucose-induced insulin secretion (Dawson et al., 1986 and Aschcroft, 1988). Sodium channels have also been identified in b-cells from human, rat and dog pancreas (Hiriat and Matteson, 1988 and Misler et al., 1992). Although there is evidence that extracellular Na^+ and changes in the membrane permeability to Na^+ are required for normal insulin secretion (Sehlin and Taljedal, 1974 and Donatsch et al., 1977 and Henquin, 1987), the importance of Na^+ channels for the glucose-induced insulin secretion were evidenciated by Toyama et al., 2000, 2001a. Our results showed that Cro 2 induced a dose dependent manner of insulin secretion, where maximum dose was -15μg/ml. Tetrodoxin has been reported as inhibitor of glucose-induced insulin secretion as reported by Donatsch et al., (1977), which suggest that about 40% of of the glucose-induced insulin secretion depend on the activation of voltage-dependent Na^+ channels and the interaction of toxins bound to different Na^+ channel sites is complex and certainly influenced by time and the state of channel activation. The TsTx-V (scorpion Na^+-neurotoxin) and Veratridine did not affect K^+-permeability in the absence of glucose (Marangoni et al., 1995), a condition in which ATPsensitive K^+ channels are open and maintain the membrane hyperpolarized (Cestele and Catterall, 2000). The results of Cro 2 and tetrodoxin suggest that Cro 2 binding to open state of the Na+ channels following glucose-induced despolarization (Glucose 16.7mM). In low glucose concentration (Glucose 2.8 and 5.6) did not observed any effect of Cro 2 on the insulin secretion out flow rate. Thus it is also possible that Cro 2 act on Na^+ channels modified by glucose-induced changes in metabolism.

Delineating which portions of Cro 2 are responsible for the interaction with sodium channel and insulin secretion and to try defining the mechanism of Cro 2 action. Fragments of the reduced Cro 2 generated by CNBr and SV8 cleavage were assayed for their propensity to increase the insulin secretion. Previous work delineated by Baker et al., (1991) and Nicastro et al., (2003) showed that N-terminal and C-terminal region of the crotamine or myotoxin a are responsible for the biological effect of these protein and these region were involved in the ability of these molecule to membrane neural receptors. The effects of the CNBr and SV8 fragment showed that short N-terminal segment corresponding to 1^{st} - 15st amino acid residue and the C-terminal region from the CNBr (CNBr-2) or SV8-2 showed similar values in the insulin secretion. These results suggest the most important region for the binding of Cro 2 to the sodium channel was involving the C-terminal region. The amino acid sequence of fragment to C-terminal fragments of myotoxin a showed high amino acid similarities to SV8-2 fragment of Cro 2. Thus

we conclude that DCRWKRKCCKKGSG region is the principal region involved in the biological activity of Cro 2. The precise mechanism of action of Cro 2 or the other crotamine like protein remain unknown. But, the first step of Cro 2 association with sodium channel and the presence of the positively charged amino acid residues in the C-terminal play important role for this interaction as for some protein as natural cell-penetrating peptides (CPPs).

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Table I. Amino acid composition

aa	Cro 2	CNBr-1	CNBr-2	SV8-1	SV8-2	SV8-3
Asp	02	01	01	00	01	01
Glu	01	01	00	01	00	00
Ser	03	02	01	00	01	02
Gly	05	03	02	02	03	00
His	02	02	00	02	00	00
Arg	02	01	01	01	01	00
Thr	00	00	00	00	00	00
Ala	00	00	00	00	00	00
Pro	03	03	00	01	00	02
Tyr	01	01	00	01	00	00
Val	00	00	00	00	00	00
Met*	01	01	00	00	01	00
Cys**	06	03	03	02	03	01
Ile	01	01	00	00	00	01
Leu	02	02	00	00	01	01
Phe	01	01	00	01	00	00
Lys	11	06	05	04	06	01
Trp	02	00	02	00	02	00
Total	42	28	15	15	19	09

* and ** detemined after oxidation of Cro 2 in presence of formic acid

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Captions of Figures.

Figure 1. Analytical reverse phase HPLC profile of the fractionation of *Crotalus durissus cascavella* venom from Maranhão (*C.d. cascavella* – MA), Ceará (*C.d. cascavella* – CE) and Bahia (*C. d. cascavella*- BA). The figure insert is the PAGE-SDS Tricine at 12.5%. The abbreviations used were: convulxin (Cvx), gyroxin (Gyr), crotoxin (Crtx), crotamine (Crot), Phospholipase A_2 (PLA_2), crotapotin (Crpt).

Figure 2. In 2a we showed the molecular exclusion HPLC chromarography profile of fractionation of *Crotalus durissus cascavella* whole venom to isolate the crotamine isoform fraction. The elution of crotamine peak was carried out with ammonium bicarbonate buffer at constant flow rate of 0.2ml/ min and the chromatographic run was monitored at 280nm. In Fig 2b. In 2b we showed the chromatographic profile of purification of Cro 1 and Cro 2 by reverse phase HPLC. The purification of both Cro 1 or Cro 2 was done in the discontinuous gradient of concentration of Acetonitrile 66 in aqueous solution of 0.1% (TFA 0.1%). The elution of sample was done at constant flow rate. The fractions after elution were immediately lyophilized.

Figure 3. In 3a we showed the two dimensional electrophoresis of Cro 2 where we detemined the pI of protein, after purification on the RP HPLC. In 3b we observed the Tricine PAGE-SDS profile of protein purified on the reverse phase HPLC. In this condition we estimated its molecular mass at 5kDa and we evaluated its molecular homogeneity. In Fig 3c. We showed the MALDI TOFF MS spectrometry of Cro 2, which showed a 4.89 kDa mass for Cro 2.

Figure 4. In 4a we showed the purification of peptide fragments of RC Cro 2 digested with cyanogen bromide where we observed two major peaks that are named as CNBr-1 and CNBr-2. In 4b we showed

the reverse phase HPLC profile of purification of peptide fragments from the enzymatic digestion of RC Cro 2 with protease V8 (SV8). In this figure (4b) we observed three main fraction named as SV8-1, SV8-2 and SV8-3.

Figure 5. In Fig. 5a. Determination of the primary structure of the protein where the N-terminal is the amino acid sequence of the whole Cro 2 and amino acid alignment of the novel crotamine like from Ceará compared to other crotamines and small basic myotoxins. In Fig. 5b. The amino acid sequences were obtained from the BLAST and Swiss prot protein data bank. Crotamine isoform precursors from *C. durissus terrificus* (this work); MYX1_CRODU, MYX2_CRODU, MYX3_CRODU and MYX4_CRODU crotamine sequences from *C. durissus terrificus* (Smith and Schmidt, 1990); MYXC_CRODU - crotamine from *C. durissus terrificus* (Laure, 1975); CRO_Ile-19 - crotamine Ile-19 from *C. durissus ruruima* (Dos Santos et al., 1993); MYX1_CROVV - myotoxin-a from *C. viridis virids* (Fox et al., 1979); MYX_CROAD CAM- toxin from *C. adamanteus* (Samejima et al., 1987); MYXC_CROVH - peptide C from *C. v. helleri* (Maeda et al., 1978); MYX1_CROVC and MYX2_CROVC - myotoxin I and II from *C. viridis concolor* (Bieber et al., 1987).

Figure 6. In 6a. Insulin secretion dose-response curve of Cro 2 on the pancreatic isolated β-cell in two different glucose concentration at at high (16.7mM) and in low glucose concentration we used only one toxin concentration (10μg/ ml). In 6b. We observed the effect of Cro 2 at 15μg/ ml on insulin secretion at rwo different conditions: in presence of 16.7mM of glucose only, in presence glucose 16.7mM and Tetrodoxin (TTX, 6mM). All experiments were performed using a minimal n=12 and error bars indicated the standard error of the mean. *P, 0.001 compared to the respective control.

Figure 7. Effect of Cro 2 and peptide fragments derived of the digestion of Cro 2 with cyanogen bromide (CNBr) and protease V8 (SV8). The protein and respective fragments were incubated with isolated pancreatic β-cell in presence of glucose 16.7mM. All experiments were performed using a minimal n=12 and error bars indicated the standard error of the mean. *P, 0.001 compared to the respective control.

Figure 8.Amino acid sequence of Cro 2 and the respective peptide with active peptide fragments of myotoxin a (Baker et al., 1991) and determination of consensus region after comparing of all protein.

Figure 1

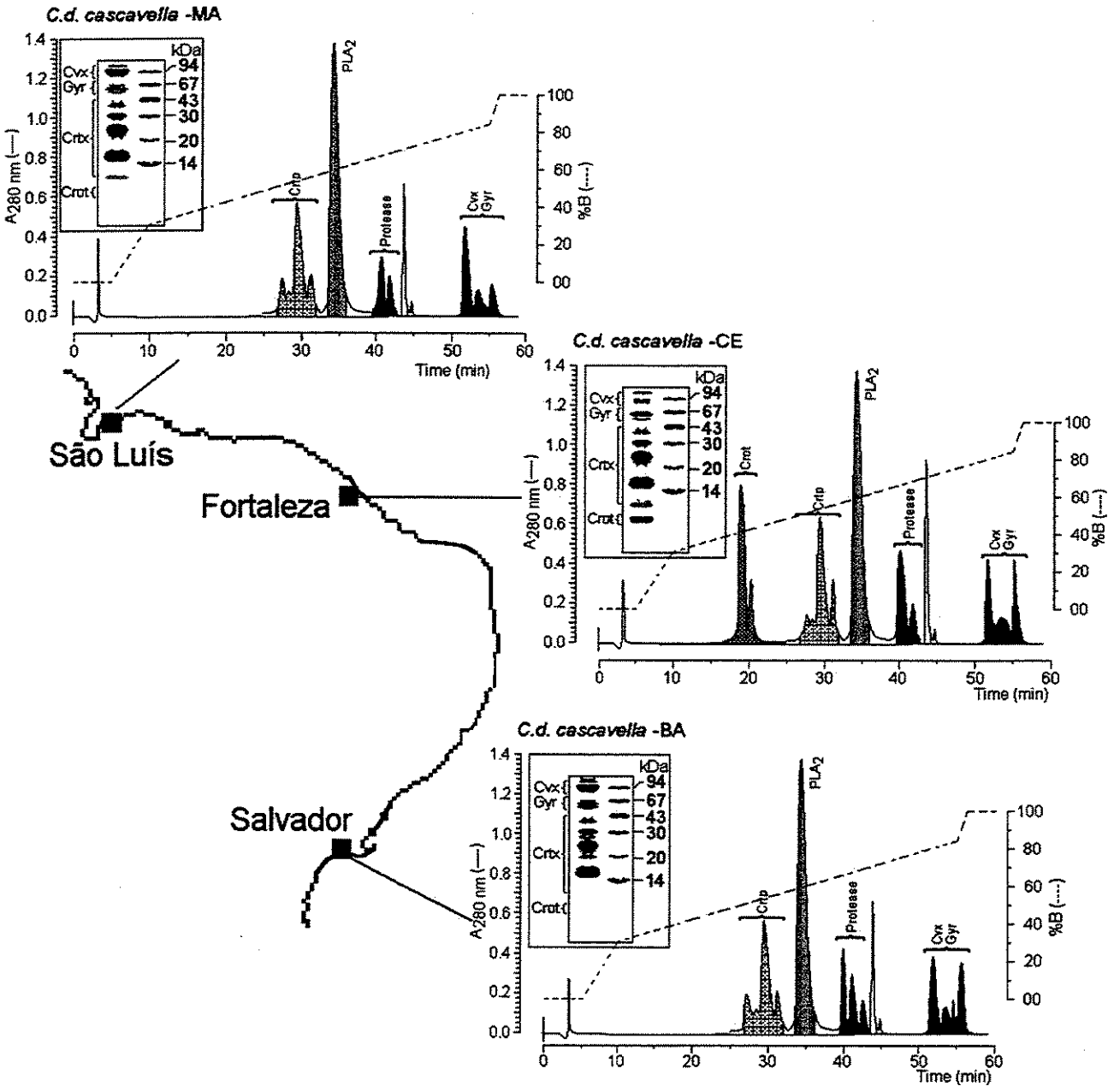
C.d. cascavella -MA
C.d. cascavella -CE
C.d. cascavella -BA
São Luís
Fortaleza
Salvador
kDa
94
67
43
30
20
14
Cvx
Gyr
Crtx
Crot
Crtp
Crmt
PLA2
Protease
Cvx Gyr
A280 nm (—)
%B (----)
Time (min)

Figure 2

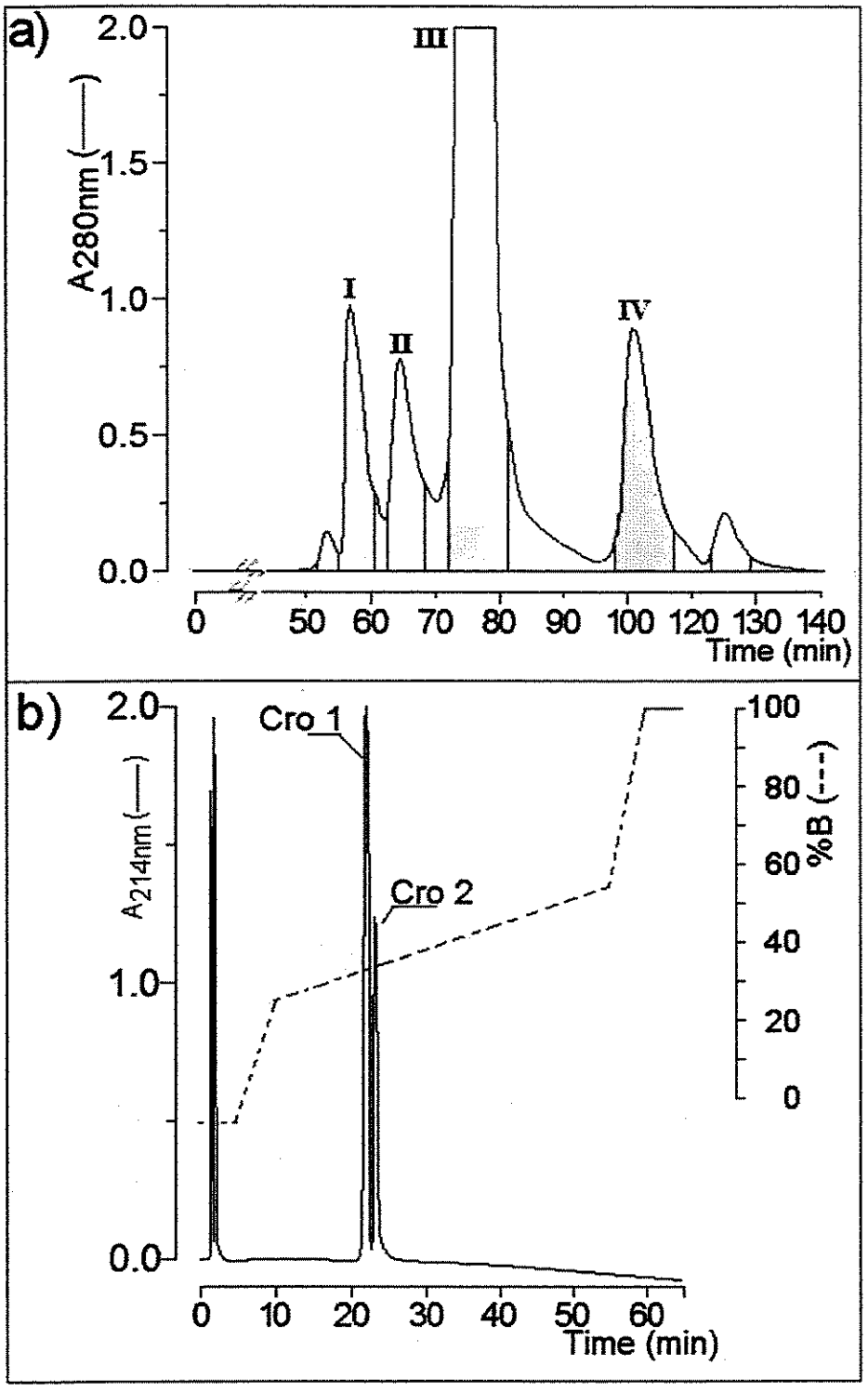
a)
2.0
1.5
1.0
0.5
0.0
A280nm (—)
I
II
III
IV
0
50
60
70
80
90
100
120
130
140
Time (min)
b)
2.0
1.0
0.0
A214nm (—)
Cro 1
Cro 2
100
80
60
40
20
0
%B (---)
0
10
20
30
40
50
60
Time (min)

Figure 3

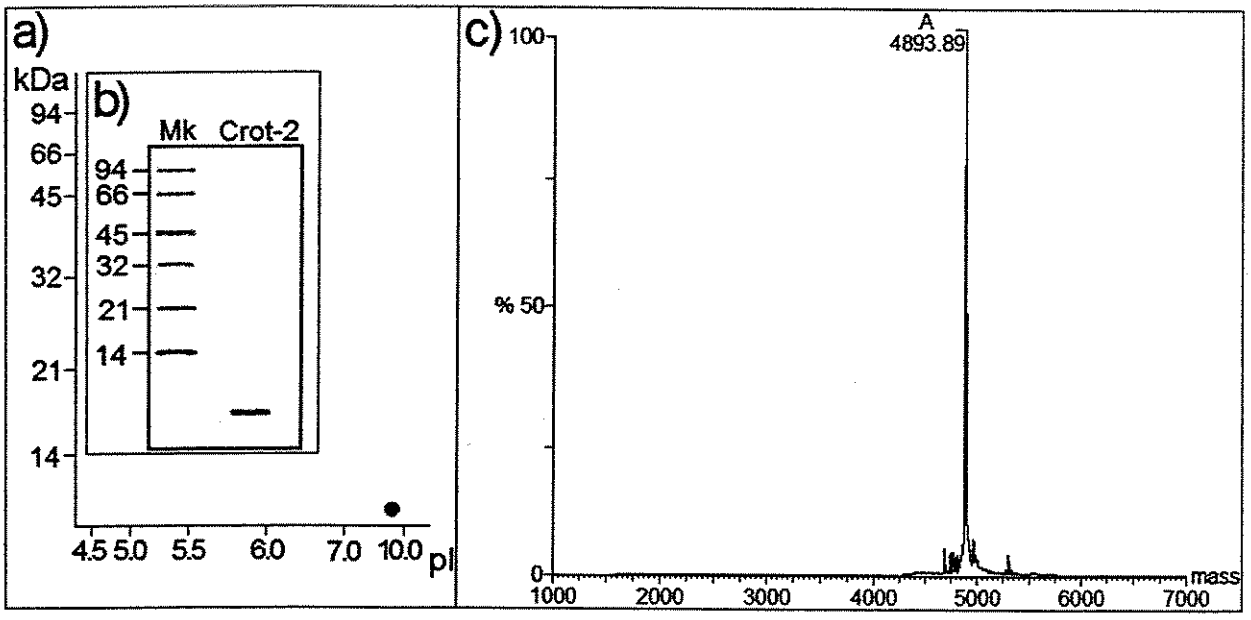
a)
kDa
94
66
45
32
21
14
b)
Mk
Crot-2
94
66
45
32
21
14
4.5 5.0 5.5 6.0 7.0 10.0
pI
c)
100
% 50
0
A
4893.89
1000 2000 3000 4000 5000 6000 7000
mass

Figure 4

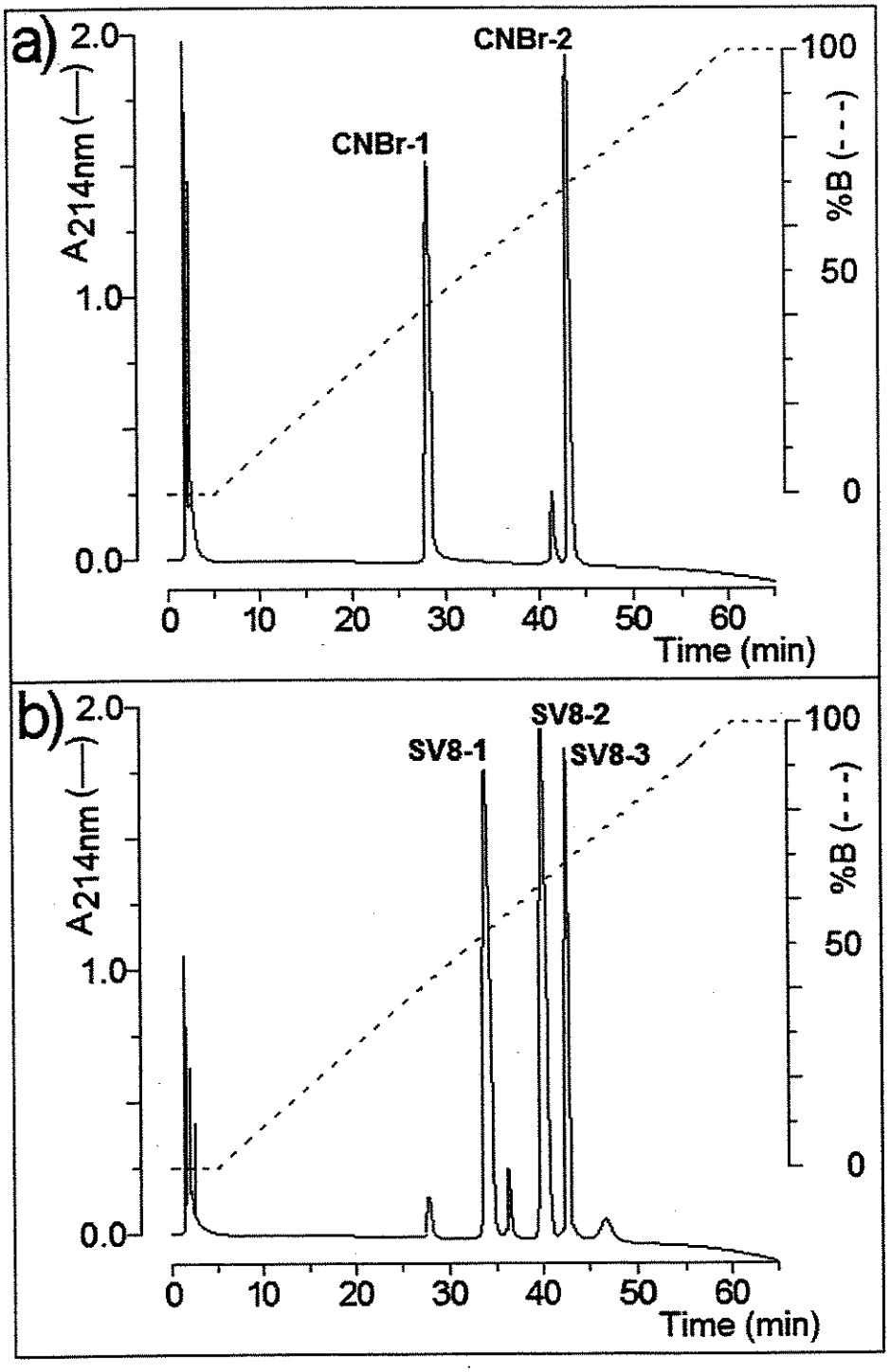
a)
2.0
1.0
0.0
A214nm (—)
CNBr-1
CNBr-2
100
50
0
%B (- - -)
0
10
20
30
40
50
60
Time (min)
b)
2.0
1.0
0.0
A214nm (—)
SV8-1
SV8-2
SV8-3
100
50
0
%B (- - -)
0
10
20
30
40
50
60
Time (min)

Figure 5

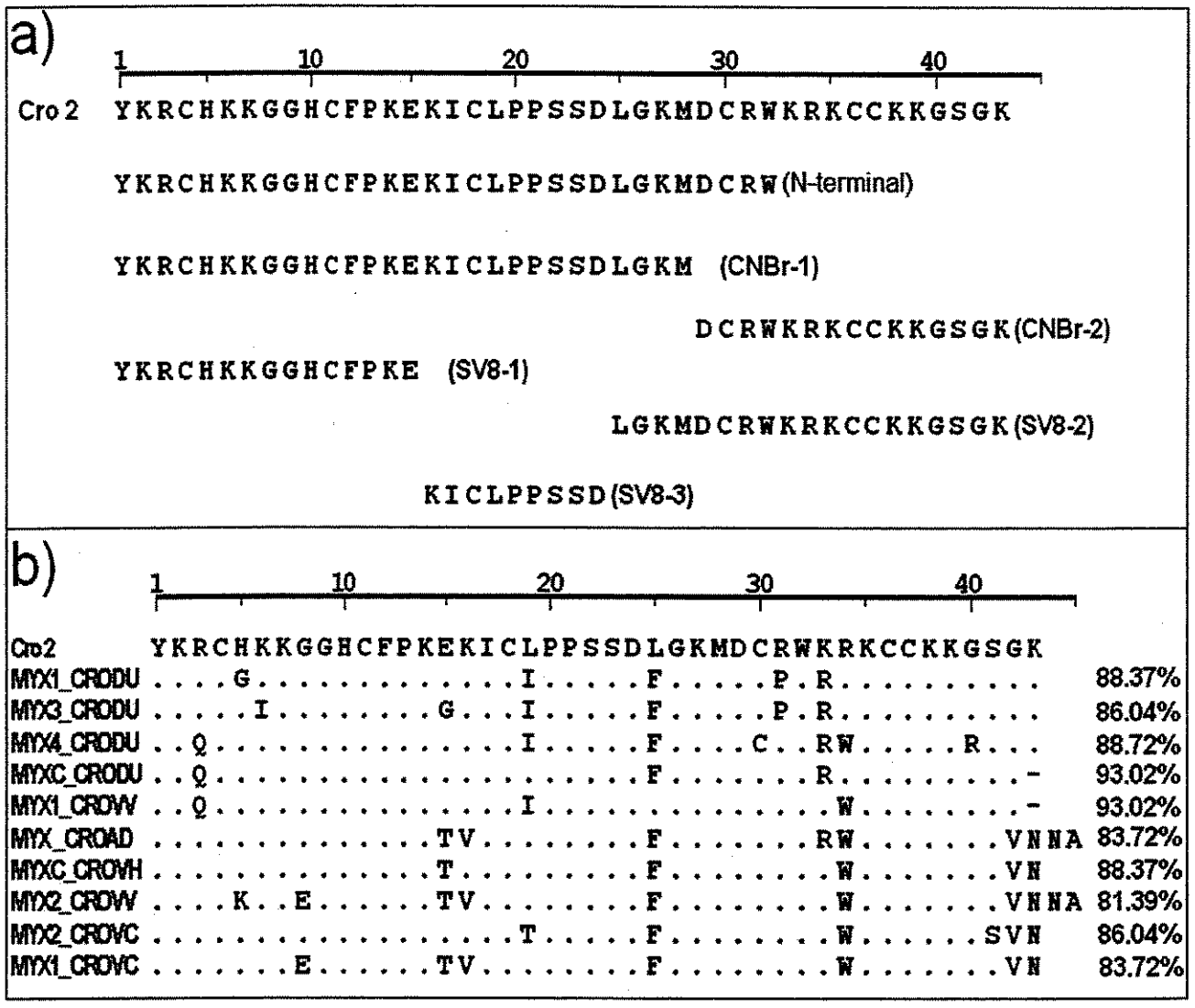
a)
1 10 20 30 40
Cro 2 YKRCHKKGGHCFPKEKICLPPSSDLGKMDCRWKRKCCKKGSGK
YKRCHKKGGHCFPKEKICLPPSSDLGKMDCRW (N-terminal)
YKRCHKKGGHCFPKEKICLPPSSDLGKM (CNBr-1)
DCRWKRKCCKKGSGK (CNBr-2)
YKRCHKKGGHCFPKE (SV8-1)
LGKMDCRWKRKCCKKGSGK (SV8-2)
KICLPPSSD (SV8-3)
b)
1 10 20 30 40
Cro2 YKRCHKKGGHCFPKEKICLPPSSDLGKMDCRWKRKCCKKGSGK
MYX1_CRODU ....G.....I.....F.....P.R..... 88.37%
MYX3_CRODU .....I.....G...I.....F.....P.R..... 86.04%
MYX4_CRODU ..Q.....I.....F....C..RW.....R... 88.72%
MYXC_CRODU ..Q.....F.....R.....- 93.02%
MYX1_CROVV ..Q.....I.....W.....- 93.02%
MYX_CROAD .....TV.....F.....RW.....VNNA 83.72%
MYXC_CROVH .....T.....F.....W.....VN 88.37%
MYX2_CROVV ....K..E.....TV.....F.....W.....VNNA 81.39%
MYX2_CROVC .....T.....F.....W.....SVN 86.04%
MYX1_CROVC .....E.....TV.....F.....W.....VN 83.72%

Figure 6

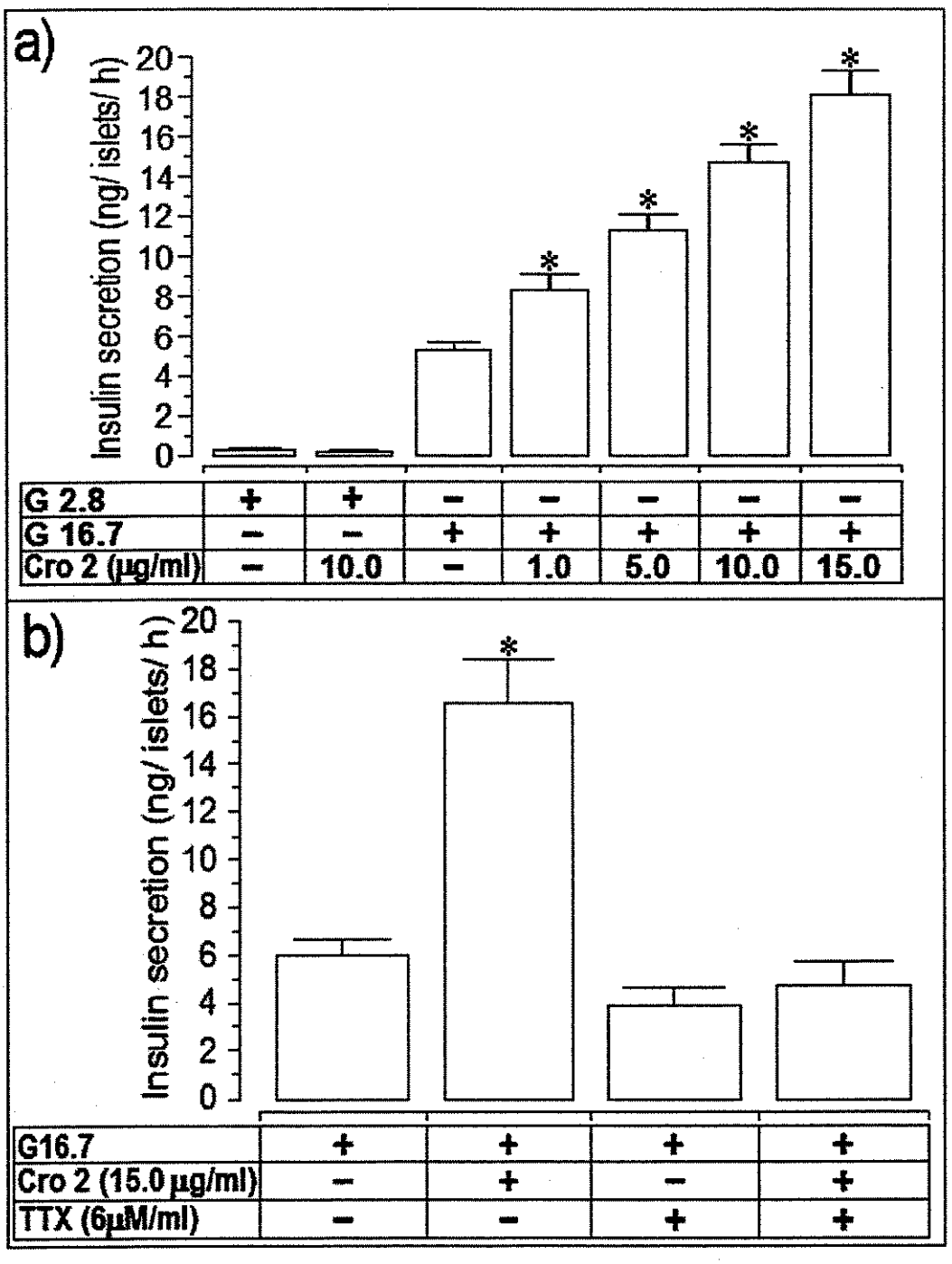
a)
Insulin secretion (ng/ islets/ h)
0
2
4
6
8
10
12
14
16
18
20
*
G 2.8 + + − − − − −
G 16.7 − − + + + + +
Cro 2 (µg/ml) − 10.0 − 1.0 5.0 10.0 15.0
b)
Insulin secretion (ng/ islets/ h)
0
2
4
6
8
10
12
14
16
18
20
*
G16.7 + + + +
Cro 2 (15.0 µg/ml) − + − +
TTX (6µM/ml) − − + +

Figure 7

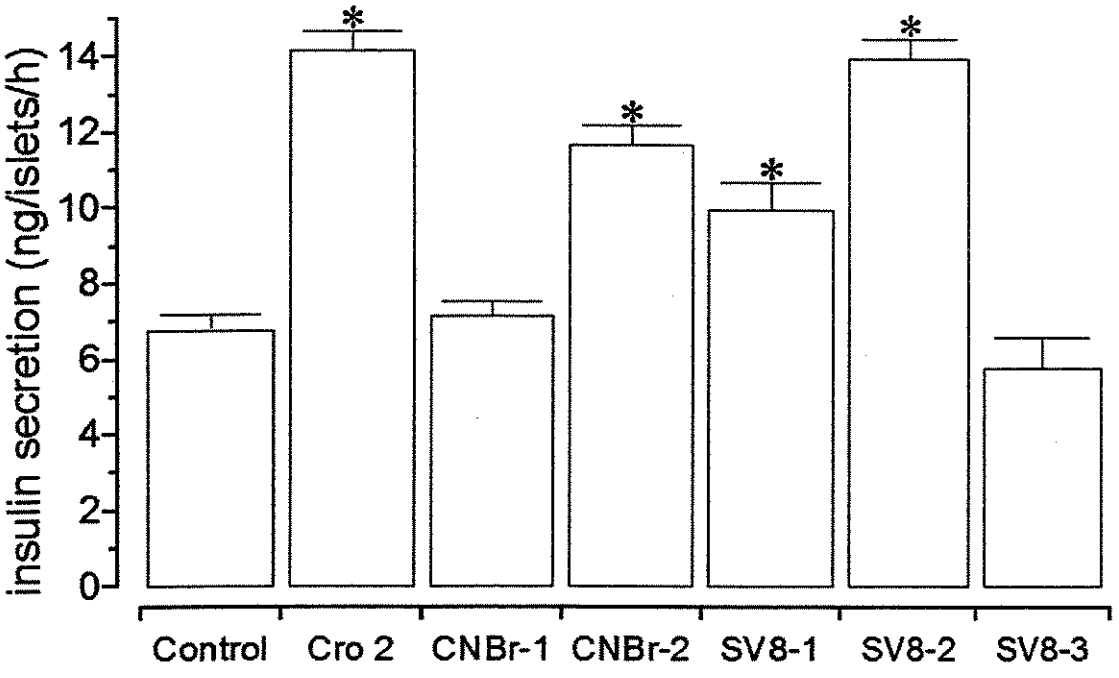
insulin secretion (ng/islets/h)
0
2
4
6
8
10
12
14
*
*
*
*
Control
Cro 2
CNBr-1
CNBr-2
SV8-1
SV8-2
SV8-3

Figure 8


1
10
20
30
40
MYX2_CROCA YKRCHKKGGHCFPKEKICLPPSSDLGKMDCRWKRKCCKKGSGK
Myotoxina YKQCHKKGGHCFPKEK LGKMDCRWKWKCCKKGSG
Myotoxina KGGHCFPKEK DCRWKWKCCKKGSG
CNBr-1 YKRCHKKGGHCFPKEKICLPPSSDLGKM
CNBr-2 DCRWKRKCCKKGSGK
SV8-1 YKRCHKKGGHCFPKE
SV8-2 LGKMDCRWKRKCCKKGSGK
SV8-3 KICLPPSSD
CONSENSUS xxxxxxKGGHCFPKExxxxxxxxxxxxxxDCRWKRKCCKKGSG

4. TRABALHOS SUBMETIDOS À PUBLICAÇÃO

CROTACETIN, A NOVEL SNAKE VENOM C-TYPE LECTIN, IS HOMOLOG OF CONVULXIN

Gandhi Rádis-Baptista, Frederico Bruno Mendes Batista Moreno, Lucas de Lima Nogueira, Alice M.C. Martins, Daniela de Oliveira Toyama, Marcos H. Toyama, Walter Filgueira de Azevedo Jr., Benildo Souza Cavada, Tetsuo Yamane.

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UNIVERSIDADE FEDERAL DO CEARÁ - UFC
CENTRO DE CIÊNCIAS
DEPARTAMENTO DE BIOOUÍMICA E BIOLOGIA MOLECULAR

September 21, 2004

Dear editor of the Biochemical and Biophysical Research Communications:

We are very glad to submit for publication our manuscript entitled "CROTACETIN, A NOVEL SNAKE VENOM C-TYPE LECTIN, IS HOMOLOG OF CONVULXIN".

In this work, we report (a) the cloning of a novel C-type lectin gene from *C. durissus terrificus*, (b) the isolation and characterization of its gene product, crotacetin β subunit, from the venom of three *C. durissus* subspecies (c) its predominant presence in the venom of *C. durissus cascavela*, and (d) the prediction of its three dimensional structure. Importantly, we demonstrated that crotacetin induces platelet aggregation, erythrocytes agglutination, and growth restrain of bacterial cells.

So, we are sure that our research article is noteworthy for the scientific community and it is in the scope of this journal.

Looking forward to hear from you.

Yours,

Dr. Gandhi Rádis Baptista
(Scientific Researcher)

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CROTACETIN, A NOVEL SNAKE VENOM C-TYPE LECTIN, IS HOMOLOG OF CONVULXIN

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Abstract

Snake venom (sv) C-type lectins encompass a group of hemorrhagic toxins, which are capable of interfering with blood stasis. They share significant similarity in their primary structures with C-type lectins of other animals, and also present a conserved carbohydrate recognition domain (CRD). In contrast, snake venom C-type lectins do not necessarily bind carbohydrate molecules nor requires calcium ions for their biological activity. A very well studied svC-type lectin is the heterodimeric toxin, convulxin (CVX), from the venom of South American rattlesnake, *Crotalus durissus terrificus*. It consists of two subunits, alfa (CVXα, 13.9 kDa) and beta (CVXβ, 12.6 kDa), joined by inter and intra-chain disulfide bounds, and it is arranged in a tetrameric $\alpha_4\beta_4$ conformation. Convulxin is able to activate platelet and induce their aggregation, by acting via p62/GPVI collagen receptor. By PCR homology screening, several cDNA precursors homolog of CVX subunits were cloned. One of them, named crotacetin β subunit, predicted a polypeptide with a topology very similar to the tri-dimensional conformations of other subunits of convulxin-like snake toxins, as determined by computational analysis. Using gel permeation and reverse phase high performance liquid chromatographies, crotacetin was purified from *C. durissus* venoms. Crotacetin is distributed among the venoms of several *C. durissus* subspecies, but its prevalence is in the venom of *C. durissus cascavella.* Importantly, crotacetin induces platelet aggregation, erythrocytes agglutination, and growth restrain of bacterial cells.

Author keywords: *Crotalus durisssus* venom, snake venom C-type lectin, platelet aggregation, erythrocyte agglutination, and anti-microbial avtivity,

1. Introduction

Snake venoms are complex mixtures of polypeptides and non-protein components with the ability to disturb the homeostais of prey organisms. The venom composition is diverse, differing among species (inter-specific venom variations) and individuals (intra-specific venom variations) [1]. Hereditary and epigenetic factors (e.g., geographical distribution, diet, snake maturity) contribute to the venom variability [2-6]. Based on victim's symptom of snakebite, snake venom can basically be classified as neurotoxic or hemorrhagic. Neurotoxins act on ion-channels and neural receptors [7]. Hemorrhagic toxins interfere with blood stasis and major classes of these toxins encompass metalloproteases, metalloproteases, phospholipases, disintegrins, and C-type lectins, as recently reviewed [8, 9].

C-type lectins are animal proteins of about 130 amino acids long, containing at least a carbohydrate recognition domain (CRD) capable of mediating sugar and calcium binding. The carbohydrate recognition is directly related to some biological activities, like cell-cell adhesion, serum glycoprotein turnover, and innate immune responses against potential pathogens [10]. Snake venom C-type lectins also contain the conserved CRD and share significant primary structure similarities with the animal C-type lectins, but they not necessarily bind carbohydrate molecules nor require calcium ions for their activity [9, 10]. A very well studied snake venom C-type lectin is a heterodimeric toxin, convulxin (CVX), from the venom of South American rattlesnake, *Crotalus durissus terrificus*, [11]. This protein consists of two subunits, alfa (CVXα, 13.9 kDa) and beta (CVXβ, 12.6 kDa), joined by inter and intra-chain disulfide bounds, and arranged in a tetrameric $\alpha_4\beta_4$ conformation [12, 13]. Convulxin is able to activate platelet and induce their aggregation, by acting via p62/GPVI collagen receptor [14]. Some other snake toxins with similar properties of activating and aggregating platelets are also known. They are bitiscetin, from the venoms of *Bitis arietans* [15], botrocetin, from *Bothrops jararaca* [16], flavocetin A [17], ophioluxin, a protein from *Ophiophagus hannah* [18], and mucrocetin, a platelet-agglutinin from *Trimerusurus mucrosquamatus* [19]. Recombinant convulxin (rCVX) has been cloned and expressed in *Drosophila* cells. Purified rCVX from cell culture supernatants binds strongly to human platelet GPVI in western blot assay when using whole platelet proteins or recombinant human GPVI as target. Importantly, rCVX induces

alpha, AF541881 and Y16349, convulxin subunit beta) one oligonucleotide primer forward CVXA/B-FW1 (5'-TCTCTCTGCAGGGAAGGAAG-3'), and two reverse primers, CVXA-RV1A (5'-TCCTTGCTTCTCCAGACTTCA-3') and CVXB-RV2B (5'-ACTTCACACAGCCGGATCTT-3') were synthesized, which correspond to 5'-UTR of CVX subunit α and β (forward primer), and to 3'-UTRs of CVX subunit α (reverse primer RV1A) and of CVX subunit β (reverse primer RV2B), respectively. The gene of one subunit of crotacetin (CTC subunit alpha) was amplified with the same primer pair used to amplify CVX subunit α, in the following way: phage particles (10^7 - 10^8 pfu) of *C. durissus terrificus* venom gland cDNA library, 10 pmoles of each primer, and 25 µl of ExLONGase enzyme mix (Invitrogen Life Technologies, San Diego, CA), were mixed and the PCR performed according to the manufacturer. The LD-PCR products were purified from the gel slice and cloned into pCR2.1-TOPO (Invitrogen Life Technologies, San Diego, CA).

The gene was sequenced with ABI Prism Big Dye Terminator (Perkin Elmer, Foster City, CA) in an automated sequencer (ABI Prism 373 or 377, Perkin Elmer) using synthetic oligonucleotides designed for CXA/B sequences (this work). The cloned crotacetin mRNA (accession number AF541884) was compared against nucleotide sequences on the GenBank at NCBI (*http://www.ncbi.nlm.nih.gov*), using the BLAST algorithm, and the Biocomputing software Lasergene (DNAStar, Inc., Madison, WI).

2.4. Similarity search and homology modeling

The amino acid sequence predicted from the corresponding *Crotalus durissus terrificus* precursor cDNA was compared against the Protein Data Bank (http://www.rcsb.org/pdb/) using BLASTP as a tool for protein search and alignment [23]. Several sequences, chosen as the best score after alignment, were used to identify conserved residues among homologous sequences. Snake venom C-type lectin sequences from the venoms of *Trimeresurus flavoviridis* (pdb entry code 1C3A, chain B) [17], *Trimeresurus mucrosquamatus* (1V4L, chain B) [19] and *Crotalus durissus terrificus* (1UOS, chain B and 1UMR, chain B) [12, 13] were aligned by CLUSTALW [24] applying default parameters.

the aggregation of platelets in platelet-rich-plasma, indicating that the recombinant CVX subunits can assemble into a functionally competent complex [20].

During the procedures for cloning convulxin α and β subunits, we have isolated several cDNA precursors, which are homologous of CVX subunits. In this work, we have characterized one of them, a new member of snake venom C-type lectin family, named crotacetin (CTC).

2. Methods

2.1. Snake and snake venom

For the construction of venom gland cDNA library, an adult specimen of *Crotalus durissus terrificus*, captured in São Paulo state was provided by the Laboratory of Herpetology, Instituto Butantan, São Paulo, Brazil. The snake was milked to collect the venom and to induce maximum level of RNA synthesis in venom gland [21]. The crude venom was vacuum dried and kept at –20°C until protein purification. The venom of *Crotalus durissus cascavella* was a gift from the Regional Snake Laboratory of Fortaleza (LAROF), Ceará, Brazil and the venom of *Crotalus durissus collilineatus* was purchased from the Bio-Agents Serpentarium in the city of Batatais, São Paulo, Brazil.

2.2. *C. d. terrificus* venom gland cDNA library construction

The cDNA library was constructed as described elsewhere [22]. Briefly, poly(A^+) RNAs were purified from a pair of venom gland excised from a single specimen of South American rattlesnake, *C. d. terrificus* (*Cdt 9706*). The complementary DNAs (cDNAs) were synthesized, selected by size and cloned into a phagemide vector – a lambda phage derivative (λ ZapII, Strategene). Recombinant phagemids were packed into viable phage particles and used to infect *E.coli* cells XL1 Blue MRF' (Stratagene, La Jolla, CA). The venom gland cDNA library was titrated, amplified and stored at - 80°C in 7% DMSO for posterior utilization.

2.3. PCR homology cloning

Based on nucleotide sequences of subunit α(or A) and β(or B) of convulxin (CVX) from *C. durissus terrificus* (GenBank accession no. AF541882 and Y16348, convulxin subunit

The alignment was used to build a tree dimensional structure of the CTC β subunit C-type lectin. One thousand models were generated throught PARMODEL, a web server at http://www.biocristalografia.df.ibilce.unesp.br/tools/parmodel [25], which paralelizes the MODELLER [26] sofware in a Beowulf cluster (16 nodes). The best model, that was selected according to the Modeller objective function. The model was evaluated by PROCHECK [27], WHATCHECK [28], and 3DANALYSIS [29]. The permissible angles of amino acid residues in the spatial strucuture of CTC β subunit were also evaluated by RAMACHANDRAN plot [30].

2.5. Crotacetin purification

Whole venom (35mg) was dissolved in a 0.2 M ammonium bicarbonate (pH 8.0) buffer and then clarified by high-speed centrifugation (4500 x g for 1 min). The supernatant was injected onto a molecular exclusion HPLC column (Superdex 75, 1 x 60cm, GE Healthcare), previously equilibrated with the same buffer used for solubilization of the whole venom. Chromatography process was performed with a flow rate of 0.2 mL/min, and monitored at 280nm. Fractions corresponding to crotacetin were pooled and lyophilized. After this first step of purification, crotacetin was re-purified by reverse phase HPLC. Approximately 1mg of the purified protein was dissolved in the buffer A (TFA 0.1% in aqueous solution), the same used for equilibration of the analytical μ-Bondapack C18 column (0.39 x 30 cm). The elution of high purified crotacetin was carried out using a linear and discontinuous buffer B gradient (66% of Acetonitrile in buffer A). The chromatographic run was conducted at constant flow rate of 1.0 ml/min and monitored at 214nm.

2.6. Reduction, S-carboxymethylation and determination of N-termianl sequence

Two milligrams of purified crotacetin were dissolved in 200 ml of a 6.0 M guanidine chloride solution (Merck, Darmstadt, Germany), containing 0.4 M of Tris-HCl and 2 mM EDTA (pH 8.15). Nitrogen was blown over the top of the protein solution for 15 min, followed by molecular reduction with 200ml of a 6.0 M DTT and further carboxymethylated with ^{14}C-iodoacetic acid and cold iodoacetic acid. Nitrogen was again

blown over the surface of the solution and the reaction tube was sealed.This solution was incubated in the dark at 37°C for 1 hour and desalted using a Sephadex G25 column (0.7 x 12 cm) with 1.0 M acetic acid buffer. The eluted RC-Crotacetin was then applied on the reverse phase HPLC μ-Bondapack C-18 column (0.39 x 30cm), previously equilibrated with buffer A (TFA 0.1% in aqueous solution). The subunits of crotacetin were eluted from the reverse phase HPLC by non linear gradient concentration of buffer B (Acetonitrile 66% in buffer A) and the fraction corresponding to each crotacetin subunits were recovered, lyophilized and stored at -80°C. Crotacetin subunits were then sequenced by automatic Edman degradation, using a gas-liquid protein sequencer (Applied Biosystems model Precise). The amino acid phenylthiohydantoin (PTH) derivatives were identified using a PTH-analyser (Applied Biosystems model 450 microgradient).

2.7. Platelet aggregation and erythrocytes agglutination

Platelet-rich plasma (PRP) was prepared as described by Chudzinski-Tavassi et al. [31] and platelet aggregation studies were performed using a Chrono-Log Lumi aggregometer (Havertown, PA). PRP samples were incubated at 37°C for 2 min with different concentrations of purified crotacetin. Platelet agglutination was monitored by turbidometry and expressed as an increase in light transmittance. Collagen was used as control of platelet aggregation.

Rabbit red blood cells were washed and centrifuged ten times with 0.15 M NaCl to recover only intact erythrocytes. The hemagglutination activity was performed with 2 % erythrocytes isotonic suspension, and serial dilution of a purified CTC diluted with 0.1 M Tris-HCl (pH 7.6), at 37°C for 30 min. The agglutination was monitored visually after 30 min at room temperature and the activity was expressed as the minimum concentration of CTC that promoted visible microscopic erythrocytes agglutination.

2.8. Antibacterial activity and Transmission electron microscopy.

Xanthomonas axonopodis pv passiflorae (Gram-negative), or *Clavibacter michiganensis michigansis* cells were harvested from fresh agar plates and suspended in sterile distilled water (A_{650nm} = 0.3/ cc 10^3CFU/ml). Aliquots of bacterial suspension were diluted to a 10^{-5} CFU/ml and incubated with crotacetin (150 μg/ml) for 20 minutes at 37°C,

after which the survival was assayed on nutrient (Difco) plates (n=5). In both antibacterial assays, electron microscopic assessments of morphological alterations were done in absence (control) or presence of crotacetin (A_{650nm} = 0.3/ cc 10^3 CFU/ml). The bacterial samples were fixed with 1% osmium tetraoxide (Agar Scientific Ltd) for 2 h at 25°C. Sections were washed three times, dehydrated in increasing concentrations of ethanol and propylene oxide, and embedded in Epon resin (Agar Scientific). Polymerization was performed at 60°C for 48 h, and ultra-thin sections were prepared with a Sorvall MT2 ultramicrotome. The sections were placed on 5% collo-dion-coated 100-mesh grids, and stained with 4% uranyl acetate (Agar Scientific) for 15 min, followed by 2.6% lead citrate (Agar Scientific) for 15 min. Samples were observed under a Hitachi 1100 transmission electron microscope (Hitachi Scientific Instruments, Japan) operating at 100 kV.

3. Results

3.1. PCR homology cloning of crotacetin β subunit

The gene coding for crotacetin β subunit, CTCβ, (GenBank accession number **AF541884**) was isolated from *Crotalus durissus terrificus* venom gland cDNA library by PCR-homology screening with specific primers for the 5'-UTR and for 3'-UTR of both convulxin subunit α (CVXα) and β (CVXβ), as described in methods.

The cloned CTCβ gene is 513 bp long with a 5'-untranslated region (5'-UTR) strictly conserved in all convulxin-like and anti-thrombin like precursors, isolated so far (Fig. 1). In CTCβ gene, the ATG start codon is located 28 bp downstream, and the stop codon (TGA) is located 496 bp. A sequence corresponding to the signal peptide lies between the nucleotides 28 and 96 (Fig 1, underlined).

At nucleotide level, the similarity between CTCβ and CVXβ is higher than between CTCβ and CVXα or CTCβ or ATLs. In contrast the similarity of CVXα and ATLs is proportionally high. The CTCβ gene precursor predicts a polypeptide of 148 residues of amino acids, 23 corresponding to the leader sequence, and a molecular mass of 14.3 kD.

3.2. Sequence alignment and tri-dimensional moddeling

Comparison of predict amino acid sequence of CTCβ gene against protein data bank reveals a high similarity of CTCβ with C-type lectin subunits from the venom of *Trimeresurus flavoviridis* (pdb entry code 1C3A, chain B), *Trimeresurus mucrosquamatus* (1V4L, chain B) and *Crotalus durissus terrificus* (1UOS, chain B and 1UMR, chain B) (Fig. 2A). The similarity of CTCβ and these other snake venom aggregating toxins is around 50%. Whether conserved substitutions are considered, then the similarity exceeds 70%. The eight residues of cysteine in primary structure of CTCβ are loacted in the same position of all aligned sequences. This positions include the six residues invoveld in intra-chain disulfide bridges, and the two other "extra" cysteines in carboxi- (Cys3) and amino- (Cys77) terminals.

The computer generated model of CTCβ shows an overall topology of five principal β-strands and two α-helices, forming a globular structure with a lateral loop similar to the other convulxin-like C-type lectins (Fig. 2B). The lateral loop contributes to the formation of heterodimers that is stabilized by the "extra" cysteine residue.

Ramachandran diagram of CTCβ modell gives indication that psi (ψ) and phi (ϕ) angles of the majority of amino acid residues are located in thermodinamically favorable region (Fig. 2C).

3.3. Isolation, reduction and N-terminal sequence of crotacetin from *C. durissus*

By exclusion chromatography, crotacetin appear as a minor fraction peak in the venom of diverse *Crotalus durissus* subspecies, namely, *Crotalus durissus cascavella, Crotalus durissus terrificus and Crotalus durissus collilineatus* (Fig. 3A). In the venom of *Crotalus durissus cascavella*, the amount of crotacetin is significantly abundant (represents around 0.8% of the proteins in the crude venom) and this protein was isolated and characterized from this venom. Purified crotacetin from *Crotalus durissus cascavella* shows an apparent molecular mass of 70 kDa, by gel permeation chromatography, and it appears as two subunits of different size by reverse phase HPLC (Fig. 3B). Furthermore, by molecular exclusion chromatography it appears as a high molecular weight, indicating the presence of oligomeric forms.

The partial N-terminal amino acid sequence of purified crotacetin (β subunit) corresponds to that predicted from the gene sequence (Fig. 3C).

3.4. Platelet agglutination and erythrocyte aggregation assays

Crotactin is capable of aggregating human platelet, in platelet rich plasma (PRP), in a dose-dependent manner: 22% at concentration of 32.8 μg/ml (47 μM), 36 % at 49.3 μg/ml (70 μM), and 84 % at 65.5 μg/ ml (94 μM), (Fig. 4A).

Agglutination of rabbit red blood cells was also detected by optical microscopy when using concentrations of crotacetin in the range of 160 μg/ml (23 μM), independent whether calcium ions were present in the mixture of reaction (Fig 4B and 4C).

3.5. Anti-microbial activity of native crotacetin and of isolated α and β subunits

The whole (intact) crotacetin protein decreased the bacterial growth of two plant pathogen: *Xanthomonas a. pv. passiflorae*, a Gram-negative and *Clavibacter m. michiganensis*, a Gram-positive. The inhibition rates were in the range of 87.8% and 96.4%, respectively. The isolated chains did not show significant anti-microbial activity (Figs. 5a and 5b) for both species of bacteria tested. In case of *Xanthomonas a. pv. passiflorae*, crotacetin induced a massive vacuolization of cell cytoplasm and in some case the rupture of membrane (Figs. 5c and 5d).

4. Discussion

Using specific forward primer for the 5'-UTR and reverse for 3'-UTR of both convulxin subunit α (CVXα) and β (CVXβ), we have isolated several cDNA precursors from *Crotalus durissus terrificus* venom gland cDNA library that are homologues of convulxin (CVX) subunits. The nucleotide sequence of two of these precursors, precisely of their subunits, and both CVXα and CVXβ subunit sequences, and the deduced amino acid sequence of CTCβ, are shown in Figure 1. These cDNA sequences present conserved nucleotides starting at – 27 bp, from the ATG initiation codon, and extending several bases along the precursor. All of the isolated genes, coding for convulxin-like subunits, encompass open reading frames (ORFs) of approximately 500 nucleotides (CVXβ, 490 nt;

CTCβ, 513 nt; CVXα, 537 nt; ATLs, 522 nt). Crotacetin β and CVXβ genes are almost identical (82 % of similarity, 439/537 nt), whereas CVXα and ATLs genes are more related to each other (circa of 83%, 409/490 nt). When CTCβ mRNA is compared with other sequences in gene databank, identities of over 86% are found. For example, mucrocetin β chain mRNA (**AY390534**), from *Protobhotrops mucrosquamatus*, shares 87% of identity (439/537 nt). Almost two hundred mRNA sequences of snake venom C-type lectin share some similarity with crotacetin β subunit and with one another.

The high homology of *C. durissus* C-type lectin genes, and with some others from different snakes, points out to their own evolutionary history. These genes seem to have arisen by more than one single event of gene duplication, what probably occurred after the division of Viperidae and Colubridae. The α- and β- chains of the C-type lectins are restricted to viper and pit viper snakes and, therefore, compose a monophiletic gene clade [32]. However, duplication is not the only event for genes to evolve. Nucleotide substitution (transversion and transition) and deletion play a role in gene evolution, consequently generating a family of homologous genes and polypeptides with diverse functions or targets. In fact, toxin genes present conserved non-coding regions, whereas having hyperdiverse coding region [33-36]. In general, the coding region of a given toxin gene lies in exons which are separated from the exon encompassing the leader sequence of the toxin and its 5'-UTR [37-39]. Thus, the gene organization has influence not only in its own evolution, but also in the protein diversity. For instance, gene organization allows accelerated evolution of polypeptides, where gene hypervariation favor evolutionary advantage, as in the case of prey and predator relationship and, at molecular level, of agonist/antagonist and cellular receptor interactions [40-42].

Comparison of deduced amino acid sequence of crotacetin β subunit with members of snake venom hemorrhagic toxins have corroborated that CTCβ belongs to snake venom C-type lectin family. A consensus structural characteristic of convulxin-like proteins rely on eight residues of cysteine in chain B, which are also conserved in CTC β subunit. Six cysteine residues are involved in intra-chain disulfide bridges, linking at 4-15, 32-121 and 98-113 residues. The other two cysteines are recruited in the formation of the heterodimer in a head-to-head fashion, and consequently in the $\alpha_4\beta_4$ quaternary structure, as previously observed [12, 19]. Cysteine located at position 77 in β subunit participate in an inter-chain

disulfide bridge with Cys81 from α subunit, which stabilizes the heterodimer. In heterodimer, subunits are linked by additional disulfide bridge between cysteines, that are present in the N-terminal of β subunit and C-terminal of α subunit, resulting in a conserved tetrameric $\alpha_4\beta_4$ conformation [12]. To explore the structural conformation adopted by crotacetin β subunit and to build a 3-D model of CTCβ, we had utilized amino acid sequences and coordinates data of other already crystalized convulxin-like snake venom C-type lectins. It is very well known that the two most critical problems in homology modeling are the degree of similarity among target sequences and templates, and evidently the fidelity of the alignment [26]. In case of crotacetin β subunit, its predicted 3-D structural model is practically identical to the experimentally resolved ones. The overall topology of five principal β-strands and two α-helices, forming a globular structure with a lateral loop similar to the other convulxin-like C-type lectins, is maintained. It is now known that the lateral loop contributes to the formation of heterodimers that is stabilized by the "extra" cysteine residued located at position 3 (Cys3) and 77 (Cys77) in the amino acid sequence. , Thus, in case of CTCβ and, in general, of snake venom C-type lectins, not only the primary sequences are significantly consrved but also their folds and topologies.
Based in the fact that CTC β subunit belongs to the class of convulxin-like protein with the characteristic motif of C-type lectin domains (CTLDs), including the carbohydrate recognition domain (CRD), we decide to conducte several biological assays with purified crotacetin. Although, crotacetin was cloned from *Crotalus durissus terrificus* venom gland, it can also be purified from the venom of diverse *Crotalus durissus* subspecies, appearing as minor fraction peak in the chromatograms of the venom of *Crotalus durissus cascavella, Crotalus durissus terrificus and Crotalus durissus collilineatus*. In the venom of *Crotalus durissus cascavella*, the amount of crotacetin is significantly abundant (represents around 0.8% of all proteins in the crude venom). The observation that crotacetin is expressed in the venom of several subspecies of South American rattlesnakes, inhabiting distinct geographical locations, confirm the phenomena of hypervaryability widely seen in snake venom composition [3, 4]. Interestingly, even if snake venom C-type lectins are diverse, they are restricted to the venom of Viperidae, the family that *Crotalus durissus* species belong, as mentioned above and studied elsewhere [32].
Thus far, crotacetin was isolated and characterized from the venom of *C. d. cascavella*.

Purified crotacetin from *Crotalus durissus cascavella* appears as two subunits of different size (apparent molecular mass of 70 kDa) and, by gel permeation, a high molecular weight oligomeric form protein is evidenced.

Using human platelet rich plasma, it was observed that CTC is capable of aggregating platelets in a dose-dependent manner and, in higher dosis, the platelet aggregation is more sustainable than collagen. Surprinsingly, agglutination of rabbit red blood cells was also detected by optical microscopy. Crotacetin is able to agglutinate erytrhrocytes independent whether calcium ions were present in the mixture of reaction. Actually, platelet aggregation is a common phenomenon of snake venom C-type lectins, usually mediated by glycoprotein receptors on platelet membranes. Until now, we have not studied whether crotacetin acts on GPVI receptor, as convulxin do. Whether or not, Kaniji *et al* [43] demonstrated that in addition to GPVI, convulxin bind to native human GPIbα, exhibiting dual specificity to both platelet receptors. Thus, it remains to be verified the receptor specificit of this novel member of convulxin-like family, that is, whether crotacetin has the ability to bind and activate platelet via GPVI, GPIbα or other collagen receptor on platelet, like GPIa-IIa ($\alpha_2\beta_1$ integrin), and CD36. Furthermore, glycoprotein receptors on erythrocyte membranes appear as specific molecular target for convulxin-like snake toxins, as we have demonstrated in the present work and in an recent study on recombinant BJcuL, a C-type lectin of *Bhotrops jararacussu* venom [44].

Noteworthy, crotacetin also showed a significant anti-microbial activity against two different bacterial strains, *Xanthomonas axonopodis pv passiflorae* and *Clavibacter michiganensis michiganesis*. The oligomeric form of crotacetin reduced the overall growth of both bacteria. However, the separation of intact oligomeric protein into their isolated chains significantly abolish the anti-microbial activity. As seen before, tetrameric form is a typical structure adopted by several snake venom convulxin-like C-type lectins, and this quaternary arrangement seems to be essential for anti-microbial activity. The microscopic effect of crotacetin on *Xanthomonas a. pv. passiflorae* involved the induction of citoplasmic vacuolization and membrane rupture. From our point of view, these latter results are of particular relevance, since they concern to anti-microbial activities of an snake venom C-type lectin. The most studied CTLDs-containing proteins with the property of binding to microorganisms are the mannose binding lectins (MBLs) and the lectins

receptors located on antigen-presenting cell membranes. MBLs are involved in first-line defense by binding to bacteria, viruses, protozoa and helminths and then initiating a range of host response [45]. C-type lectin receptors (CLRs) on dendritic cells are type II transmembrane proteins implicated in the pattern recognition of pathogens and in the distinction of self and no-self antigen recognition in mammals [46].

Taken these results together, it is evident that snake venom C-type lectins have their structural domains derived from a common ancestral precursor, which comprise not only a multi-gene family, but also homologous polypeptides possessing conserved C-type lectin domains with the ability to interact with glycoproteins on blood cell membranes, and with microbial cell as well.

In this work, we report (a) the cloning of a novel C-type lectin gene from *C. durissus terrificus*, (b) the isolation and characterization of its gene product, crotacetin β subunit, from the venom of three *C. durissus* subspecies (c) its predominant presence in the venom of *C. durissus cascavela*, and (d) the prediction of its three dimensional structure. Importantly, we demonstrated that crotacetin induces platelet aggregation, erythrocytes agglutination, and growth restrain of bacterial cells.

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Figure Legends

Figure 1. Comparison of nucleotide sequence of crotacetin subunit β, convulxin subunits α and β, and anti-thrombin-like subunit precursors

All genes were isolated from *C. d. terrificus* venom gland cDNA phage library by PCR homology screening. Sequences were aligned using CLUSTALW, disponible at EBI (http://www.ebi.ac.uk/clustalw/index.html) with parameters in default. The numbers in the left side of each precursor correspond to the GenBank accession number (**AF541881**, convulxin subunit b mRNA; **AF541882**, convulxin subunit a mRNA, **AF541883**, anti-thrombin like mRNA, and **AF541884**, crotacetin subunit b mRNA, all from *Crotalus durissus terrificus*). Star and stop codons are boxed in gray color. Strictly conserved 5'-untraslated sequences (5'-UTR), where the primer CVXA/B FW exactly anneals, are underlined. Dots represent conserved nucleotides of homologous sequences. Deduced amino acid sequence is under the nucleotide sequences in bold capital letters. The signal peptide is underlined.

Figure 2. Structural analysis of crotacetin subunit β

A.- Comparison of deduced aminoacid sequence of crotacetin (CTC) subunit β, from *C. d. terrificus* (this work), with other snake venom C-type lectin subunit sequences from PDB. The alignment was performed by CLUSTALW. Aminoacid sequences are from *Trimeresurus flavoviridis* (PDB entry 1C3A, chain B), *Trimeresurus mucrosquamatus* (1V4L, chain B), and *Crotalus durissus terrificus* (1UOS, chain B and 1UMR, chain B). Cysteine residues are represented in gray and disulfide bridges by dotted lines.

B.- Computer generated model of Crotacetin subunit β.

The 3-D structure was generated throught PARMODEL, a web server at http://www.biocristalografia.df.ibilce.unesp.br/tools/parmodel. Non-pairing intra-chain or "extra" cysteine residues at positions 3 and 77 are indicated by arrows.

C.- Ramachandran plot of CTCβ.

Figure 3. Isolation, reduction and N-terminal sequence of crotacetin from *C. durissus spp*

Crotacetin was purified from the most abundant source, the crude venom of *C. durissus cascavella*, by a combination of gel permeation HPLC (Superdex 75, 1 x 60 cm) and sequential reverse phase HPLC, as described in methods. **A.-** gel permeation chromatography of the crude venom of three different subspecies of *Crotalus durissus* (*C. d. terrificus, C. d. collilineatus, and C. d. cascavella*). The peaks corresponding to crotacetin and other toxins are indicated. **B.-** Reverse phase HPLC (μ-Bond pack C-18 column) profile of reduced crotacetin, where the peaks of subunit α and β are separated. **C-.** N-terminal sequencing of crotacetin α and β subunit, and comparison with predicted amino acid sequence (this work) and with other snake venom C-type lectins.

Figure 4. Platelet agglutination and erythrocyte aggregation assays

Platelet agglutination assay **(A)** were carried out with human platelet rich plasma (PRP) and increasing quantity of pure crotacetin (32.8 μg/ml, blue curve; 49.3 μg/ml, yellow; 65.5 μg/ml, magent). Collagen was used as control (black curve). Agglutination response were monitored by turbidmetry and represented by percent of light transmittance (Y-axis) versus time in minutes (X-axis). Erythrocyte aggregation assay was conducted with isotonic suspensions of two percent of rabbit blood cells. The agglutination was visually monitored after incubation with serial dilutions of pure crotacetin. Images of optical microscopy of non-treated (B) and crotacetin-treated erythrocytes (C) were taken.

Figure 5. Anti-microbial activity of native crotacetin and isolated α and β subunits

Xanthomonas axonopodis pv passiflorae (panel A) and *Clavibacter michiganensis michiganensis* (panel B) were incubated with native crotacetin or with isolated α and β subunits. The reduction of anti-bacterial activity is compared. Transmission electron microscopy of untreated *Xanthomonas axonopodis pv passiflorae* (panel C) and treated with crotacetin (panel D).Vacuolization of bacterial cell is observed in crotacetin-treated bacterial cells (D).

Figure
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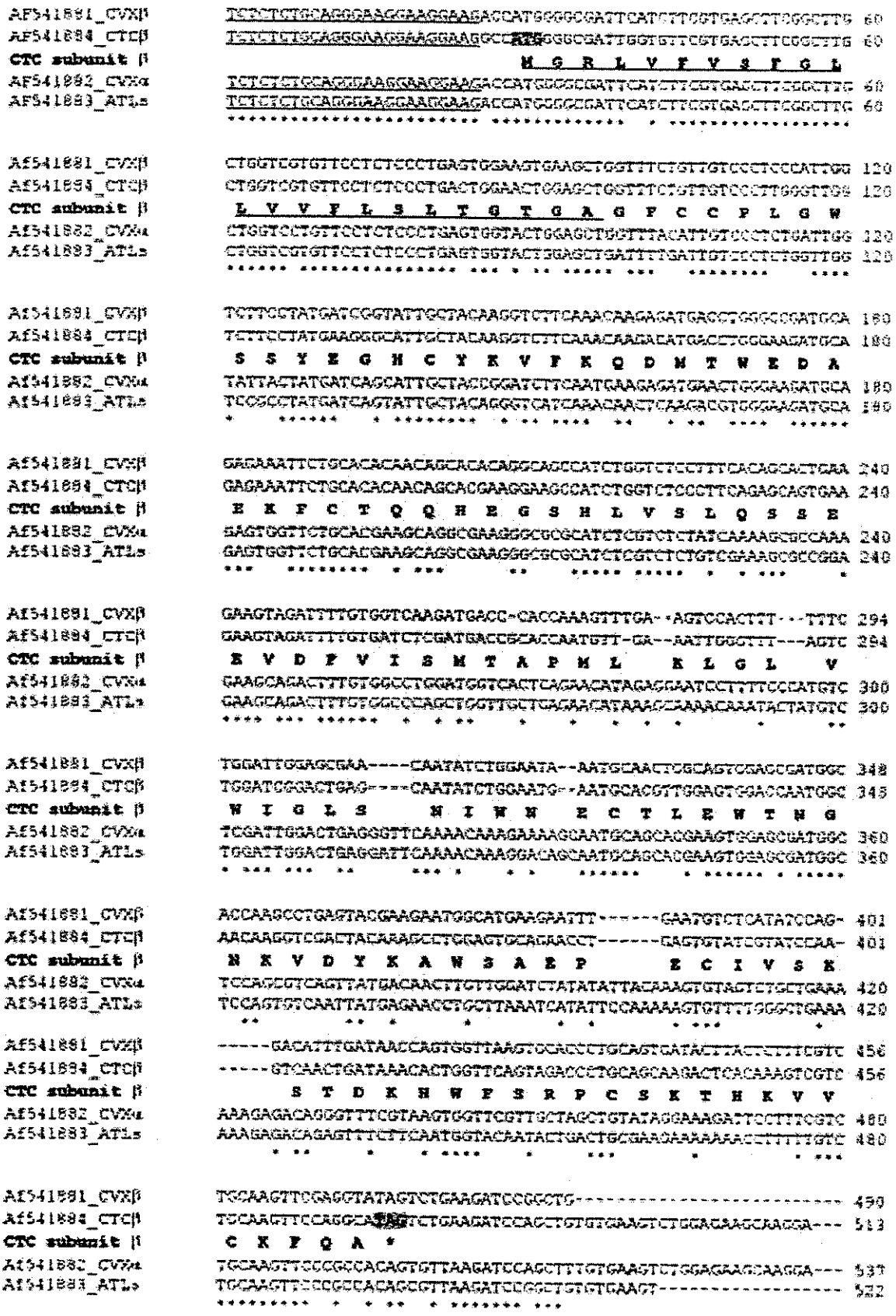

Figure 1. Rádis-Baptista et al., 2004

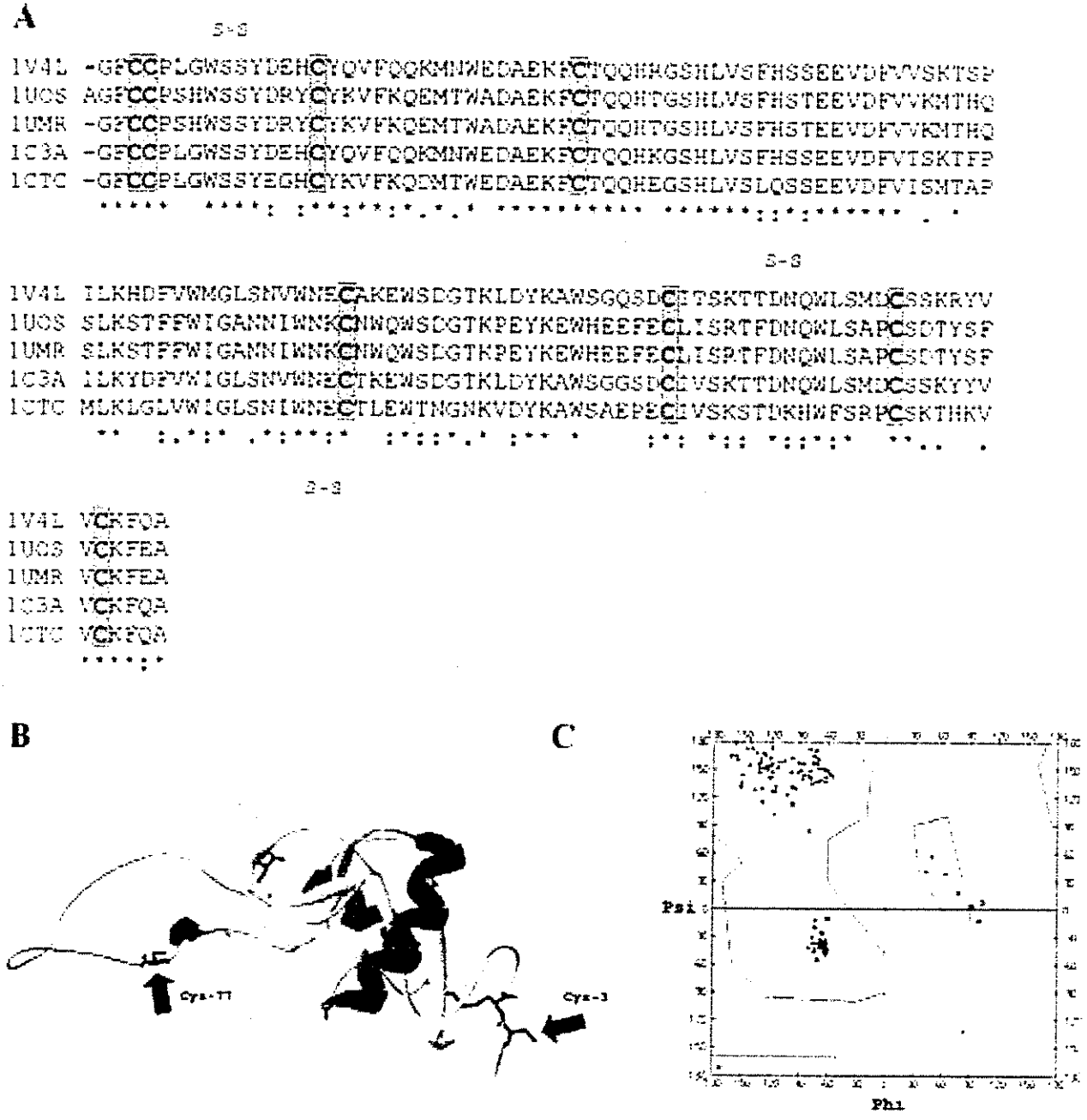

Figure 2. Rádis-Baptista et al., 2004

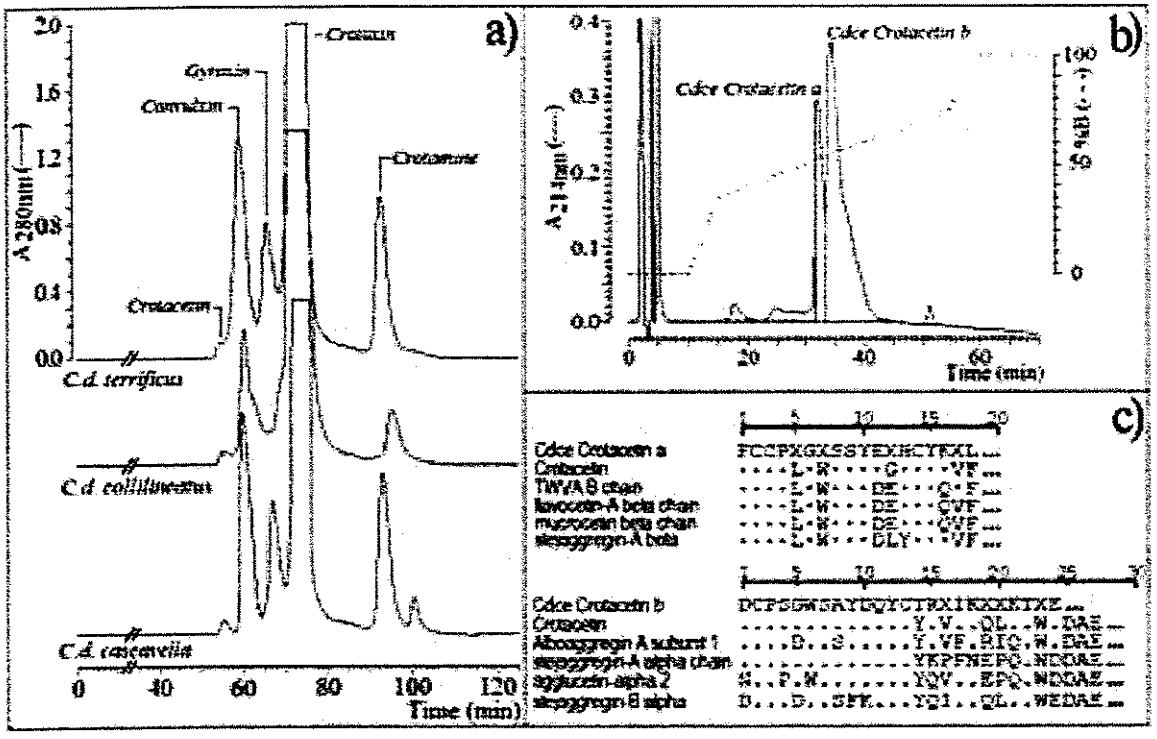

Figure 3 Radis-Baptista et al., 2004

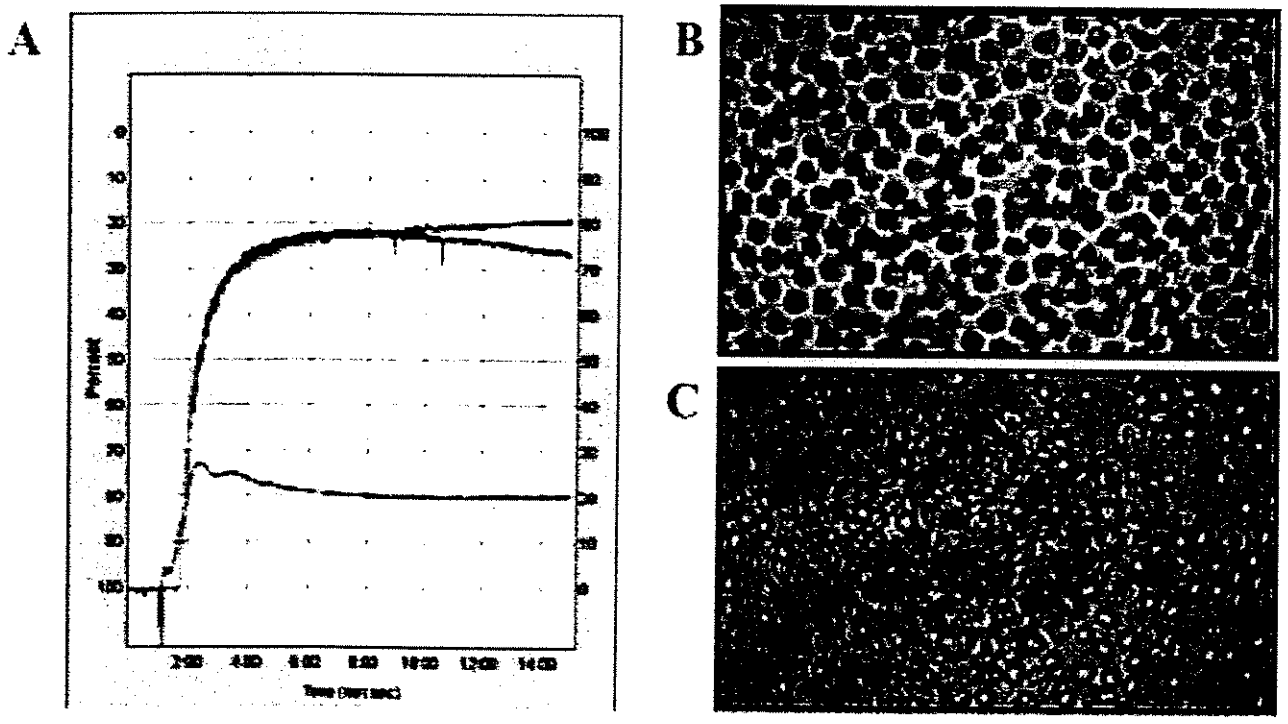

Figure 4. Rádis-Baptista et al., 2004

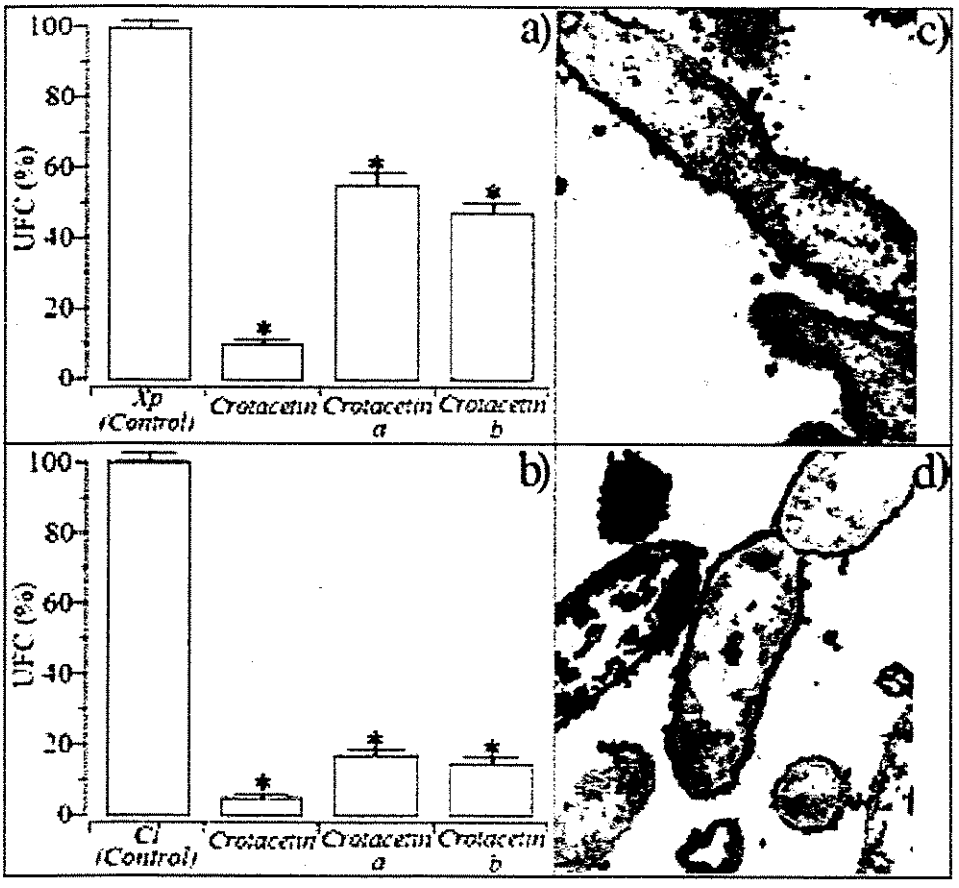

Figure 5 Badis-Baptista et al., 2004

PURIFICATION AND CHARACTERIZATION OF A LECTIN FROM *Lonchocarpus sericeus* SEEDS

B.S. Cavada, K.B.L. Servulo, D.P. Bezerra, K.S. Aragão, E.S. Marinho, D.O. Toyama, T. Santi-Gadelha, C.A.A. Gadelha, N.M.N. Alencar, A.M.S. Assreuy, V.P.T. Pinto, C.S. Negano, A.H. Sampaio, M.H. Toyama, H. Debray.

Protein and Peptides Letter

PURIFICATION AND CHARACTERIZATION OF A LECTIN FROM *Lonchocarpus sericeus* SEEDS

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A new N-acetylglucosamine-specific lectin from *Lonchocarpus sericeus* seeds (LSL) was purified by a combination of affinity chromatography (chitin) and ion exchange chromatography (Mono-Q). The lectin showed a major band of 26 kDa by SDS-PAGE and 23 kDa by MALDI-TOF, and exhibited different physicochemical properties as compared to the lectins from *Lonchocarpus capassa* (GalNAc), *Pterocarpus angolensis* (Man/Glu), and *Vatairea macrocarpa* (Gal), belonging to the same group.

INTRODUCTION

Lectins are a heterogeneous group of (glyco) proteins containing at least one carbohydrate-binding site [1] that make them able to develop various biological activities, some of them with amazing potential and practical applications [2]. These proteins have been purified from virus, microorganisms, animals and plants [3]. However, the main source of new lectins until now is the world flora, although the interest for animal lectins, especially those from mammals, has been receiving enormous attention [4]. The potential of a lectin is based on their variety of biological applications, from agricultural to medicine proposes [5]. Interestingly, they possess many advantages, including easy availability, distinct specificity, and high stability, and more importantly, their reactions with glycoconjugates in solutions and cells can be reversed by simple sugars. On the other hand, it is well accepted now that these proteins play an important role on decodification of cellular glycocodes [6] and are essential for cell-cell communications. Moreover, except for a few exceptions, the lectins in use in a vast field of application are derived from plants. Different plant lectins are grouped in families and the most studied of them is the legume lectin family, comprising specially those purified from species belonging to the Papilionoideae subfamily [7]. However, the Dalbergieae tribe from this subfamily, is until now, poorly explored as a source of lectins, such as the

galactose-specific lectin from seeds of *Vatairea macrocarpa*, which has been purified and well characterized [8, 9,10,11]. The present paper describes the purification and partial molecular characterization of a new *N*-acetylglucosamine-specific lectin from *Lonchocarpus sericeus* seeds.

MATERIALS AND METHODS

Mature seeds from *L.sericeus* were collected in the state of Ceará (NE Brazil) and grounded in a coffee mill. The flour was defatted with n-hexane and air-dried at room temperature for further use. Soluble proteins were extracted by continuous stirring with 1:10 (w/v) 0.15 M NaCl solution for 3 hours at room temperature. Insoluble material was separated by centrifugation at 10,000 g for 20 min at 5 °C and the resultant supernatant applied to a chitin-column (2.0 x 20 cm) equilibrated with 0.150 M NaCl. After removing unbound material (Peak 1), the lectin was eluted with 0.1 M glycin buffer, pH 2.6, containing 0.15 M NaCl, at a flow rate of 30 mL h^{-1}. The retained fraction (Peak 2) containing all the haemagglutinating activity was submitted to an ion-exchange chromatography on Mono-Q column. (1.0 x 17 cm), equilibrated with 0.02 M phosphate buffer, pH 7.5. The adsorbed proteins were eluted with a linear gradient of 0-1M NaCl in PBS buffer. The elution was monitored at 280 nm and 3 mL fractions were collected manually and tested for haemagglutinating activity towards trypsinized rabbit erythrocytes [12]. Active fractions were pooled, dialyzed extensively against distilled water, freeze-dried, and stored at -30 °C for further investigations. Protein was measured by the method of Bradford [13], using bovine serum albumin (Sigma) as standard. The protein purity and molecular mass were assessed by polyacrylamide gel electrophoresis (12.5%) after heating the native lectin in presence of SDS and 2-mercaptoethanol [14]. N-terminal analyses were performed using an Applied Biosystems Procise f gas-liquid sequencer. The phenylthiohydantoin (PTH) amino acids were identified by comparing their retention times to that of the 20 PTH amino acid standards. The amino acid analysis was carried out with PICO-TAG amino acid analysis (Waters) after sample hydrolysis in sealed, evacuated ampoules at 110°C with 6 M HCl for 24 h. Searches for similarity were performed against a non-redundant protein databank using the program FASTA 3 [15] (available at http://www.ebi.ac.uk/fasta33/). Molecular mass of the native *Lonchocarpus sericeus* lectin was determined by MALDI-TOF mass spectrometry using a PE Biosystems Voyager DE-PRO instrument operating at a 20-kV accelerating voltage in linear mode. Sinapinic acid (10 mg/ml in 50% acetonitrile, 0.3% TFA) was used as sample matrix .

RESULTS

The lectin was easily isolated in one step by affinity chromatography on a chitin column (Figure 1), and purified by ion exchange chromatography (Figure 2a, Table 1). LSL agglutinated preferentially rabbit erythrocytes, with no differences in activity upon enzymatic treatment. The apparent molecular mass of LSL determined by SDS-PAGE yielded an electrophoretic pattern with an apparent molecular mass of 26 kDa similar to the major band of *Vatairea*

macrocarpa lectin (VML) [8] (Figure 2b) and 23.52 kDa determined by MALDI-TOF mass spectrometry. Amino acid analysis of LSL showed high values of aspartic acid/asparagine and glutamine/glutamic acid residues (Table 2). Analysis of the first 40 N-terminal amino acids residues of LSL exhibited extensive identity with the N-terminal sequence of the lectins I (AAB51457), II (P93538) and III (AAB5142) of *Sophora japonica* [16], *Maakia amurensis* lectin [17], *Vatairea macrocarpa* lectin [9], and *Pterocarpus angolensis* lectin [18] (Figure 3).

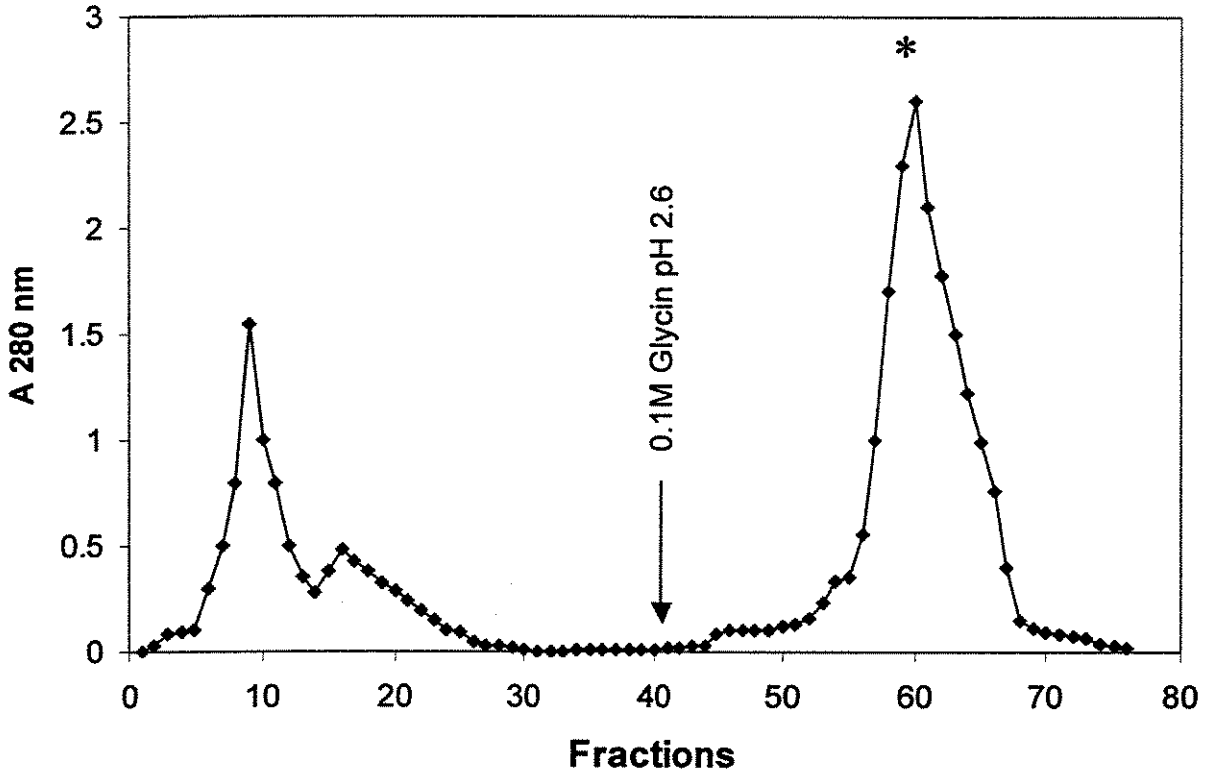

Figure 1. Affinity chromatography of crude extract from *L.sericeus* seeds. Aqueous extract prepared with 0.15M NaCl was applied to a chitin-column (2.0 x 20 cm). The retained fraction was eluted with 0.1 M glycin/HCl, 0.15M NaCl, pH 2.6, at the flow rate of 30 mL h^{-1}. The eluate was monitored at 280nm. * Indicates peak with hemagglutinating activity.

Table 1. Purification of the lectin from *Lonchocarpus sericeus* seeds.

Steps	Specific activity* units/mgP x 10	Purification (fold)
Crude extract	9.5	1
Affinity chromatography	36	3.7
Ion-exchange chromatography	39.5	4.2

*Units of hemagglutinating activity by mg of protein.

Table 2. Amino acid composition of the lectin from *Lonchocarpus sericeus* and other *Dalbergieae* lectins.

Amino acid	(%Mol)		
	L.sericeus	*L.capassa**	*V.macrocarpa**
Asx	16.7	14.6	10.8
Thr	3.6	11.3	8.6
Ser	7.2	10.4	9.3
Glx	19.7	5.8	7.5
Pro	6.6	5.0	5.3
Gly	6.3	8.3	8.6
Ala	6.0	5.8	11.6
Cys	-	-	-
Val	4.8	7.9	6.9
Met	0.2	0.4	0.3
Ile	3.4	6.7	3.9
Leu	11.6	6.7	7.0
Tyr	-	2.9	-
Phe	5.1	5.4	6.7
His	1.7	0.4	2.1
Lys	6.5	4.1	6.0
Arg	0.6	2.5	2.4
Trp	N.D.	1.7	N.D.

* [19]

** From sequence of *Vatairea macrocarpa* lectin [9]

N.D- not determined

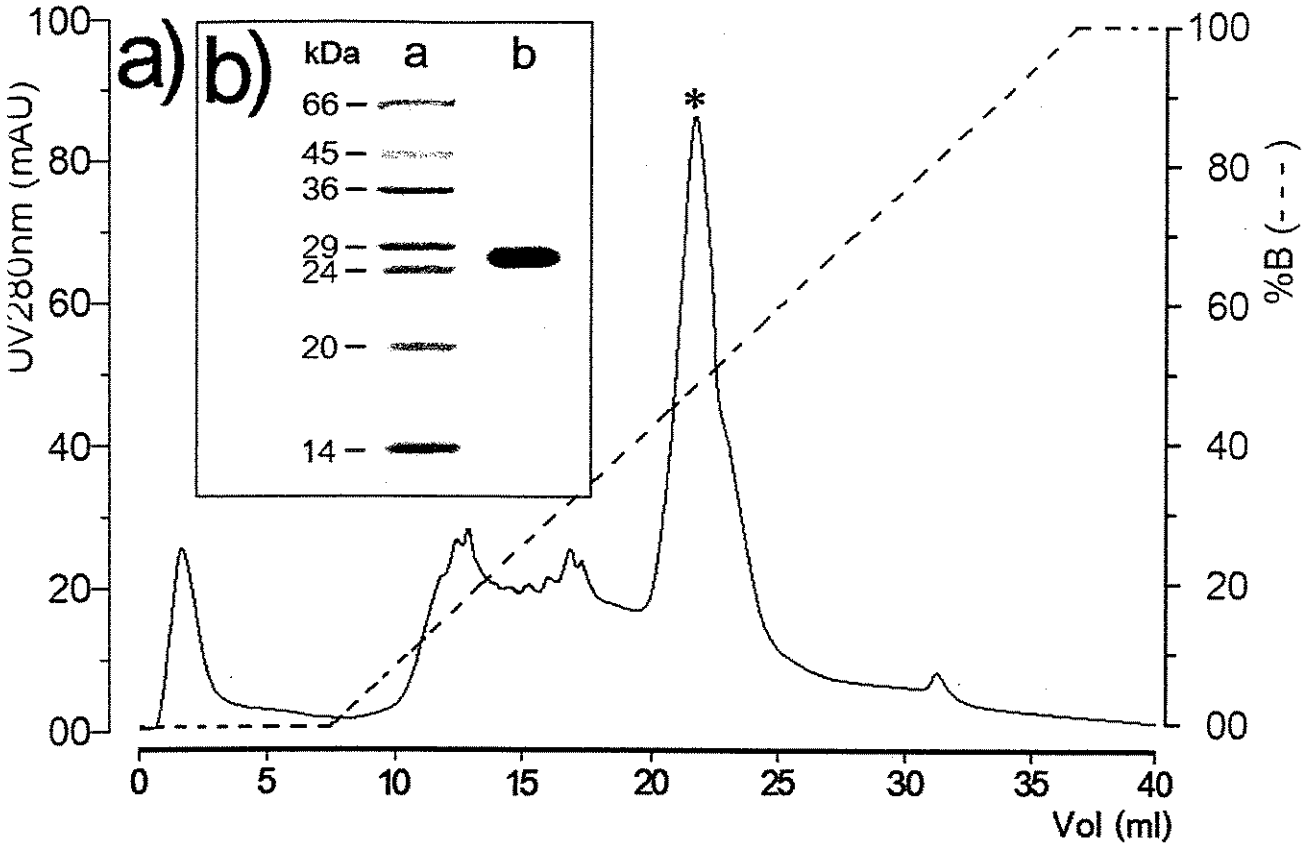

Figure 2a. Ion-exchange chromatography of the *L.sericeus* lectin. The retained fraction from affinity chromatography was load on a Mono-Q HR 5/5 column. Elution was carried out at a flow rate of 1.0 mL/min with a linear gradient from 0 to 1M NaCl in 0.02 M phosphate buffer, pH 7.5. Protein elution was monitored at 280 nm. *Indicates peak with hemagglutinating activity. 2b. SDS-PAGE of the purified lectin from *Lonchocarpus sericeus*. Lane a: molecular mass markers (bovine serum albumin 66 kDa, chicken egg ovalbumin 45 kDa, glyceraldehyde 3-phosphate dehydrogenase 36 kDa, carbonic anhydrase 29 kDa, bovine pancreas trypsinogen 24 kDa, soybean trypsin inhibitor 20 kDa, bovine milk lactalbumin 14.2 kDa). Lane b: LSL.

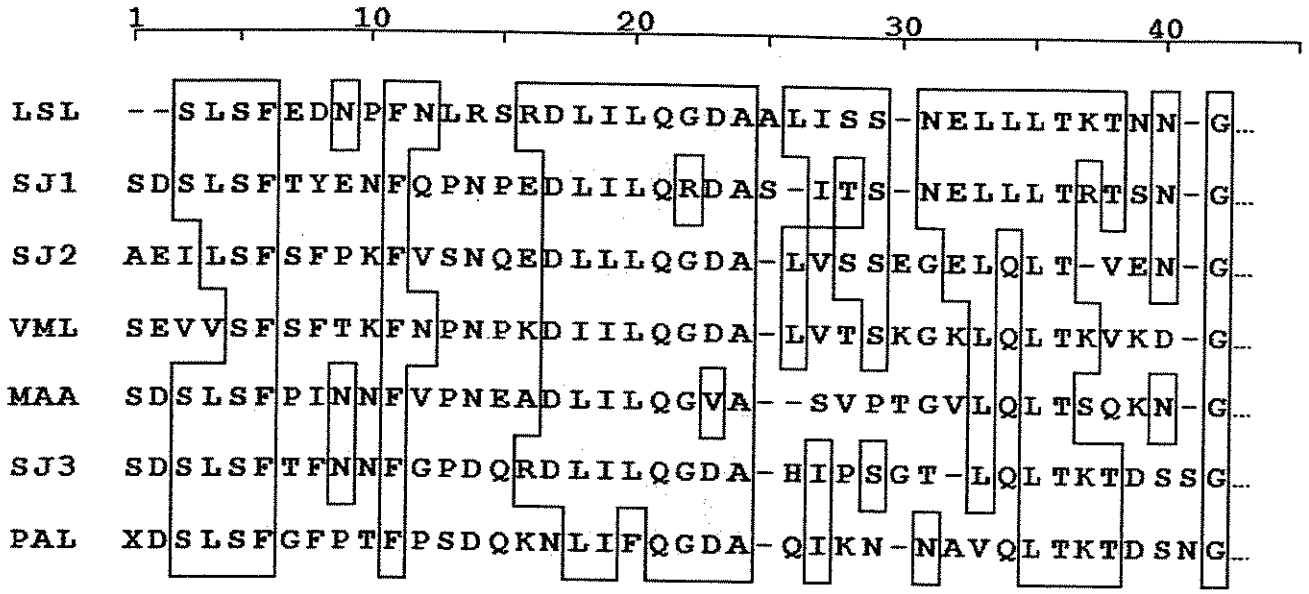

Figure. 3. Alignment of the N-terminal sequence of LSL with those of the lectins of *Sophora japonica* - SJ1, AAB51457 [16]; SJ2, P93538 [16]; SJ3, AAB5142 [16]; *Vatairea macrocarpa* - VML, P81371 [9]; *Maackia amurensis* -MAA, AAB39932 [17]; *Pterocarpus angolensis* - PAL, 1UKGA [18]. Amino acids of LSL conserved in the other N-terminal sequences are boxed.

DISCUSSION

The purification of the *Lonchocarpus sericeus* lectin (LSL), performed by a combination of affinity chromatography on chitin-column and ion-exchange chromatography on Mono-Q column, yielded a pure lectin as observed by SDS-PAGE, MALDI-TOF and partial N-terminal sequence. The specificity for N-acetylglucosamine exhibited by LSL differs from lectins of the same Dalbergieae tribe, such as the *Lonchocarpus capassa* lectin , specific for N-acetylgalactosamine [19], the mannose/glucose-specific lectin from *Pterocarpus angolensis* seeds [18], and the galactose-specific lectin from *Vatairea macrocarpa* seeds (VML) [8]. Apparently, the significant difference in sugar specificity makes the Dalbergieae lectins peculiar and therefore, structural studies will be necessary to explain these differences. Moreover, only the primary structure of VML and the three-dimensional structure of the mannose/glucose *Pterocarpus angolensis* lectin have been resolved [18]. In general, leguminous species belonging to a same tribe contain lectins that express similar specificities towards monosaccharides, such as the glucose/mannose Con A-like lectins from the Diocleinae tribe [20, 21]. The comparison of the first 40 N-terminal residues of LSL with N-terminal residues of other legume lectins showed a high similarity with *Sophora japonica* lectin I (AAB51457, 50 %), *Sophora japonica* lectin II (P93538, 45 %), *Sophora japonica* lectin III (AAB5142, 56.1 %), *Vatairea macrocarpa* lectin (P81371, 42.5 %), *Maackia amurensis* agglutinin (AAB39932,

37.5 %), and *Pterocarpus angolensis* lectin (1UKGA, 46.4 %). These results are in accordance with the botanic systematic used with these groups of plants, indicating that subtribe Dalbergieae was derived from tribe Sophoreae. However, there are a lot of questions to be answered such as why lectins from the tribe Dalbergieae express different sugar specificities, although they exhibit great N-terminal similarity. Even more, no similarity was observed between the N-terminal amino acids from LSL and *L. capassa* lectin. [19].

The specificity for GlcNAc expressed for LSL might suggest an antifungal activity, since this sugar is always present on the fungal wall surface. For example, the specific for N-acetyl-glucosamine lectin from *Urtica dioica* (UDA), [22], present in high concentrations in subterranean organs, is able to inhibit growth of fungi *in vitro* [23]. The apparent molecular mass of LSL determined by SDS-PAGE is similar to others lectins isolated from tribe Dalbergiae: VML has a major band with apparent molecular mass of 26 kDa and *L.capassa* lectin with 29kDa. Amino acid analysis indicates that LSL has a high content of Glu/Gln, Asp/Asn and Leu differing from VML [9] and *L.capassa* lectin [19] by a significant amount of Glu/Gln and Leu. Another interesting point is that LSL agglutinates only rabbit erythrocytes while VML [8] and *L.capassa* lectin [19] agglutinate human red blood cells too. Furthermore,it is important to quote that only the *L.capassa* lectin is a metalloprotein, while VML and LSL do not require metals to express biological activity. Finally, the results obtained with LSL compared to those of VML and *L.capassa* lectin strongly indicate that there is much work to be done, especially structural studies that will explain their differences. All these data will be valuable to understanding the mechanisms that govern distinct biological activity expressed by these lectins.

Acknowledgements

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Determination of Amino acid sequence of a new Phospholipase A_2 (MIDCA 1) isolated from *Micrurus dumerilli carinicauda* venom

Chárlston A. Dal Belo, Marcos H. Toyama, Daniela de O. Toyama, Sergio Marangoni, Frederico B. Moreno, Benildo S. Cavada, Marcos D. Fontana, Stephen Hyslop, Everardo M. Carneiro and Antônio C. Boschero

The Protein Journal

Determination of Amino acid sequence of a new Phospholipase A_2 (MIDCA1) isolated from *Micrurus dumerilii carinicauda* venom.

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Abstract

A new Phospholipase A_2 (PLA_2) from *Micrurus dumerilii carinicauda* venom was isolated and its primary structure determined. This new PLA_2 showed a low enzymatic activity when compared with other PLA_2 and showed a moderate basic character with an isoelectric point of 8.0. It amino acid sequence showed the presence of 120 amino acid residues and its sequence was: NLIQFLNMIQCTTPGREPLVAFANYGCYCGRGGSGTPVDELDRCCQVHDNCYDTAKKVFGCSPYFTMYS YDCSEGKLTCKDNNTKCKAAVCNCDRTAALCFAKAPYNDKNYKIDLTKRCQ. The structural modeled view of the MIDCA1 when compared with other strong neurotoxic PLA_2 as *Naja naja* showed significant differences in the β-wing and neurotoxic sites, despite the high amino acid sequence similarities. These observations indicate dissociation between biological and catalytic activity of this new PLA_2 supporting the view that other regions of the protein are involved in the biological effects.

Key words: Phospholipase A_2, amino acid sequence, Micrurus, neurotoxic and myotoxic

1. Introduction

Phospholipase A_2 (PLA_2) are large family of related enzyme that have been classified into groups I-XII according to several criteria which includes catalysis, protein sequence, molecular mass, several disulfide bonds, requeriment of Ca^2. The $sPLA_2$s are also found in abundance in snake and bee venoms (Kini and Evans, 1989 and Hawgood and Bon, 1991). These enzymes have conserved many important features with mammalian $sPLA_2$, including a common catalytic mechanism, the same calcium requirement, and much conserved primary and tertiary structures (Davidson and Dennis, 1990, Dennis, 1994 and Van den Bergh et al., 1989) In addition to their probable roles in the digestion of preys, snake venom $sPLA_2$s have evolved into extremely potent toxins displaying neurotoxic, myotoxic, anticoagulant, and proinflammatory effects (Denni et al., 1994). The diversity of the pathophysiological effects of venom $sPLA_2$s is probably linked to the presence of specific high affinity receptors for these enzymes (Valentin and Lambeau, 2000). A first type of receptors initially identified in brain is called N (for neuronal)-type PLA_2s receptors. It recognizes with high affinity a large number of toxic $sPLA_2$s including OS_2, a new highly neurotoxic $sPLA_2$ purified from the Taipan snake venom, the bee venom $sPLA_2$s, and the neurotoxic $sPLA_2$s CM-III from *Naja mossambica mossambica* (Lambeau, et al., 1989). Nontoxic venom $sPLA_2$s as well as the porcine pancreatic $sPLA_2$ display very low affinities for these receptors (Lambeau, et al., 1989). A second type of receptors initially identified in rabbit skeletal muscle and thus referred to as M (for muscle)-type PLA_2 receptors recognizes with very high affinities OS_2 and OS_1, a new non toxic $sPLA_2$s also purified from the Taipan snake venom (Lambeau et al., 1990). This M-type PLA_2 receptor does not bind the bee venom $sPLA_2$ or the CM-III $sPLA_2$s from *N. mossambica mossambica* (Lambeau et al., 1990). This receptor binds the porcine pancreatic group I $sPLA_2$ as well as the human inflammatory group II $sPLA_2$, both with fairly high affinity (Lambeau et al., 1994). It is now known that M-type as well as N-type receptors have different subunit constitutions and are not exclusively present in brain or muscle (Valentin and Lambeau, 2000). It is believed that these receptors are normal binding targets for endogenous $sPLA_2$s, which, by binding to them, could work as hormones or growth factors. Molecular cloning of rabbit (Lambeau et al., 1994) and bovine (Valentin and Lambeau, 2000) M-type $sPLA_2$s receptors has recently been achieved and has revealed that these receptors are structurally related to the macrophage mannose receptor, a protein involved in the endocytosis of mannose-bearing glycoproteins and microorganisms (Valentin and Lambeau, 2000). Elapid snake venom has been extensively studied as source of highly presynaptic or postsynaptic neurotoxic PLA_2 (Francis et al., 1997). But this protein differently of the other previously isolated Micrurus or coral PLA_2 did not have high neurotoxic activity, but MIDCA1 showed a high myotoxic

activity. In this article we isolated this PLA_2 enzyme from *Micrurus dumerilii carinicauda* and investigated some structural differences to try explaining the low neurotoxic activity of this PLA_2.

2. Material and Methods

2.1. Purification

The whole venom (10mg) form the *Micrurus dumerilli carinicauda* were dissolved in 250µl of 0.1% TFA and the result sample was clarified by centrifugation at 4,500 xg for 2 min. The neurotoxic PLA_2 was purified by preparative reverse phase C18 HPLC column (0.78 x 30cm) (Waters Corp). Initially, the reverse phase column was equilibrated with buffer A (0,1% trifluoroacetic acid, in water) and the samples were eluted with a gradient of buffer B (0.035% TFA in 66% acetonitrile). The column was eluted at a flow rate of 2.0 ml/min, the absorbance measured at 280 nm and the enzymatic activity was then lyophilized and stored at -40°C. The purity degree of PLA_2 was assayed using two dimensional (2D) electrophoresis and MALDI TOFF mass spectrometry. 2D electrophoresis was conducted as described by Anderson and Anderson (1996). The spectrometry used proteins purified by HPLC, with the PLA_2s spotted on a sample plate and introduced into the MALDI TOFF mass spectrometer.

2.1. Amino acid analysis.

The amino acid analysis was carried out with PICO-TAG amino acid analysis (Waters) after sample hydrolysis in sealed, evacuated ampoules at 110°C with 6 M HCl for 24 h.

2.2. Amino acid sequence.

Three milligrams of purified protein were dissolved in 200 µl of 6M guanidine chloride (Merck, Darmstadt, Germany) containing 0.4mM Tris-HCl and 2 mM EDTA (final pH 8.15). Nitrogen was flushed over the top of the protein solution for 15 min, which was then reduced with DTT (6M, 200 µl) and carboxymethylated with ^{14}C–iodoacetic acid and cold iodoacetic acid. Nitrogen was again flushed over the surface of the solution and the reaction tube sealed. This solution was incubated in the dark at 37°C for 1 h and desalting was done on a Sephadex G 25 column (0.7 x 12 cm) in 1mM acetic acid buffer. The eluted reduced and carboxymethylated (RC) protein was then lyophilized and stored at -20°C. One sample of this RC-protein was then digested by *Staphylococcus aureus* protease V8 for 16 h

at 37°C, using an enzyme to substrate ratio of 1:30. The reaction was stopped by lyophilization. A second sample of the RC-PLA_2 was digested with Clostripain for 8 h at 37 °C and then lyophilized (Toyama et al., 2000), with products separated by reverse phase HPLC using a Waters PDA 991 system with a C-18 μ-Bondapack column. The peptide peaks were isolated using a linear gradient (0-100% of acetonitrile in 0.1% TFA (V/V)). A third sample (2mg) was cleaved with a 15-fold molar excess of cyanogens bromide (CNBr) over methionine residues in 70% formic acid (4ml) under nitrogen for 24 h at room temperature, after which the reaction mixture was diluted with 40 ml of water and lyophilized. Excess reagents were removed by gel filtration on a Sephadex G-25 column (1 x 20 cm) equilibrated with 10% acetic acid. The CNBr peptide fragments were separated by reverse phase HPLC, using analytical μ-Bondapack C18 column (0.39 x 30cm; Waters), with0.1% TFA as solvent A and acetonitrile containing 30% of solvent A (solvent B). The elution profile was monitored at 214nm. Analysis of the amino acid sequence of the RC-protein was well as that of the enzymatically digested fragments, were performed with an Applied Biosystems model Procise f gas-liquid protein sequencer. The phenylthiohydantoin (PTH) derivatives of the amino acids were identified with an Applied Biosystems model 450 microgradient PTH-analyser.

2.3. Molecular modeling.

Searches for similarity were performed against a non-redundant protein databank using the program FASTA 3 (available at http://www.ebi.ac.uk/fasta33/). In this work we determined the three dimensional structure (3D) of MIDCA1 by homology modeling. Next, we carried out several rounds of molecular dynamics calculation. The preliminary 3D model was first obtained using the MSI Insight II software and its Homology and Modeler modules using the alignment retained in the previous step. The atomic coordinated of neurotoxic PLA_2 from the *Naja naja* venom were obtained from the Protein Data Bank. The complete model structure in this way obtained was refined by several energy minimization rounds (1000 steps of steepest descents followed by conjugate gradient until convergence). The model visualization was made using graphical software VMD.

3. Results.

Micrurus dumerilii carinicauda coral snake venom was acquired from SIGMA and the purification of neurotoxin was made on the preparative RP HPLC, using a μ-Bondapack C18 (0.3 x

30cm, Waters). This protein showed a molecular mass of 15kDa in the PAGE-SDS electrophoresis, a pI value of 8.0 (2D electrophoresis) and a molecular mass of 15.55 kDa as determined by MALD-TOFF (Figure 1). This new PLA_2 showed high amount of Asp, lower of Asp and Arg and Lys as well as Cys showed similar values to found by other PLA_2 (table I). The complete structure of the MIDCA1 PLA_2 was determined by sequencing of reduced and carboxymethiled protein (RC-PLA_2) and the RC-PLA_2 digested with Clostripain (Clt) and protease V8 (SV8) and Cyanogem bromide). The most important peptides used for the determination of the complete amino acid sequence of MIDCA1 were: SV8-2, SV8-4, SV9-6, CNBr-3, CNBr-1, Clt3 and Clt-5 (Figure 2). This protein showed high amino acid sequence homology at calcium binding loop and catalytic site with other enzymatic and non-enzymatic active enzymes, but this protein showed several significant and important amino acid replacements at neurotoxic and β-wing in comparison with other neurotoxic PLA_2 such as *Naja naja* and like proteins (Figure 3). The amino acid sequence analysis showed that presence of several basic amino acid residues in the C-terminal region and other important amino acid residues responsible for myotoxic activity that were pointed as narrow (Figure 3).

MICDCA1 PLA_2 showed conserved amino acid residue found for class I PLA_2 (elapide loop, placed at position 52 and 60^{th} amino acid residue, we found also a conserved residues in the catalytic network (H48, Y52 and D107), the calcium binding site (Y28, G32, G34 and D49). Also this PLA_2 showed highly conserved basic and hydrophobic amino acid responsible for myotoxic effect of elapidae PLA_2 (Figure 3).

In this work we determined the three dimensional structure (3D) of MIDCA1 by homology modeling. Next, we carried out several rounds of molecular dynamics calculation. The preliminary 3D model was first obtained using the MSI Insight II software and its Homology and Modeler modules using the alignment retained in the previous step. The complete model structure in this way obtained was refined by several energy minimization rounds (1000 steps of steepest descents followed by conjugate gradient until convergence). The model visualization was made using graphical software VMD. During the molecular modeling the MIDCA1 PLA_2 showed good amino acid sequence alignments with neurotoxic PLA_2 but in the model some structural changes was observed, mainly in the β-wing and neurotoxic sites (Figure 4).

4-Discussion.

This protein showed high acid and lower basic amino acids that probably involved in the lower pH value if compared to other basic PLA_2 (table I). Several attempts have been made to predict the neurotoxic site of PLA_2 enzymes by theoretical methods (Dufton & Hider, 1983). It is widely accepted that each presynaptic neurotoxin of PLA_2 type from the snake venom has an individual neurotoxic site separated from the PLA_2 catalytic site (Yang, 1994 and Kini & Evans, 1989). Such a neurotoxic site enables the neurotoxin to specifically bind to the receptor on the membrane of nerve. However, the location of neurotoxic site on the neurotoxin molecule and details of its interactions with the receptor have not yet been clearly understood. Presynaptic neurotoxic potential is predicted by hydropathy profiles and phospholipases A_2 have distinct hydrophobic regions around residues 80 to 110 in group I PLA_2s (Kini & Iwanaga, 1986). Whether the more recently discovered mammalian $sPLA_2$s bind to Ntype, M-type or structurally distinct receptors and the ability of the PLA_2 to interact with this receptor is very important for development of several pharmacological activities of PLA_2 (Valentin and Lambeau, 2000). Recently, several PLA_2 receptors have been characterized and molecular model showed that interaction between secretory PLA_2 for the receptor is dependent on tandem CRD-like domains (Valentin and Lambeau, 2000). Subsequent molecular analysis of pancreatic secretory PLA_2 showed that molecular regions near to the Ca^{2+} binding loop were critical for binding on the N or M-type receptor. Chemical modifications have indicated that segment 59 to 90, that contains the anticoagulant region (Toyama et al., 2000) and the β-wing loop (75 – 85) is responsible for the neurotoxic activity of PLA_2 (Yang, 1997). The amino acid sequence alignment of MIDCA1 PLA_2 with other PLA_2 showed several conserved domain such as calcium binding, catalytic network but the neurotoxic site as well as the calcium binding loop of this PLA_2 showed several differences. These analyses suggest that the lower potency of MIDCA1 involve this modification on the neurotoxic site and the calcium binding loop. The neurotoxic site of MIDCA1 showed presence of Lys86 that involved in the structural modification of the neurtoxic loop and consequent lost of the neurotoxic potency. The presence of Lys76 in the β-wing seen inovolved in the significant modification of the β-wing loop as observed in the figure 4. Thus the lost of neurotoxic effect of MIDCA1 probably involved this two Lysine amino acid residue 76 and 86, which decrease the hydrophobicity of this region and decrease the capacity of this toxin to interact to M or N-type receptor and decrease its neurotoxicity. Singh et al., showed that some replacement of neutral or polar amino acid residues by basic amino acids in the neurotoxic site was sufficient to induce drastic modification of the hydropath of this region and consenquely decrease the ability of this region to bind to the receptor (Singh, et al., 2001). Nigroxins A and B have been

characterized a myotoxic PLA_2 isolated from the American elapidae *Micrurus nigrocinctus* and their amino acid sequence showed some highly conserved amino acid residues involved in the myotoxic activity such as Arg15, Val90, Ala103, Asn110 and other several basic amino acid placed in the C-terminal region (Alape-Girón et al., 1999. The amino acid sequence of MIDCA1 showed the presence of same conserved amino acid residues, except Arg15 that was replaced by G15 in *Micrurus dumerilli carinicauda*, also we found several basic amino acids as Arg and Lys. Thus, we conclude that enzymatic potency did not responsible for the neurotoxic or myotoxic activity, but β-wing, C-terminal and neurotoxic site will be involved in the both activities.

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Figure Captions.

Figure. 1. Repurification of active PLA_2 fraction from the *Micrurus dumerilli carinicauda* whole venom on the reverse phase HPLC and insert of Figure 1a showed the PAGE-SDS of MIDCA1 PLA_2. Two dimension electrophoresis of the purified fraction and the molecular homogeneity of MIDCA1 were evaluated by MALDI-TOFF mass spectrometer.

Figure 2. Determination of the amino acid sequence of MIDCA1 PLA_2. N-terminal was obtained by direct sequencing of RC-PLA_2 and CNBr, Clt and SV8 correspond the peptides obtained from the treatment of RC-PLA_2 with cyangem bromide, clostripain and protease V8, respectively.

Figure 3. Amino acid aligment of MIDCA1 PLA_2 from the *Micrurus dumerilii carinicauda* coral snake with other myotoxic (MICNI A and MICNI) *Micrucus nigrocinctus* PLA_2 (**) and with Neurotoxic PLA_2 from the other species (*). These amino acid sequences were obtained from the Protein Data Bank and PubMed.

Figure 4. The 3D structure of MIDCA1 PLA_2, the amino acid sequence of PLA_2 was fitted to the high-resolution structure of to *Naja naja* neurotoxic PLA_2. The initial model was then subjected to energy minimization and molecular dynamic processing. The MIDCA1 PLA_2 is depicted in β-wing and neurotoxic site.

Table I. The amino acid composition of MIDCA1 PLA_2, determined here and comparison with other PLA_2 from the different source described in the literature.

	Micrurus	*Crotalus durissus*			*Bothrops*			
aa	MIDCA1	Cdca F6	Cdt F15	Cdco F6	BthTx-I	Bj IV	Bj V	PrTx-I
Asp	20	12	12	12	12	12	11	14
Glu	06	10	08	09	08	15	15	09
Ser	04	06	08	08	07	02	02	07
Gly	08	12	12	11	12	10	10	11
His	01	02	02	02	03	01	01	03
Arg	04	09	07	09	06	06	06	05
Thr	10	07	08	08	04	08	08	06
Ala	08	06	07	07	08	07	07	07
Pro	05	06	05	05	05	09	09	05
Tyr	08	09	08	07	09	10	10	08
Val	04	04	05	04	04	04	04	05
Met*	02	02	02	02	01	02	02	01
Cys**	07	07	07	06	07	07	07	07
Ile	04	05	04	05	05	03	03	05
Leu	06	08	10	10	10	07	07	11
Phe	05	04	03	03	04	04	04	03
Lys	11	11	12	12	14	12	13	13
Trp***	00	02	02	02	02	02	02	01
Total	120	122	122	122	121	121	121	121

Figure 1.

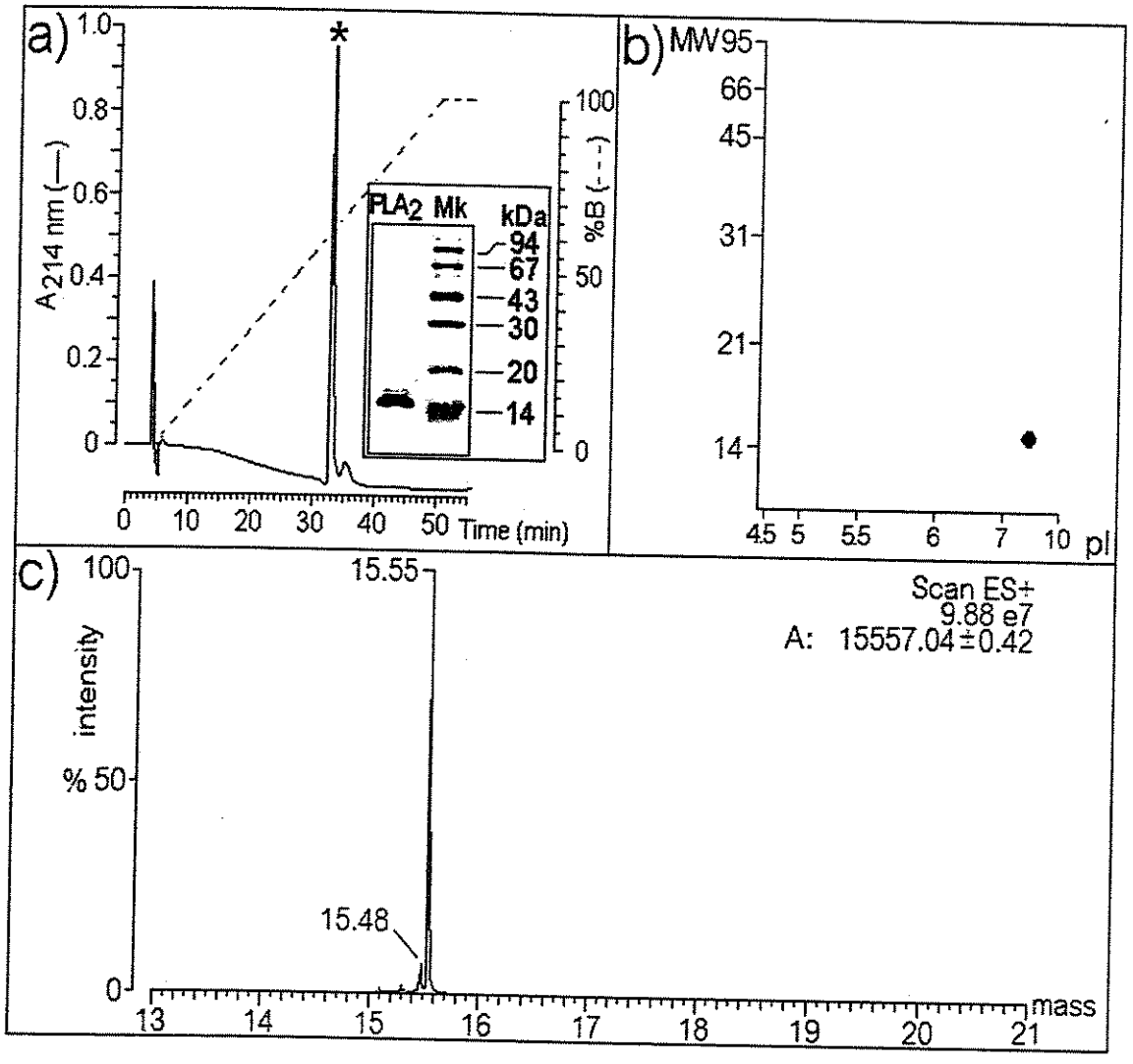

a)
1.0
0.8
0.6
0.4
0.2
0
A214 nm (—)
*
100
50
0
%B (- - -)
PLA2 Mk
kDa
94
67
43
30
20
14
0 10 20 30 40 50 Time (min)
b) MW95
66
45
31
21
14
4.5 5 5.5 6 7 10 pI
c)
100
% 50
0
intensity
15.55
15.48
13 14 15 16 17 18 19 20 21 mass
Scan ES+
9.88 e7
A: 15557.04±0.42

Figure 2.

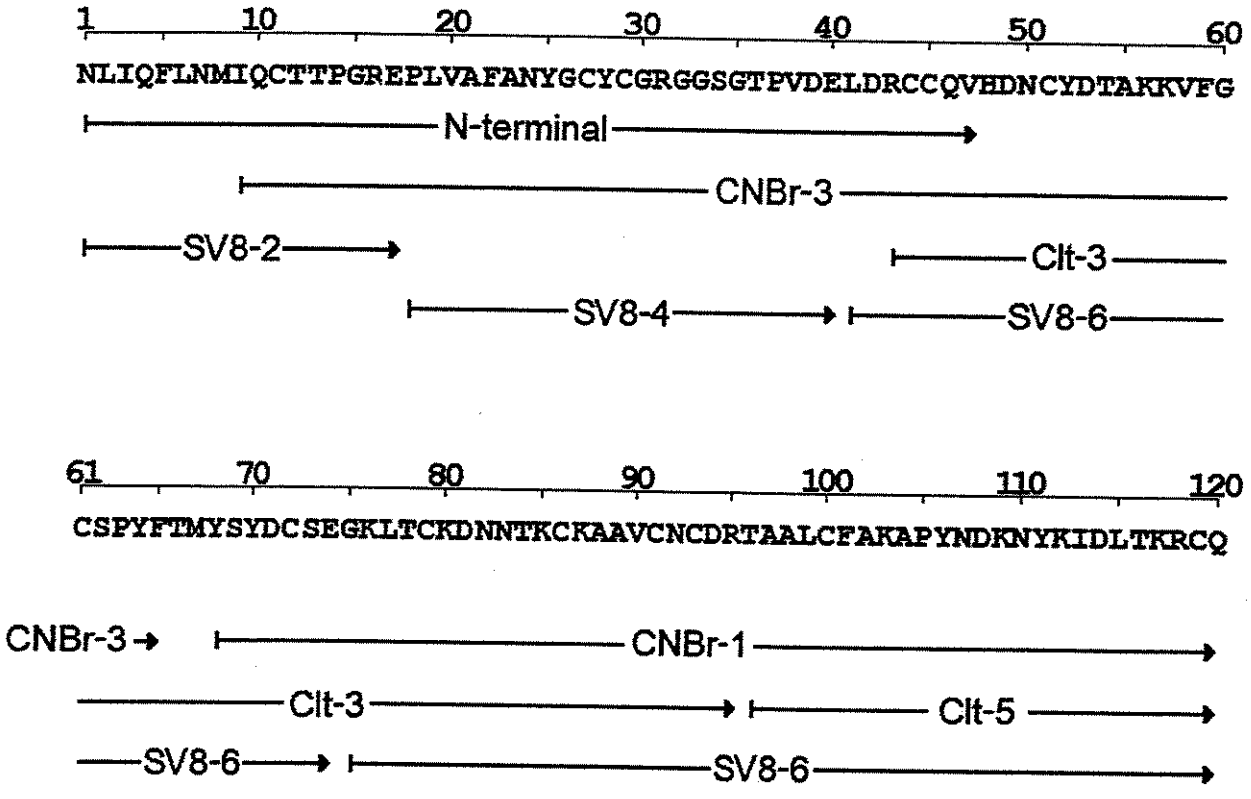

1 10 20 30 40 50 60
NLIQFLNMIQCTTPGREPLVAFANYGCYCGRGGSGTPVDELDRCCQVHDNCYDTAKKVFG
N-terminal
CNBr-3
SV8-2
Clt-3
SV8-4
SV8-6
61 70 80 90 100 110 120
CSPYFTMYSYDCSEGKLTCKDNNTKCKAAVCNCDRTAALCFAKAPYNDKNYKIDLTKRCQ
CNBr-3
CNBr-1
Clt-3
Clt-5
SV8-6
SV8-6

Figure 3.

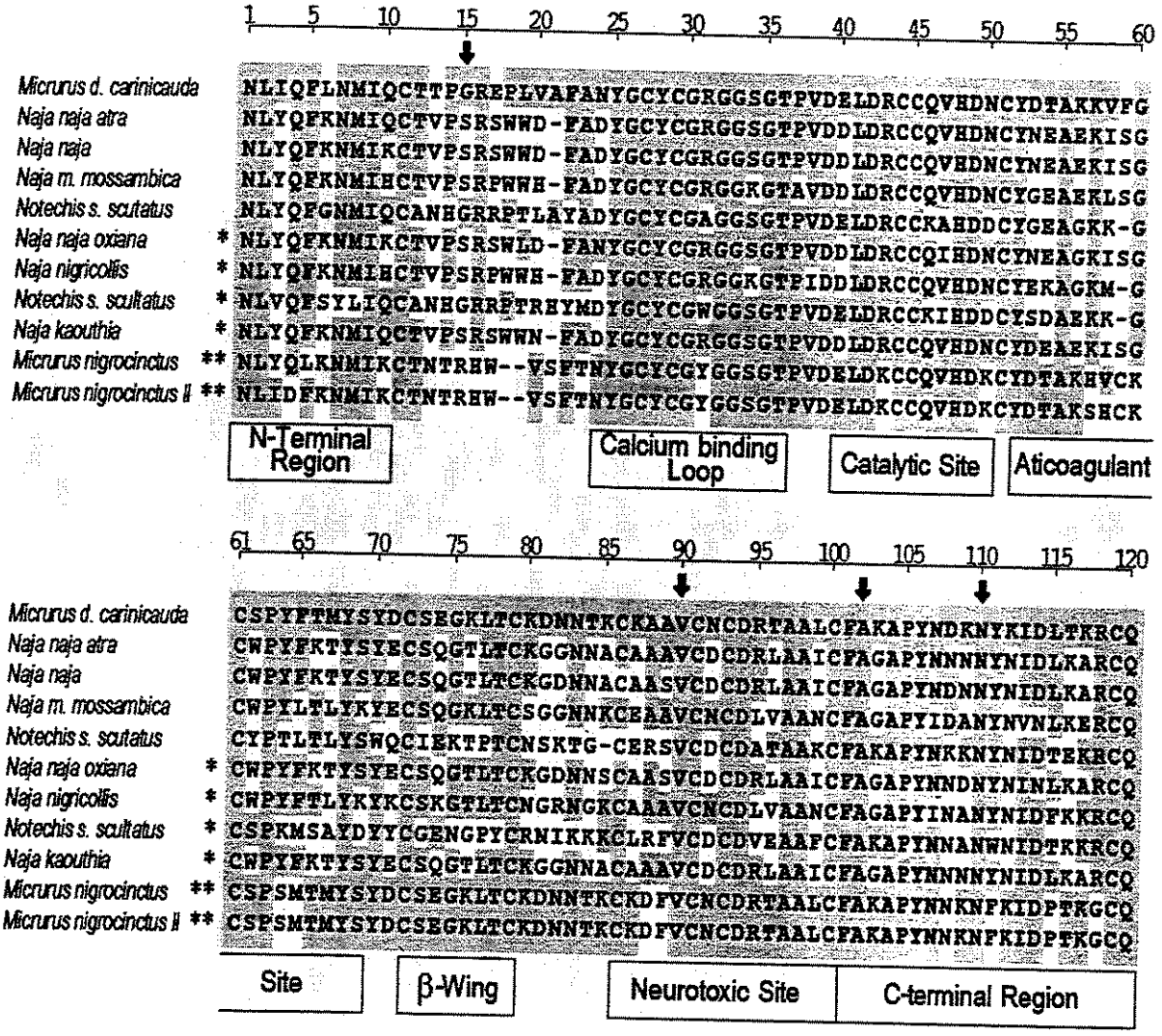
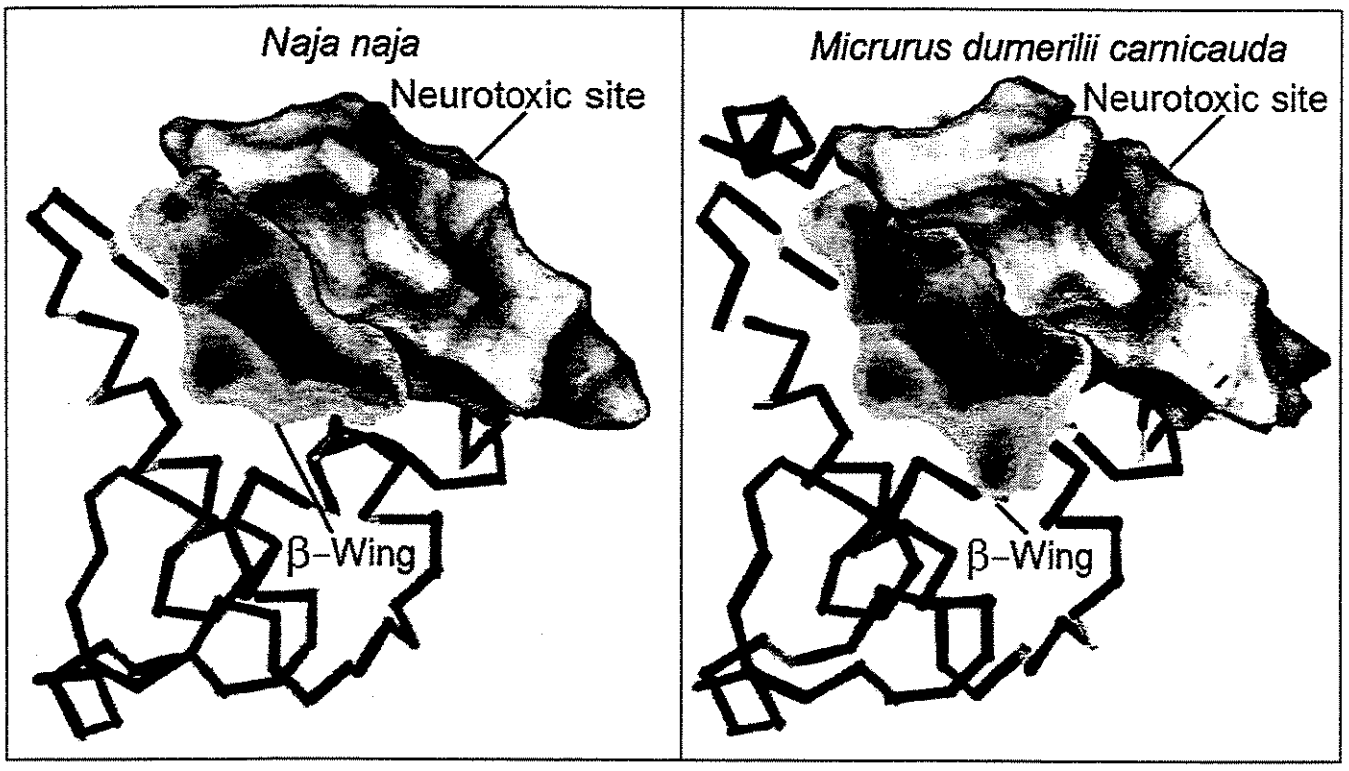

1 5 10 15 20 25 30 35 40 45 50 55 60
Micrurus d. carinicauda NLIQFLNMIQCTTPGREPLVAFANYGCYCGRGGSGTPVDELDRCCQVHDNCYDTAKKVFG
Naja naja atra NLYQFKNMIQCTVPSRSWWD-FADYGCYCGRGGSGTPVDDLDRCCQVHDNCYNEAEKISG
Naja naja NLYQFKNMIKCTVPSRSWWD-FADYGCYCGRGGSGTPVDDLDRCCQVHDNCYNEAEKISG
Naja m. mossambica NLYQFKNMIHCTVPSRPWWH-FADYGCYCGRGGKGTAVDDLDRCCQVHDNCYGEAEKLSG
Notechis s. scutatus NLYQFGNMIQCANHGRRPTLAYADYGCYCGAGGSGTPVDELDRCCKAHDDCYGEAGKK-G
Naja naja oxiana * NLYQFKNMIKCTVPSRSWLD-FANYGCYCGRGGSGTPVDDLDRCCQIHDNCYNEAGKISG
Naja nigricollis * NLYQFKNMIHCTVPSRPWWH-FADYGCYCGRGGKGTPIDDLDRCCQVHDNCYEKAGKM-G
Notechis s. scutatus * NLVQFSYLIQCANHGRRPTRHYMDYGCYCGWGGSGTPVDELDRCCKIHDDCYSDAEKK-G
Naja kaouthia * NLYQFKNMIQCTVPSRSWWN-FADYGCYCGRGGSGTPVDDLDRCCQVHDNCYDEAEKISG
Micrurus nigrocinctus ** NLYQLKNMIKCTNTRHW--VSFTNYGCYCGYGGSGTPVDELDKCCQVHDKCYDTAKHVCK
Micrurus nigrocinctus II ** NLIDFKNMIKCTNTRHW--VSFTNYGCYCGYGGSGTPVDELDKCCQVHDKCYDTAKSHCK
N-Terminal Region
Calcium binding Loop
Catalytic Site
Aticoagulant
61 65 70 75 80 85 90 95 100 105 110 115 120
Micrurus d. carinicauda CSPYFTMYSYDCSEGKLTCKDNNTKCKAAVCNCDRTAALCFAKAPYNDKNYKIDLTKRCQ
Naja naja atra CWPYFKTYSYECSQGTLTCKGGNNACAAAVCDCDRLAAICFAGAPYNNNNYNIDLKARCQ
Naja naja CWPYFKTYSYECSQGTLTCKGDNNACAASVCDCDRLAAICFAGAPYNDNNYNIDLKARCQ
Naja m. mossambica CWPYLTLYKYECSQGKLTCSGGNNKCEAAVCNCDLVAANCFAGAPYIDANYNVNLKERCQ
Notechis s. scutatus CYPTLTLYSWQCIEKTPTCNSKTG-CERSVCDCDATAAKCFAKAPYNKKNYNIDTEKRCQ
Naja naja oxiana * CWPYFKTYSYECSQGTLTCKGDNNSCAASVCDCDRLAAICFAGAPYNNDNYNINLKARCQ
Naja nigricollis * CWPYFTLYKYKCSKGTLTCNGRNGKCAAAVCNCDLVAANCFAGAPYINANYNIDFKKRCQ
Notechis s. scutatus * CSPKMSAYDYYCGENGPYCRNIKKKCLRFVCDCDVEAAFCFAKAPYNNANWNIDTKKRCQ
Naja kaouthia * CWPYFKTYSYECSQGTLTCKGGNNACAAAVCDCDRLAAICFAGAPYNNNNYNIDLKARCQ
Micrurus nigrocinctus ** CSPSMTMYSYDCSEGKLTCKDNNTKCKDFVCNCDRTAALCFAKAPYNNKNFKIDPTKGCQ
Micrurus nigrocinctus II ** CSPSMTMYSYDCSEGKLTCKDNNTKCKDFVCNCDRTAALCFAKAPYNNKNFKIDPTKGCQ
Site
β-Wing
Neurotoxic Site
C-terminal Region

Figure 4.


Naja naja
Neurotoxic site
β-Wing
Micrurus dumerilii carnicauda
Neurotoxic site
β-Wing

Antimicrobial effect of new crotamine like protein (Cdru Cro2) isolated from the *Crotalus durissus ruruima*

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The Protein Journal

Antimicrobial effect of new crotamine like protein (Cdru Cro2) isolated from the *Crotalus durissus ruruima.*

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Abstract

In this work we isolated a novel crotamine isoform from the *Crotalus durissus ruruima* rattlesnake venom and its antibacterial effect was characterized. This protein was named as Cdru Cro2 and this protein was purified by combination of two chromatographic steps on molecular exclusion chromatography on Superdex 75 and reverse phase HPLC (μ-Bondapack C18). The reverse phase HPLC of this fraction give three crotamine isoform Cdru Cro1, Cdru Cro2 and Cdru Cro 3 but only Cdru Cro2 showed a potent antimicrobial effect. The homogeneity of crotamine isoform was evaluated by Tricine SDS-PAGE that showed one protein band with a molecular mass of 5kDa approximately. The primary structure of crotamine was determined after sequencing of the reduced and carboxymethiled protein (RC-protein) and the peptides obtained after chemical or enzymatical cleavage of RC-protein. This protein showed the presence of 43 amino acid residues and its primary structure was: YKRCHK KGGHCFPKEKICLPPSSDFGKMDCRWRRKCCKKGSQK. This protein showed high molecular amino acid sequence identity with other crotamine like proteins from the *Crotalus durissus terrificus*. This new crotamine induced a high antimicrobial effect against both kind of bacteria and its activity was modulated by the presence of positively charged amino acid residues similar to found in the presence and this effect was carried out by destruction of the membrane as shown in the electron microscopy. The amino acid alignment of Cdru Cro2 with bacteriocins and β-defensins suggest that C-terminal domain of this protein was involved for antimicrobial region. Several peptide fragment of Cdru Cro2 was assayed against both bacterial strain and only the C-terminal region peptide showed higher antimicrobial effect.

Key words: crotamine, β-defensins, *Crotalus durissus cascavella* and antimicrobial

1. Introduction

Antibacterial peptides have been found in a wide rage of species (Wade, et al., 2001). These peptides are known to be important components of innate immunity and the host defense system of insects, amphibians and mammals (Boman, 1991 and Boman, 1995). Mellitin exhibits potent hemolytic and cytolytic actions against mammalian cells and a broad spectrum of antibacterial, antifungal, antiviral and antiprotozoal properties. Melittin related peptides was grouped into the melittin-related peptides that has been classified as antibacterial peptides with cytolytic activity that can lyses mammalian cells as well as bacteria cells (Conlon et al., 2004). Recently novel scorpion pore-forming peptide has been isolated from the scorpion *Opistophtalmus carinatus* that showed strong hemolytic and lytic activities and also showed strong antibacterial activity (Moerman et al., 2002). Antimicrobial peptides can be classified into cytotoxic peptides such as melittin and peptides such as cecropin A (CA) (Lee et al., 1989) and magainin 2 (MA) (Zasloff, 1987) that are active only on bacteria cells. Both kind of peptide has been used as probe for synthesis of synthetic peptides that having more potent antimicrobial activity than of natural peptides without damaging against mammalian cells (Park, et al., 2001 and Conlon et al., 2004). Several this peptides has high account of basic and aliphatic amino acid residue which are involved in the formation of membrane pore on the membrane (Park et al., 2001).

Crotamine belongs to a group of closely related small, nonenzymatic, basic polypeptides which cause myonecrosis on snake envenomation (Radis-Baptista et al., 1999). More recently Mancin et al., 1998 described an analgesic property of crotamine, which was similar to other analgesic peptides previously described in the literature. Recently, Nicastro et al., (2003) showed that antibacterial β-defensins and crotamines able to interact with lipid membrane. Both proteins showed similar secondary and tertiary motifs with sodium α and β scorpion neurotoxins. A comparison of crotamine with human β-defensins also showed a similar net positive potential surface with presence of several basic amino acids. Despite the lithe amino acid sequence homology with β-defensins from human or murines, these proteins share highly conserved three-dimensional structure and showed the presence of three disulfide bridges (Circo et al., 2002). Sinqueira et al., (2002) also showed that crotamine showed high molecular overlapping of the structural features and some amino acid conservation with anthopleurine-β, defensin-12 and other defensin like peptides DLP-1 and DLP-2. In this article we isolated a new crotamine like (Cdru Cro2) protein from the *Crotalus durissus ruruima* venom and evaluated the antimicrobial effect of crotamine and the identification of the molecular region of the crotamine responsible for this effect.

2. Material and Menthods

2.1. Isolation of Cdru Cro2

Molecular exclusion chromatography: Approximately 35 mg of whole venom from the *Crotalus durissus ruruima* venom was dissolved in 400µl ammonium bicarbonate buffer (0.2M; pH 8.0) and homogenized at still complete dissolution, followed by clarification with high speed centrifugation (4500xg for 2min). The supernatant was recovered and injected on a molecular exclusion HPLC column (Superdex 75, 1x60cm, Pharmacia), previously equilibrated with same buffer used for dissolving the whole venom. The flow rate used for elution of the fraction was 0.2ml/ml; the chromatography was monitored at 280nm and all fraction isolated were immediately lyophilized and stored at -40°C. The crotamine from the *Crotalus durissus ruruima* venom was purified by reverse phase HPLC according to method described by Toyama et al. (2000). Briefly, three milligrams of whole crotamine was dissolved in 250µl of buffer A and centrifuged at 4500xg for 2 minutes and the supernatant was then applied on the analytical reverse phase HPLC, previously equilibrated with buffer A (0.1% trifluoroacetic acetic acid (TFA) for 15 minutes. The elution of the protein was then conducted using a linear gradient of buffer B (66.6% Acetonitrile in buffer A). After elution the fraction was lyophilized and stored at -40°C. The purity degree of Cdru Cro2 was assayed using Tricine SDS-PAGE gels at 10% electrophoresis that madding according the method described by Schagger and von Jagow (1987). The spectrometry used proteins purified by HPLC, with the crotamine was spotted on a sample plate and introduced into the MALDI TOFF mass spectrometer.

2.2. Antibacterial activity against *Xanthomonas axonopodis. pv. passiflorae and Clavibacter* subsp. *michiganensis michiganensis*

Xanthomonas axonopodis pv. passiflorae (Gram-negative) bacterial strain and *Clavibacter michiganensis michiganensis* (Gran-positive) were harvested from fresh agar plates and suspended in distilled sterilized water (A600nm = 3 x 10^8 CFU/ml). Aliquots of bacterial suspension were diluted to a 10^3 CFU/ml and incubated with the isolated crotamine (Cdru Cro2) and CNBr and protease V8 peptide fragments peptides (150 µg/ml) for 60min at 28 °C, after which the survival was assayed on nutrient agar (Difco) plates (n=5).

2.3. Electron microscopy

The transmission electron microscope (TEM) of *Xanthomonas axonopodis. pv. passiflorae* assessments of structural alterations were done with bacteria incubated with saline (control) and with native crotamine (Cdru Cro2) and CNBr and protease V8 peptide fragments of RC-Cdru Cro2 incubated with bacteria. The bacterial samples were fixed with solution establish (2,5% glutaraldeide and cacodylate buffer 0.1M pH 7.4 and 0.1 M of tanic acid) for 12 h at 4°C and refixed with 1% osmium tetroxide for 2h at 4°C. The sampes were dehydrated in increasing concentrations of ethanol and embedded in Epon resin. Polimeryzation was performed at 60°C for 48 h, and ultra-thin sections were prepared with a Sorvall MT-2 ultramicrotome. The sections were stained with 2%uranyl acetate for 20 min. fallowed by 2% lead citrate for 10 min. Samples were observed under a LEO-906 transmission electron microscope operating between 40 to 100 kV. The scanning electron microscopy (SEM) of *Xanthomonas axonopodis. pv. passiflorae* assessments of morphological alterations were done with bacteria incubated with saline (control) and with native crotamine (Cdru Cro2) and CNBr and protease V8 peptide fragments of RC-Cdru Cro2) incubated with bacteria. The samples were taken for examination after the incubation time requested for the antimicrobial activity (60 min). After centrifugation, pellets were fixed at 4°C in 0.1 M cacodylate buffer (pH 7.4) containing 2.5% (v/v) glutaraldehyde for 12 h. The bacterial samples were fixed again with 1% osmium tetraoxide for 2 h at 4°C. The samples were dehydrated in increasing concentration of ethanol. Specimens were coated with gold in vacuum using a Sputter Coater BALZERS SCD 050. Electron micrographs were obtained using a JSM-5800LV-JEOL scanning electron microscope.

2.3. Reduction and carboxymethylation; digestion and amino acid sequence determination of Cdru Cro 2

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

(RC) protein was then lyophilized and stored at -20°C. One sample of this RC-protein (4.5 mg) was then digested by *Staphylococcus aureus* protease V8 for 16 h, 37°C and pH 7.4, using an enzyme to substrate ratio of 1:30. The Cdru Cro 2 peptide fragments obtained after the treatment of RC protein with protease V8 were separated by reverse phase HPLC, using analytical μ-Bondapack C18 column (0.39 x 30cm; Waters), with 0.1% TFA as solvent A and acetonitrile containing 30% of solvent A (solvent B). The elution profile was monitored at 214nm and peptides were lyophilized. Other aliquot of RC-protein (4.5mg) was digested with a 15-fold molar excess of cyanogens bromide (CNBr) over methionine residues in 70% formic acid (4ml) under nitrogen for 24 h at room temperature, after which the reaction mixture was diluted with 40 ml of water and lyophilized. Excess reagents were removed by gel filtration on a Sephadex G-25 column (1 x 20 cm) equilibrated with 10% acetic acid. The CNBr peptide fragments were separated by reverse phase HPLC, using analytical μ-Bondapack C18 column (0.39 x 30cm; Waters), with 0.1% TFA as solvent A and acetonitrile containing 30% of solvent A (solvent B). The elution profile was monitored at 214nm and peptides were lyophilized. Analysis of the amino acid sequence of the RC-protein was well as that of the enzymatically or chemically digested fragments, were performed with an Applied Biosystems model Procise f gas-liquid protein sequencer. The phenylthiohydantoin (PTH) derivatives of the amino acids were identified with an Applied Biosystems model 450 microgradient PTH-analyser.

2.5. Statistical analyze

The results were expressed as the mean ± SEM. The data were analyzed by analysis of variance (ANOVA) followed by a Bonferroni test. The level of significance was set at $p < 0.05$.

3. Results

3.1. Crotamine purification

The fractionation of the *Crotalus durissus ruruima* venom on Superdex 75 column allowed the purification of four major fractions named as convulxin (fraction I;), gyroxin (fraction II), crotoxin (fraction III) and crotamine (fraction IV) (Fig. 1a). Gyroxin and crotoxin showed proteolytic activity, crotoxin also showed phospholipase A_2 acitivity and L-amino acid oxidase activity was found in the fraction II (gyroxin) (Fig. 1a). The reverse phase HPLC of crotamine purified on the Superdex 75 showed the presence of three different fractions named as Cdru Cro1, Cduru Cro2 and Cdru Cro3. Only

fraction Cdru Cro1 and Cro2 produced the characteristic spastic paralysis provoked by native crotamine in mice immediately after i.v. injection (1 to 2mg/ kg). RP HPLC analysis showed that Cdru Cro2 accounting for approximately 53% of whole crotamine (Fig. 1b). The Tricine SDS-PAGE electrophoresis of the purified Cdru Cro2 in presence or absence of reducing agents (DTT, 1M) showed only one electrophoretic line with a molecular mass of approximately of 5kDa (Fig. 2a). The MS spectral profiles of the crotamine isoform (Cdru Cro2) from the *Crotalus durissus ruruima* showed a molecular mass of 4967.45Da (Fig. 2b).

3.2. Antimicrobial effect

Antibacterial assay of the crude venom of *Crotalus durissus ruruima* showed strong action against both Gram-negative (*Xanthomonas axonopodis pv passiflorae*) and Gram-positive (*Clavibacter michiganensis michiganensis*) bacterial strains at 200μg/ml concentration (Fig 3a and 3b). The venom was separated on a HPLC molecular exclusion chromatography into four fractions and crotamine was then purified on reverse phase HPLC. Whole crotamine inhibited the bacterial growth rate of Gram-negative and Gram-positive in 70% and 85%, respectively (Figure 3a and 3b). Cdru Cro2 isolated from the reverse phase HPLC chromatography was two times most active than to whole crotamine and Cdru Cro3 and Cdru Cro1 showed similar values found for whole crotamine. Antibacterial activities of the crotamine peptide fragments from the treatment of crotamine with cyanogen bromide and protease V8 was also evaluated at similar mode described for native crotamine. Among then the most active antibacterial peptide was CNBr-2, which correspond to the C-terminal domain of Cdru Cro-2 (DCRWRRKCCKK RSQK), whereas SV8-2 (KICLPPSSDFGKMDCRWRRKCCKKGSQK) did not showed an expressive antibacterial activity than to CNBr-2. SV8-2 was identified by direct amino acid sequencing as whole Cdru Cro2 that did not completely digested. N-terminal fragments obtained after treatment of Cdru Cro2 with protease V8 (SV8) or cyanogens bromide (CNBr) did not showed strongly inhibited the bacterial growth rate (Figure 4).

Electron microscopy of bacteria treated with Cdru Cro2 incubated during 30 minutes demonstrated clear differences in the morphology of treated bacteria in comparison with the control. The TEM observation showed celular evidences of the potent permeabilizing activity of the Cdru Cro2 (Fig. 5b). TEM showed that Cdru Cro2 was induced aggregate of bacteria entangled with material extruded from the bacteria surfaces. The scanning electron microscopy (SEM) of *Xanthomonas axonopodis. pv. passiflorae* assessments of morphological alterations revealed

irregular bacterial surface membrane structure similar to bleb projection (Fig 5c) and in some case several membrane ruptures were observed. TEM indicates that the bacterial inner and outer membranes, as well as the peptidoglycan layer between, were extensively damaged. The cytoplasmic contents of the cells, however, did not appear radically disturbed, providing little evidence for osmotically induced cytolysis (Fig. 5b). CNBr-2 also showed similar results observed for Cdru Cro2 (Fig. 5e and 5f).

3.3. Primary structure determination.

The amino acid sequence determination was possible after sequencing of reduced and carboxymethiled (RC-Cdru Cro2) protein at 26th amino acid residue of Cdru Cro2. The protease V8 peptide fragments were purified on the RP-HPLC that allowed purification of three major fractions named as SV8-1, SV8-2 and SV8-3 (Fig. 6a). The amino acid sequence of SV8-1 and SV803 were YKRCHKKGGHCFPKE and KICLPPSSDFGKMDCRWRR KCCKKRSQK, respectively (Fig. 6c). The amino acid sequencing of SV8-2 showed similar sequence to RC-Cdru Cro2. The purification of peptide fragments of Cdu Cro2 with cyanogens bromide showed elution of two major fractions named as CNBr-1 and CNBr-2 (Fig. 6b). The amino acid sequencing of CNBr-1 corresponds to N-terminal region of Cdru Cro2 and its sequence was YKRCHKKGGHCFPKEKICLPPSSDFGKM. The amino acid sequence of CNBr-2 was DCRWRRKCCKKRSQK (Fig. 6c). The most important peptide fragment that allowed the determination of final primary structure of Cdru Cro2 was SV8-3 and CNBr-1 (Fig. 6c).

4. Discussion

In this article we characterized antimicrobial action of crotamine like protein from *Crotalus durissus ruruima* (Cdru Cro2) against *Xanthomonas axonopodis psv passiflorae* (Gran-negative) and *Clavibacter michiganensis michiganensis* (Gran-positive) and determined the molecular region involved in the antimicrobial effect of Cdru Cro2. The transmission electron microscopy (TEM) and scanning electron microscopy (SEM) of the treated bacteria with crotamine and CNBr-2 showed extensive cell surface disruption. The SEM showed that crotamine induces a complete loss of cellular component or organization, the loss of citoplasmatic membrane and several bacterial cells (Gran-negative or Gran-positive). The SEM results provide morphological evidence of the potent permeabilization of the crotamine that was similar to found by other positively charged antimicrobial peptides. Kerkis et al.,

(2004) showed that crotamine can rapidly penetrate into different cell types and mouse blastocyts in vitro. Thus the bactericidal effect of CNBr-2 or the native Cdru Cro2 was due the ability of this peptide to penetrate into the cells similarly to observe for some cationic basic peptides. Cationic antimicrobial peptides were generally defined as peptides of less than to 50 amino acid residue with an overall positive charge, imparted by the presence of multiple lysine and arginine residue, and a substantial of hydrophobic residue and these can posses antimicrobial activity against Gram-positive and Gram-negative bacterias but this protein did not has any disulphide bounds (Power and Hancock, 2003). The main action of these peptides involves the membrane destruction and the basic amino acid play important hole for the interaction with the bacterial membrane (Park et al., 2003). This peptide induces several membrane damage similar to produced by crotamine and its CNBr-2 peptide. The activity and potency of CNBr-2 that represent the C-terminal region of Cdru Cro2 is the most important region of the molecule responsible for the bactericidal effect. Sinqueira et al., (2002) and Nicastro et al. (2003) showed that crotamine C-terminal domain were the strong candidate for the antimicrobial activity of crotamine but in this work we corroborate this structural observation. All antimicrobial peptide showed several common features such as the presence of positively charged, amphipathic amino acid residue that are able to increase phospholipid membranes permeability. Dos Santo et al., (1993) has been described the amino acid sequence of crotamine like protein of *Crotalus durissus ruruima*, but our analysis showed that crotamine peak obtained by conventional our low pressure purification protocols led to isolation of non purified crotamine isoform (Toyama et al., 2003). In the article we able to isolate three new crotamine isoform, that did not show a homogeneous biological or structural properties. The crotamine isolated by dos Santos (1993) is only crotamine like proteins that showed the presence of extra Cys at the C-terminal region, but our results did not corroborate the presence of this "extra" Cys (Figure Probably the difference observed for both structural data involves the impurity of the crotamine peak.

ACKNOWLEDGMENTS

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Figure Captions

Figure 1. 1a) Chromatographic profile of purification of whole venom on the HPLC molecular exclusion chromatography. Crotamine peak was signaled as fraction IV, convulxin (fraction I), gyroxin (fraction II), crotoxin (fraction III). The elution of the fraction was made with ammonium bicarbonate buffer and the chromatographic run was monitored at 280nm of absorbance. 1b) Chromatographic profile of purification of crotamine isoforms designated as Crot 1 and Crot 2. In this work we use exclusively Crot 1 that eluted as the main isoform from crotamine purified in the HPLC molecular exclusion. 1c) Two dimensional electrophoresis of Crot 1 isoform when we determined the pI value of this fraction.

Figure 2. The MS analysis suggests that crotamine isoform (Crot 1) has a molecular mass of 4.83 kDa.

Figure 3. Antimicrobial effect of Crotamine and the chemical digest of reduced and carboxymethyled crotamine (CNBr-1, CNBr-2 and CNBr-3). 3a) Antimicrobial effect of crotamine and crotamine derived peptides incubated with *Xanthomonas axonopodis pv passiflorae*, using a single dose of 150μg/ml. 3b) Antimicrobial effect of crotamine and crotamine derived peptides incubated with *Clavibacter michiganensis michiganensis*, using a single dose of 150μg/ml.

Figure 4. Dose response curve of *Xanthomonas axonopodis pv passiflorae* and *Clavibacter michiganensis michiganensis* at several concentration of native crotamine and CNBr peptide fragments of reduced and carboxymethyled crotamine. In this figure we determined the concentration of peptide that inhibited 50% of bacterial growth rate.

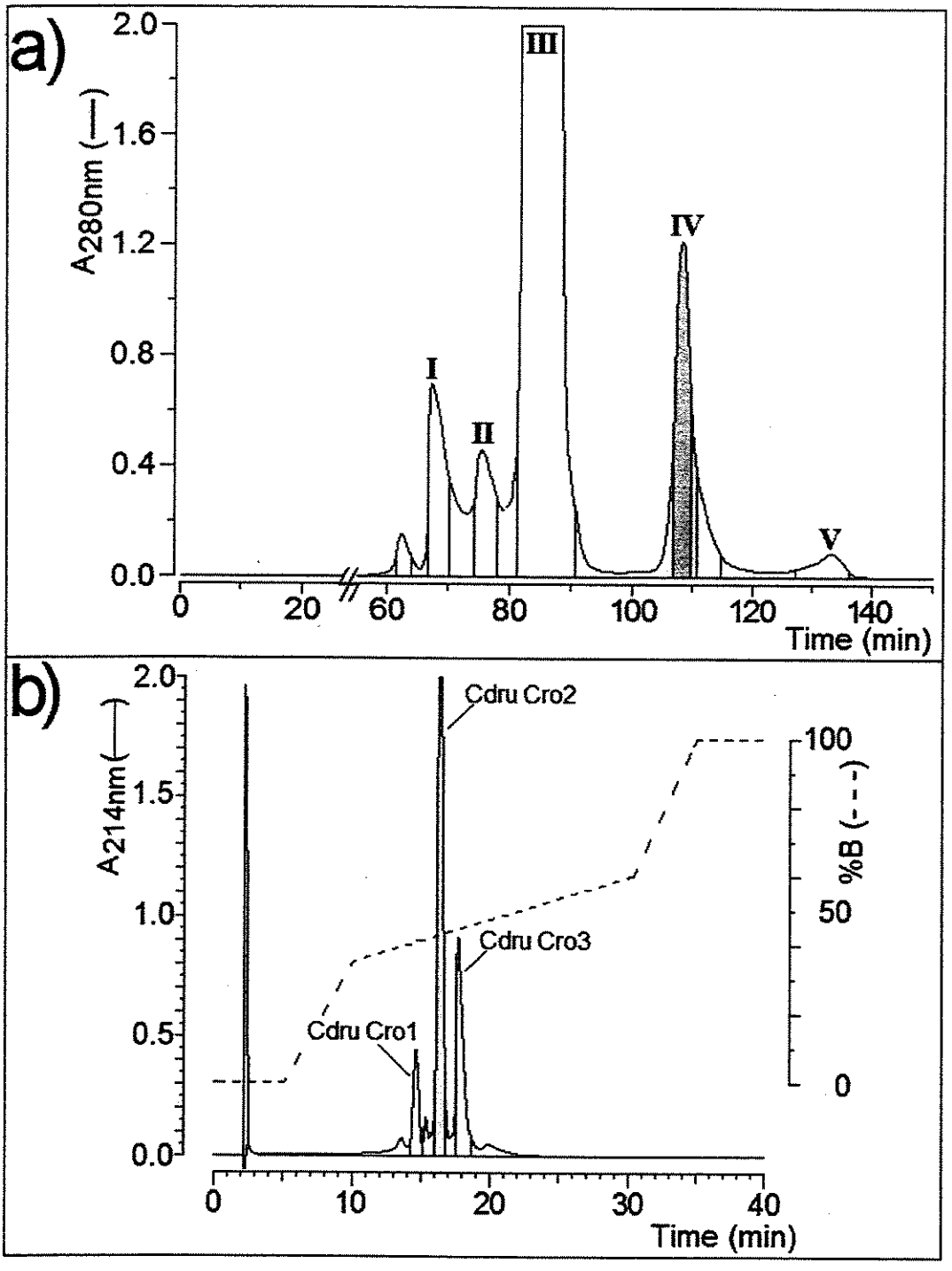
Figure 5. Transmition electron micrography of the *Xanthomonas axonopodis pv passiflorae*. In this protocol we used a single dose of 150μg/ml of crotamina and CNBr-1. 5a and 5d) bacteria no treated (control), 5b and 5e) bacteria treated with native crotamine, 5c and 5f) bacteria treated with CNBr-1.

Figure 6. Negative staining electron micrography of the *Xanthomonas axonopodis pv passiflorae* incubated with CNBr-1 at dose of 150μg/ml.

Figure 7. Determination of the complete amino acid sequence of crotamine. In 7a we showed the purification of the CNBr peptides after incubation of RC-protein with cyanogens bromide (CNBr) over methionine residues. The purification of the peptides was done using analytical reverse phase HPLC column (μ-Bondapack C18) and elution of peaks was done with aqueous solution of Acetonitrile (66%). The chromatographic run was monitored at 214nm of absorbance. The peptides were designated as CNBr-1, CNBr-2 and CNBr-3. In 7b, determination of the amino acid sequence of crotamine (Crot-1).

Figure 8. Structural analysis of crotamine from the Crotalus durissus terrificus and CNBr-1 in comparison with other small basic myotoxins and crotamines like. In this section we compared CNBr-1 with several antimicrobial peptides (β-defensins).

Figure 1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b)
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30
40
Time (min)

Figure 2

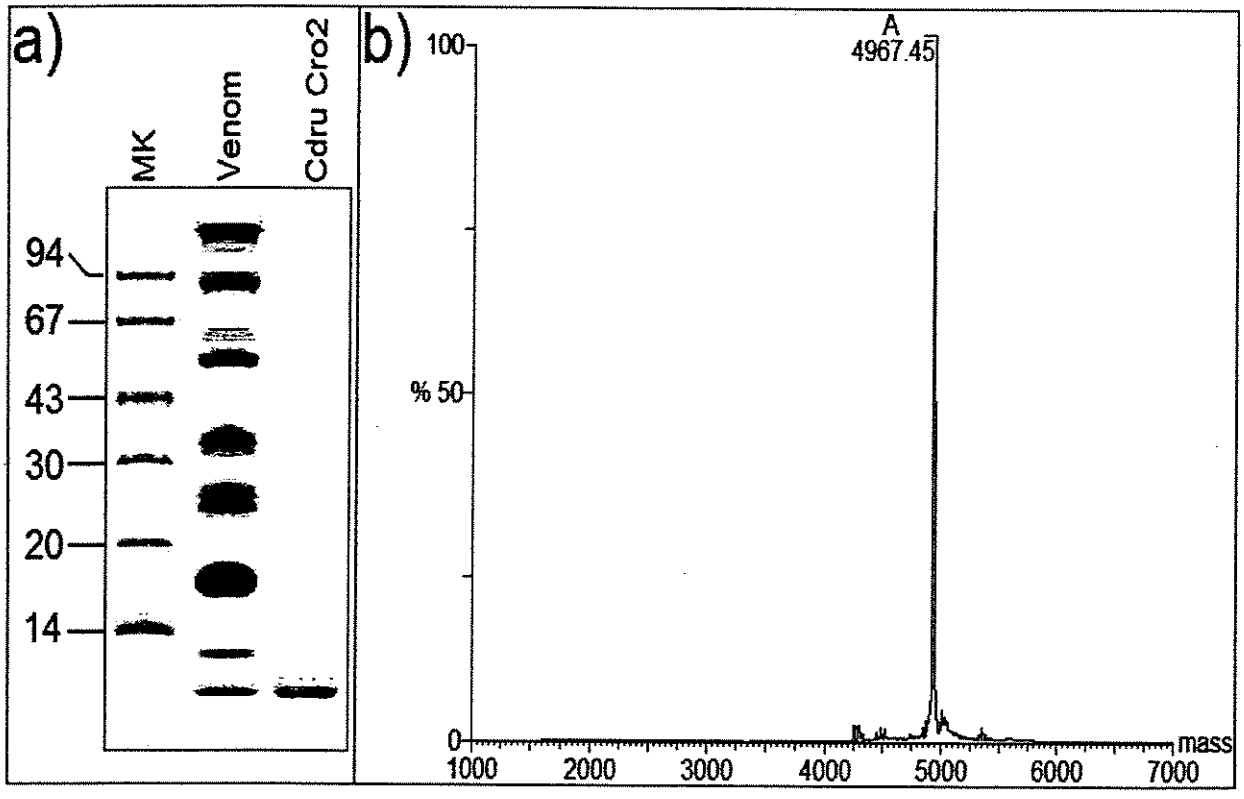
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Figure 3

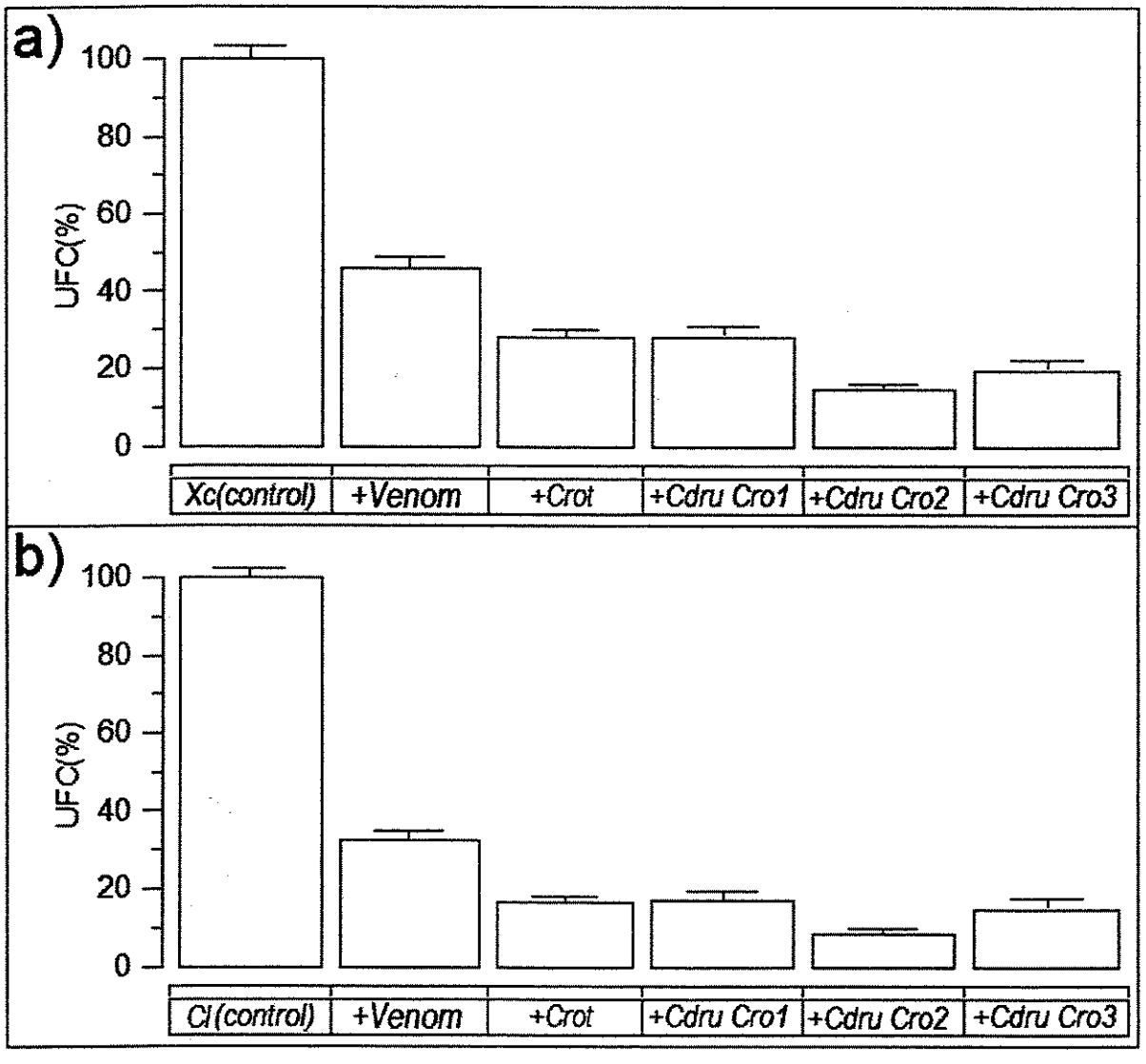
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Figure 4

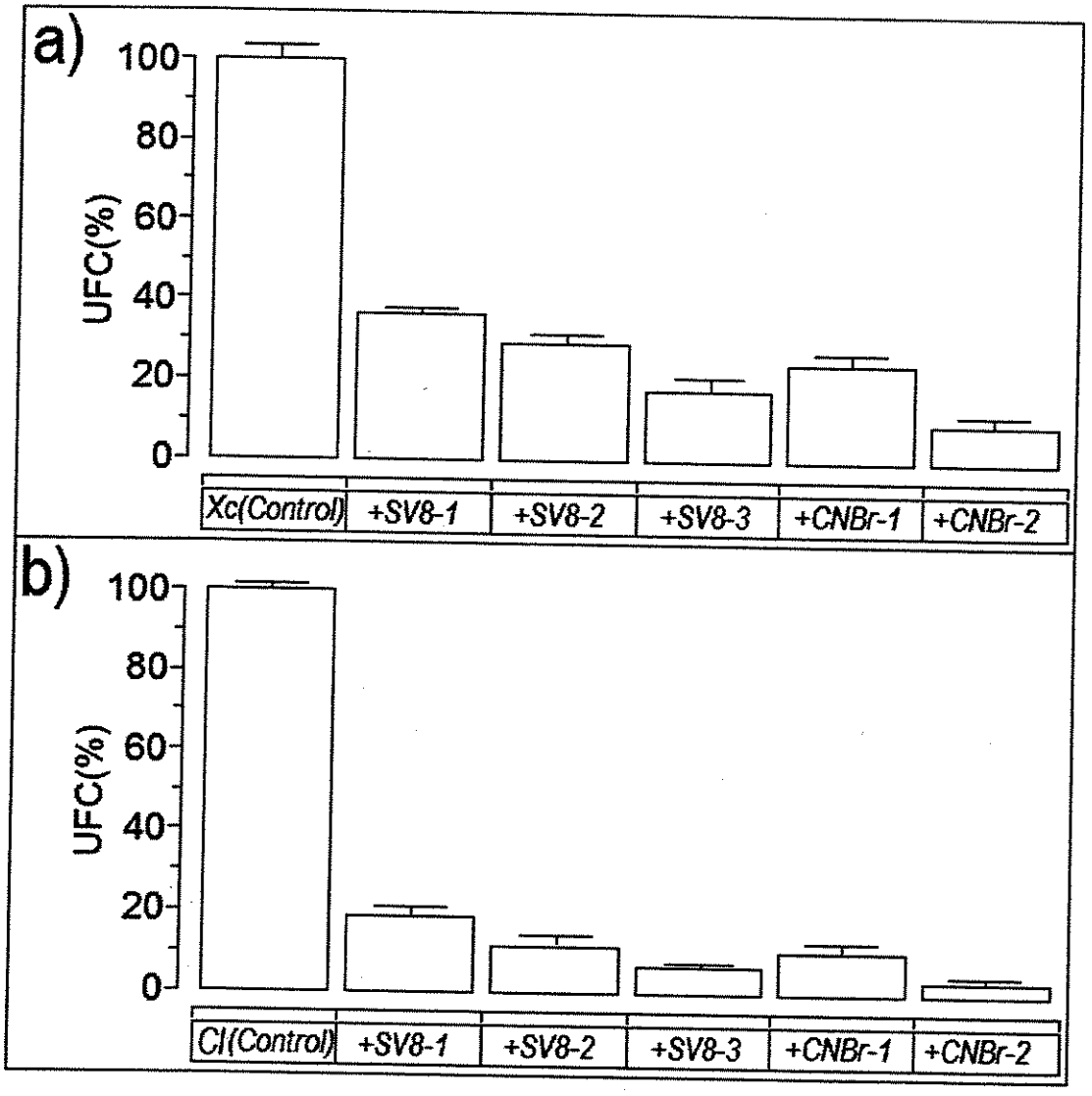
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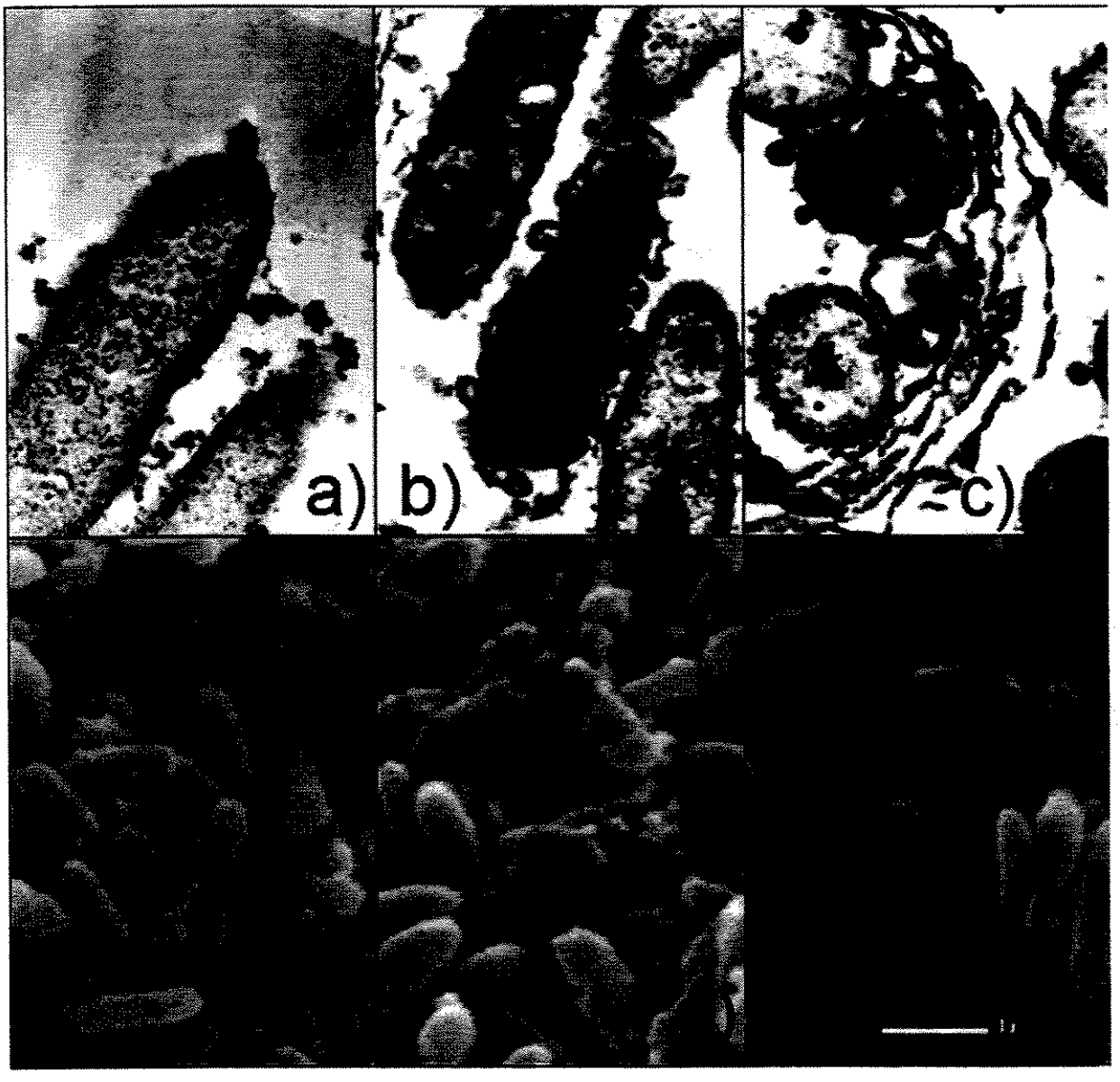
Figure 5



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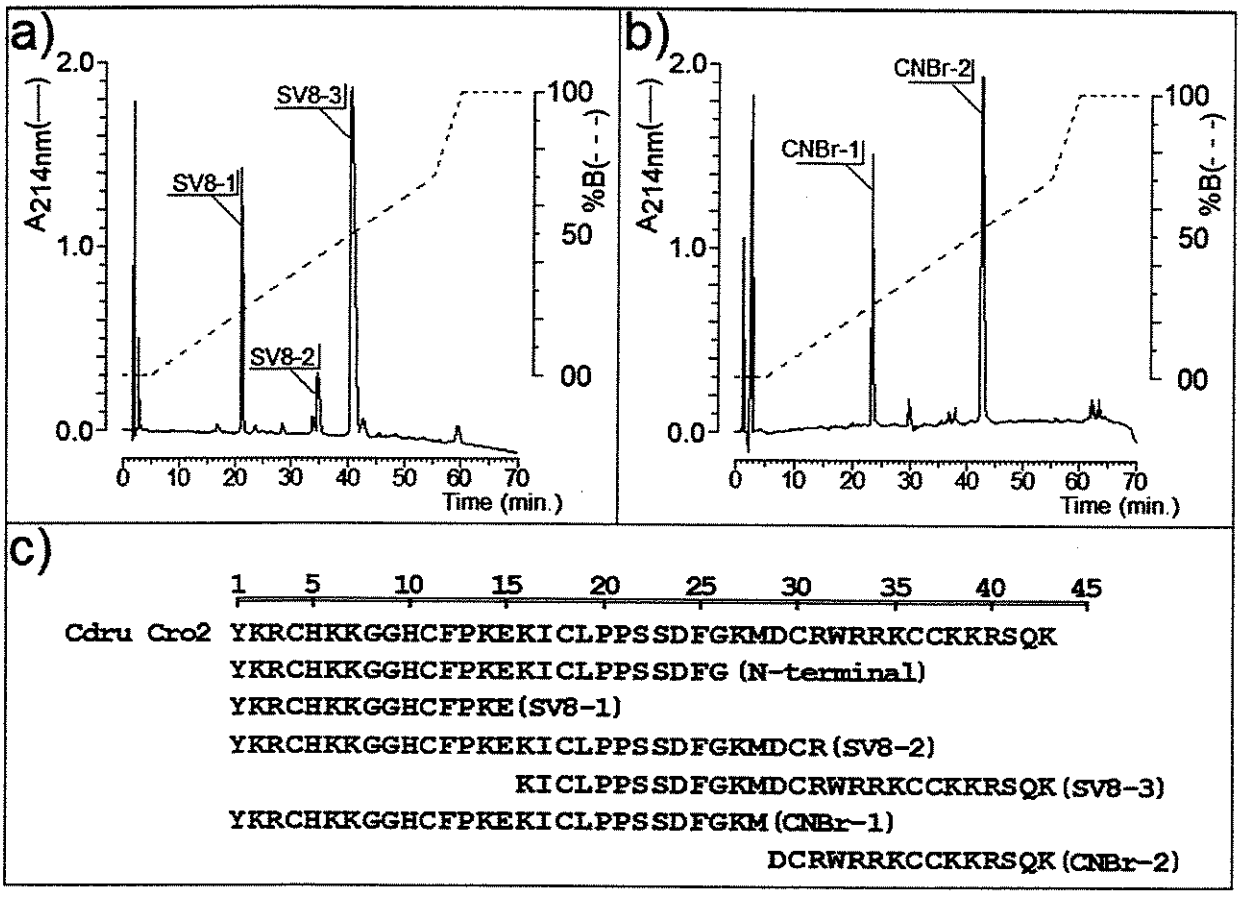
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Time (min.)
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Time (min.)
c)
1 5 10 15 20 25 30 35 40 45
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YKRCHKKGGHCFPKEKICLPPSSDFG (N-terminal)
YKRCHKKGGHCFPKE(SV8-1)
YKRCHKKGGHCFPKEKICLPPSSDFGKMDCR(SV8-2)
KICLPPSSDFGKMDCRWRRKCCKKRSQK(SV8-3)
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DCRWRRKCCKKRSQK(CNBr-2)

Figure 7

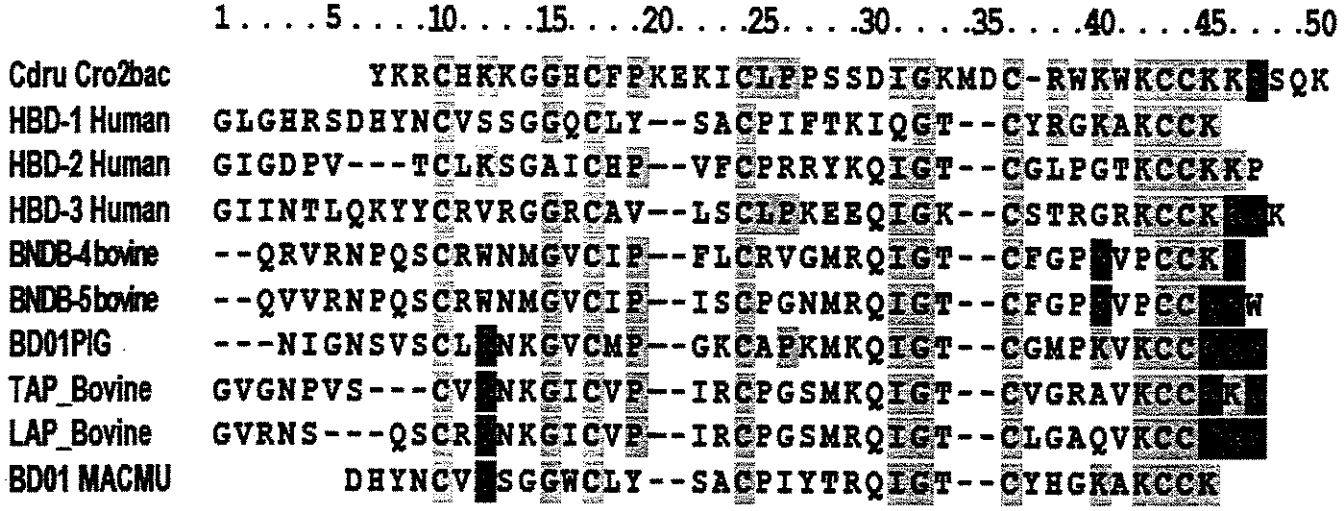
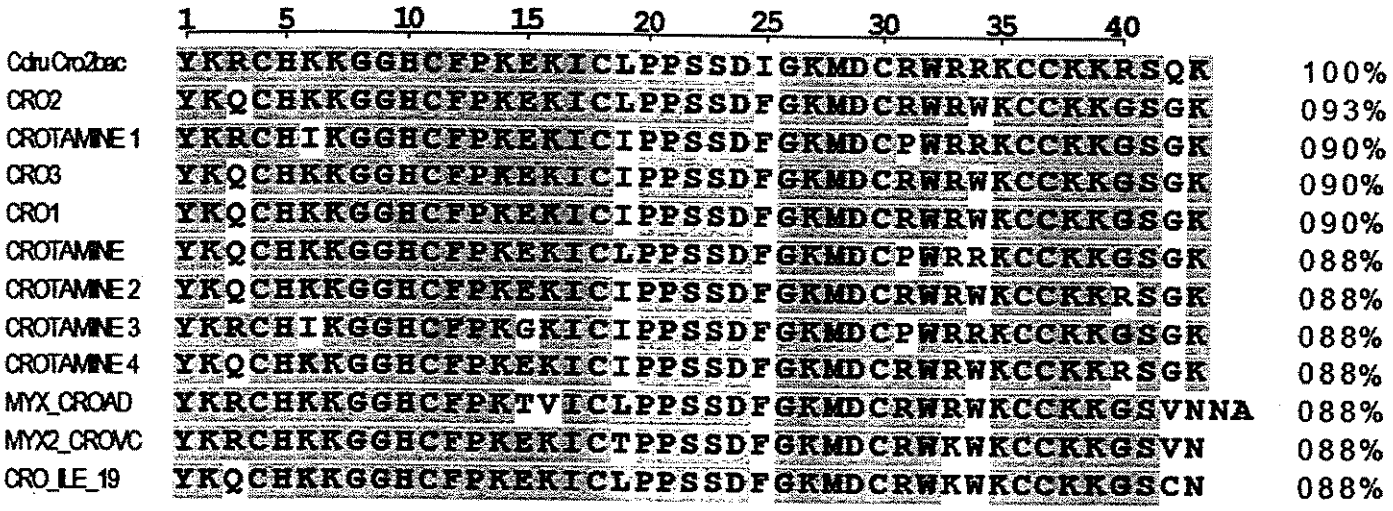

1....5....10....15....20....25....30....35....40....45....50
Cdru Cro2bac YKRCHKKGGHCFPKEKICLPPSSDIGKMDC-RWKWKCCKKSQK
HBD-1 Human GLGHRSDHYNCVSSGGQCLY--SACPIFTKIQGT--CYRGKAKCCK
HBD-2 Human GIGDPV---TCLKSGAICHP--VFCPRRYKQIGT--CGLPGTKCCKKP
HBD-3 Human GIINTLQKYYCRVRGGRCAV--LSCLPKEEQIGK--CSTRGRKCCK
BNDB-4bovine --QRVRNPQSCRWNMGVCIP--FLCRVGMRQIGT--CFGPVPCCK
BNDB-5bovine --QVVRNPQSCRWNMGVCIP--ISCPGNMRQIGT--CFGPVPCC
BD01PIG ---NIGNSVSCLNKGVCMP--GKCAPKMKQIGT--CGMPKVKCC
TAP_Bovine GVGNPVS---CVNKGICVP--IRCPGSMKQIGT--CVGRAVKCC
LAP_Bovine GVRNS---QSCRNKGICVP--IRCPGSMRQIGT--CLGAQVKCC
BD01 MACMU DHYNCVSGGWCLY--SACPIYTRQIGT--CYHGKAKCCK

Figure 8


1 5 10 15 20 25 30 35 40
CdtuCro2bac YKRCHKKGGHCFPKEKICLPPSSDIGKMDCRWRRKCCKKRSQK 100%
CRO2 YKQCHKKGGHCFPKEKICLPPSSDFGKMDCRWRWKCCKKGSGK 093%
CROTAMINE 1 YKRCHIKGGHCFPKEKICIPPSSDFGKMDCPWRRKCCKKGSGK 090%
CRO3 YKQCHKKGGHCFPKEKICIPPSSDFGKMDCRWRWKCCKKGSGK 090%
CRO1 YKQCHKKGGHCFPKEKICIPPSSDFGKMDCRWRWKCCKKGSGK 090%
CROTAMINE YKQCHKKGGHCFPKEKICLPPSSDFGKMDCPWRRKCCKKGSGK 088%
CROTAMINE 2 YKQCHKKGGHCFPKEKICIPPSSDFGKMDCRWRWKCCKKRSGK 088%
CROTAMINE 3 YKRCHIKGGHCFPKGKICIPPSSDFGKMDCPWRRKCCKKGSGK 088%
CROTAMINE 4 YKQCHKKGGHCFPKEKICIPPSSDFGKMDCRWRWKCCKKRSGK 088%
MYX_CROAD YKRCHKKGGHCFPKTVICLPPSSDFGKMDCRWRWKCCKKGSVNNA 088%
MYX2_CROVC YKRCHKKGGHCFPKEKICTPPSSDFGKMDCRWKWKCCKKGSVN 088%
CRO_ILE_19 YKQCHKKGGHCFPKEKICLPPSSDFGKMDCRWKWKCCKKGSCN 088%

Biological and Structural characterization of new PLA_2 from *Crotalus durissus colillineatus* venom

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The Protein Journal

Biological and structural characterization of new PLA_2 from the *Crotalus durissus collilineatus* venom.

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Key words: PLA_2; Crotoxin; *Crotalus durisssus collilineatus*; rattlesnake and venom, neurotoxicity, myonecrosis.

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Abstract

In the present article we reported the biological characterization and amino acid sequence of a new basic PLA_2 isolated from the *Crotalus durissus collilineatus* venom. This PLA_2 was named as Cdcolli F6 that present 122 amino acid residues with a pI value of 8.3 and molecular mass of 14 kDa. Its amino acid sequence was:HLLQFNKMIKFETRRNAIPFYAFY
GCYCGWGGRGRPKDATDRCCFVHDCCYEKLTGCNYKWDFYRYSLRSGYFQCGK
GTCEQQICECDRVAAECLRRSLSTYRKKYMIYPKSRCRKPSETC. This protein revealed an amino acid sequence identity of 80% with the crotalic PLA_2 such as Mojave B, Cdt F15, CROATOX, however, this homology drop to 50% if compared to other source PLA_2s, such as from the Bothrops snake venom. Although, Cdcolli F6 remains an amino acid sequence identity up to 93% with other crotalic venom, this protein showed a highly conserved basic amino acid residues in the C-terminal domain similar to found in the myotoxic PLA_2 from the Bothrops venom. Also this PLA_2 induced a myonecrosis, but this effect was lower than to BthTx-I or whole crotoxin. Also, this protein was able to induce a strong blockage effect on the chick biventer neuromuscular preparation, independently the presence of the acid subunid (crotapotin) and its neurotoxic activity was reduced by the treatment of this protein with heparin or the acetylating of the Lysine basic amino acid residues and the treated the PLA_2 with 4-bromophenacyl bromide (BPB) that also induced a significant reduction of the neuromuscular effects. This protein induced a strong antimicrobial activity against the *Xanthomonas axonopodis passiflorae* (Gram-Negative), which marginally reduced by treatment of PLA_2 with anhydrous acetic acid; however the p-BPB induced a strong decrease of the bactericidal effect. Our finding here allows to speculate that basic amino acid residues on the C-terminal and molecular regions near to catalytic site regions such as Calcium binding loop or β-wing region may involved in the biding of this PLA_2 to the molecular receptor to induce the neurotoxic effect. In case of the bactericidal effect, the enzymatic activity of this enzyme plays a crucial role this effect.

1. Introduction

Phospholipase A_2 form an expanding superfamily of enzyme, which catalyze hydrolysis of the catalyze hydrolysis of the ester bond at the sn-2 position of 1,2-diacyl-sn-3-phosphoglycerides and generate the arachidonic acid, which play an important biological roles. Depending on the molecular taxonomy used, intracellular and secretory PLA_2(s) are currently classified in either six or twelve different groups (Murakami and Kudo, 2002) or in six, depending of the molecular taxonomy used. The secretory PLA_2 are enzymes of 13-18kDa with 5 to 8 disulfide bound. They show much higher affinity for aggregating substrate than to non-aggregating ones, but in both case, millimolar Ca^{2+} is essential for their activity (Toyama et al., 2003). Secretory PLA_2 have been associated with many physiological and pathological processes that may occurs, independently of the enzymatic, and are highly dependent on the interaction of the $sPLA_2$ with specific receptors (Fuentes et al., 2002).

Various membranes and soluble proteins have been identified as selective and high affinity acceptors of secretory PLA_2, and there is also some evidence that the secretory PLA_2 may bind with voltage-dependent K^+ channels, pentraxins, reticulocalbins, C-type multilectins, factor Xa, and proteoglycan glypican (Dennis, 1997, Lambeau and Lazdunki, 1999, Kudo and Murakami, 2002, Fuentes et al., 2002). The increasing number of endogenous secretory PLA_2 identified in mammals and the versatility of their receptor suggest that many biological roles for the different secretory PLA_2 have not yet been discovered. The neurotoxic effect of the secretory PLA_2 from snake venom seems dependent on the interaction of the PLA_2 with a specific receptor(s) in the nerve. Several studies have been carried out to investigate the molecular basis for this event, but despite numerous efforts, this basis is still unclear.

There are various kinds of secretory PLA_2 in snake venom, with the monomeric PLA_2, such as that found in Bothrops, interacting alone, while the multimeric ones, such as those found in Bungarus toxin or *Crotalus atrox* that requires linkage with other proteins (Oliveira et al., 2002). The PLA_2 of all

subspecies of *Crotalus durissus terrificus* is crotoxin, which is a reversible protein complex consisting of a basic PLA_2 and an acidic, non enzymatic component known as crotapotin (Habermann and Breithaupt, 1978). Pharmacologically, the whole crotoxin exercise both pre- and post-synaptic actions, although the crotapotin component is generally considered to be pharmacologically inactive, serving merely as a chaperon protein for the PLA_2 and increasing its biological activity (Breithaupt, 1976; Habermann and Breithaupt, 1978; Bon et al., 1979).

Although different subspecies of *Crotalus dirussus* have been found to present slight variation in the properties of their PLA_2, the crotapotins has been considered to be the same. In the subspecies investigated here, however (*Crotalus durissus collilineatus*) the PLA_2 showed several biological activities independent the presence of crotapotin. In this article we described the purification, determination of the primary structure and some biological effect of this PLA_2.

2. Material and Methods

The *C. d. collilineatus* venom was kindly donated by the Instituto Butantan (São Paulo, Brazil). All solvents, chemicals and reagents used in this work were HPLC on sequence grade or of the highest purity available from Sigma, Aldrich Chemicals, Merck and Bio-Rad. The male Wistar rats (120-150 g) and Swiss mice (18-20 g) used in the pharmacological assay were obtained from the University's Central Animal House Services. All of the animal experiments were approved by the State University of Campinas Ethics Committee (São Paulo, Brasil).

2.1. Purification of Cdolli F6 PLA_2

The *Crotalus durissus collilineatus* whole venom (45mg) was completely dissolved in the ammonium bicarbonate buffer (0.2M, pH 7.9) at still complete homogenization followed a clarification step using a high speed centrifugation (4500xg for 2 minutes). The supernatant obtained was recovered

and applied on the HPLC molecular exclusion chromatography (1 x 60cm, Pharmacia) previously equilibrated with same buffer used for venom dissolution. The main fractions of the venom was purified by constant flow rate of 0.3ml/min and the run chromatography was monitored at A280nm and then crotoxin like protein was eluted, pooled and lyophilized for further reverse phase HPLC. The whole crotoxin were subjected to second chromatographic step using a reverse phase HPLC. This crotoxin was dissolved in 200µL of TFA 0.1% (buffer A) at still complete dissolution, followed by clarification using a high speed centrifugation (4500xg for 3 minutes). The supernatant was then injected on the µ-Bondapack C18 reverse phase HPLC column (0.3 x 30 cm). The elution of the PLA_2 was done, using a non linear gradient of buffer B (66.6% of acetonitrile in TFA 0.1%) at constant flow rate of 1.0 ml/min. The chromatographic run was monitored at A214nm and the fraction obtained was then lyophilized. The degree of the purity o Cdcolli F6 was evaluated by Tricine SDS-PAGE according to Shagger and von Jagow (1987) and mass spectrometry on a MALD TOFF mass spectrometer.

2.2. Measurement of PLA_2 activity

The PLA_2 activity along of both HPLC molecular exclusion, reverse phase and the enzymatic assaying of the native PLA_2, PLA_2 plus heparin, PLA_2 treated with anhydrous acetic acid and PLA_2 treated with 4-bromophenacyl bromide (BPB) were carried out according the method described by Holzer and Mackessy (1996), adapted for 96 well ELISA plate. The standard assay solution were composed by 200 µl of buffer (10 mM Tris-HCl, 10 mM $CaCl_2$, 100 mM NaCl, pH 8.0), 20 µl of substrate, 20 µl of water and 20 µl of PLA_2 at a final volume of 260 µl. Previously, the standard solution without PLA_2 and the PLA_2 solution were separately incubated for 30 minutes at a constant temperature of 37°C, after this previous incubation time, approximately 20µl of the PLA_2 solution was incubated in presence of substrate to monitoring the enzymatic velocity (Vo) that carried out at A405nm, at constant intervals of 10 minutes, during 20 minutes of the experiments. All assays were conducted in triplicate and the

absorbances at 425 nm were measured using a SpectraMax 340 mutiwell plate reader (Molecular Devices, Sunnyvale, CA) and enzymatic activity was calculated based on the differences in the absorbance values after 20 minutes of the incubation time.

2.3. PLA_2 inhibition and chemical modifications.

Inhibition. Inhibition by low molecular weight heparin (Fragmin, 25,000 IE/ml, *Mr* 5,000) or EDTA was evaluated after incubation at a heparin:toxin molar ratio of 2:1 or 1 mM EDTA for 30 min at 37°C.

The chemical modification of His 48 by ρ-BPB: The modification of His 48 residue was made according to the method described by (Soares et al., 2000). The Cdcolli F6 was modified by ρ-BPB by following procedure: 2mg of protein were dissolved in 20µl of 50mM of Thris-HCl (pH 8.0) and 0.9mM EDTA. An equal volume of 0.1mM ρBPB in dimethyl sulfoxide (DMSO) solution was added and small particle formed were suspended in the solution. The reaction mixture was maintained at 25°C for 30 min. and the precipitated ρ-BPB was removed by centrifugation. The supernatant was dialyzed for 24h and lyophilized (Zhang et al., 1994).

The chemical modification of Lys: The lisine amino acid residue by anhydrous acetic acid (Acetylation): Modification of lysine residues was performed at a protein: reagent molar ratio of 1:50. Protein (3mg) was dissolved in 1.5ml dos 0.2M Tris-HCl buffer, pH 8.0, and 10µl of anhydrous acetic acid (Sigma, USA) was added. The pH was adjusted again to 8.0 with NaOH (1M) after the addition of acetic anhydride. After 1hr of incubation at 25°C, protein was separated from the from the reagents by preparative reverse phase HPLC using a delta-pack C4 column (Waters), which was previously equilibrated with TFA 0.1% and the purification the modified PLA_2 was carried out by discontinuous acetonitrile (Acetonitrile 66.6% in TFA 0.1%) gradient. In addition the composition of the amino acids of unmodified and modified protein was done following the method described by Toyama et al., (1995). In this protocol one nmol of the purified protein was hydrolysed using 6N HCl at 106°C for 24h

followed by automated amino acid analysis in a PICO-TAG amino acid analysis system. The chemical modification of the PLA_2 with heparin: The isolated PLA_2 was pre-incubated for 30 min at 25°C with heparin (2.5 μg/ μg).

2.4 Sequencing procedure.

Two milligrams of the purified protein were dissolved in 200μl of a 6 mol/l guanidine chloride solution (Merck, Darmstadt, Germany) containing 0.4 mol/l of Tris-HCl and 2 mmol/l EDTA (pH 8.15). Nitrogen was blown over the top of the protein solution for 15 minutes; it was then reduced with DTT (6M, 200μl) and carboxymethylated with 14C-iodoacetic acid and cold iodoacetic acid. Nitrogen was again blown over the surface of the solution and the reaction tube was sealed. This solution was incubated in the dark at 37°C for 1 hour and desalted using a Sephadex G25 column (0.7 x 12cm) with 1mol/l acetic acid buffer. The reduced and carboxymethylated PLA_2 (RC-PLA_2) protein was digested with *Staphylococcus aureus* protease V8 for 16 h at 37°C; using a 1:30 enzyme to substrate molar ratio and the reaction was stopped by lyophilization. The RC-PLA_2 was also digested with clostripain for 8 h at 37°C and then lyophilized again. Part of the RC-PLA_2 protein was treated with cyanogen bromide for overnight, using a 1:30 enzyme to substrate molar ratio and the reaction was stopped and the digest was lyophilized. The digested products of these treatments were fractionated by reverse phase HPLC using a Waters PDA 991 system and a C18 μ-Bondapack column. The elution of peptide peaks was made using a linear gradient consisting of 0–100% of acetonitrile in 0.1% trifluoroacetic acid (v/v).

The sequencing of the N-terminal was conducted for the RC-PLA_2 protein, using a Procise f automatic sequencer. The phenylthyoidantoin (PTH) amino acids were identified by comparing their retention times with that of the 20 PTH amino acid standards. Peptides containing 14C-CM-Cys were monitored by detecting the radioactivity label using a liquid scintillation counter (Beckman model L-

250). The primary structure of the Cdcolli F6 protein was building mainly based on the purified peptides from the protein digested by cyanogen bromide, protease V8 and clostripain.

2.5. Myotoxic activity

The plasma creatine kinase (CK) activity was measured using the CK-UV kinetic kit (Sigma Chemical Co). The unmodified or modified PLA_2 (2 μg/μl) was injected intramuscularly in the gastrocnemius of 18-22 g male Swiss mice (50 μl, n=5). Mice used as negative controls were injected with PBS. After 3 h, a blood sample was collected from the tail in heparinized capillary tubes and centrifuged for plasma separation. CK activity was determined using 4 μl of plasma according to the manufacturer's instruction, and its activity was expressed in U/l.

2.6. Neurotoxic effect assay

Male chicks (4-8 days old) were killed with ether and the biventer cervicis muscle was removed (Ginsborg and Warriner, 1960) and mounted under a resting tension of 1g in a 4ml organ bath containing aerated ($95\%O_2$ + $5\%CO_2$) Krebs solution (pH 7.5, 37°C) of the following composition (mM): 118.7 NaCl, 4.7 KCl, 1.88 $CaCl_2$, 1.17 KH_2PO_4, 1.17 $MgSO_4$, 25.0 NaHCO and 11.65 Glucose. A bipolar platinum ring electrode was placed around the tendon, which ran the nerve trunk supplying the muscle. Indirect stimulation was applied with a Grass S4 stimulator (0.1 Hz, 0.2 msec, 3-4 mV). Muscle contractions and contractures were recorded by reparation was connected to a force displacement transducer (Narco Biosystems Inc) coupled to a Gould RS 3400 recorder. Contractures to exogenously applied acetylcholine (ACh, 55 or 110 μM for 60 s) and KCl (5 mM for 120–130 s) were obtained in the absence of nerve stimulation prior to the addition of modified and non modified PLA_2

(10 μg/ml) and at end of the experiment. The preparations were allowed to stabilize for at least 20 min before the addition of ACh or KCl and a single concentration (10 μg/ml) of the compounds.

2.7. Antibacterial Activity

Xanthomonas axonopodis. psv. passiflorae (gram-negative) bacterial strain were harvested from fresh agar plates and suspended in distilled sterilized water (A_{600nm} = 3 x 10^8CFU/ml). Aliquots of the bacterial suspension were diluted to 10^3 CFU/ml and incubated with PLA_2, treated and modified PLA_2 (250μg/ ml) for 1 h at 28°C; after incubation, survival was assayed on nutrient agar (Difco) plates (n=5). In both antibacterial assays, electron microscopic assessments of morphologic alterations were done in absence (control) or presence of PLA_2 and treated and modified PLA_2 (treated). The bacterial samples were fixed with solution establish (2,5% glutaraldeide and cacodylate buffer 0.1M pH 7.4 and 0.1M tanic acid) for 12 h at 4°C and refixed with 1% osmium tetroxide for 2h at 4°C. The sampes were dehydrated in increasing concentrations of ethanol and embedded in Epon resin. Polimeryzation was performed at 60°C for 48 h, and ultra-thin sections were prepared with a Sorvall MT-2 ultramicrotome. The sections were stained with 2%uranyl acetate for 20 min. fallowed by 2% lead citrate for 10 min. Samples were observed under a LEO-906 transmission electron microscope operating between 40 to 100 kV.

2.8 Statistical analyses

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

3. Results

The HPLC molecular exclusion chromatography of the *Crotalus durissus collilineatus* venom allowed the fractionation of several biologic active proteins and crotoxin fraction was eluted as the main fraction (approximately 60% of the total of venom). Crotoxin from the *C. d. collilineatus* venom present a moderate phospholipase A_2 activity as well as the discrete photolytic activity (Figure 1a). The purification of the acid component (Crotapotin) and the basic component (Phospholipase A_2) was carried out by non linear gradient concentration of Acetonitrile (Figure 1b).

The purified PLA_2 showed a moderate enzymatic activity that was strongly reduced by the treatment of PLA_2 with ρ-BPB, whereas the treatment of this enzyme with anhydrous acid induced a slight enzymatic reduction. Although in presence of heparin we observed a significant increasing of the enzymatic activity (Figure 2a). The native PLA_2 induced a significant myotoxic activity, but this effect was two fold little if compared to the native crotoxin. The ρ-BPB did not reduced significantly the myotoxic activity, but the anhydrous acetic acid treatment and heparin pre-incubation of PLA_2 reduced significantly the myotoxic effect induced by PLA_2 (Figure 2b).

This PLA_2 induced similar blockage effect observed to PLA_2 incubated with crotapotin, thus the neurotoxic effect induced to PLA_2 occur independently the presence of acid subunit (crotapotin). This neurotoxic effect was strongly reduced by pre-incubation of heparin, whereas anhydrous acetic acid and ρ-BPP showed similar reduction of the neurotoxic effect (Figure 3a and 3b, respectively).

The PLA_2 from the *C. d. collilineatus* also induced a strong bactericidal effect against *Xanthomonas axonopodis pv passiflorae* (Xc) and *Clavibacter michiganensis michiganensis* (Cl) and this effect was most effectively against Gran-negative bacterial strain. In both condition the treatment of this PLA_2 with ρ-BPB strongly reduced the bactericidal effect, whereas Heparin and anhydrous acetic acid showed similar reduction bactericidal activity of PLA_2 (Figure 4a and 4b, respectively).

PLA_2 induced several morphological alteration of the *Xanthomonas axonopodis pv passiflorae*, we observed in the incubation of bacteria with PLA_2 severe membrane damage, the lost of cytoplasmatic content, the cytoplasm appear with high electron dense material probably because the cytoplasmatic protein prescipitation. In the treatment of PLA_2 with ρ-BPB, Heparin or anhydrous acetic acid we observed some cytoplasmatic vacuolization and some membrane rupture (Figure 5).

The N-terminal region of PLA_2 was obtained by the direct sequence to RC-PLA_2 on the automatic sequencer at 37^{th} amino acid residue (Figure 6a). The rest of the primary structure of RC PLA_2 was obtained from the sequencing of the peptide obtained after the treatment of RC PLA_2 with specific digestion, among then the most important peptide fragment used to elucidate the complete amino acid sequence were: SV8 7, SV8 2 and SV8 5 (from the enzymatic treatment of RC-PLA_2 with protease V8) and Clt 5, Clt 9, Clt 2, Clt 1 (from the enzymatic treatment of RC-PLA_2 with Clostripain) (Figure 6).

This novel PLA_2 from the *C. d. collilineatus* was designated as Cdolli F6, which showed a high amino acid sequence identity with other crotalic PLA_2 such as Cdt F15, CROTOXB and MOJAVEB (Figure 7). This protein showed a high amino acid sequence identity to calcium biding, catalytic site and β-wing. This protein showed high conservation of the Lysine amino acid residue in the C-terminal domain similar to found to Bothropic venom (BthTx-I, Mtx II, PrTx-I), which are found in the other crotalic neurtoxic enzyme (Cdt F15) (Figure 7).

4. Discussion

The analysis of the chromatography suggests that crotoxin is the main toxin found the whole venom with approximately 60% of total. Thus the neurotoxic, myonecrotic or other biological activities may due by crotoxin. Our results also showed the elutions of two distinct proteases peaks, one of then correspond to gyroxin describe by Aguiar et al, (1996) and other found in the crotoxin peaks. Thus the native crotoxin beside the neurotoxic effect present a proteolytic activity that not still described for *Crotalus durissus collilineatus*. Landucci et al., (1994) showed that crotoxin from the *Crotalus durissus terrificus* induced the platelet aggregation and that it is not clearly dependent of the PLA_2 activity. Thus we may speculate that protease activity found inside of crotoxin play important role for the platelet aggregation and other haemostatic alterations.

The crotoxin from the *Crotalus durissus collilineatus* showed the presence of two major crotapotin and three PLA_2 isoforms. In the figure 1 we indicated the most abundant fraction of crotapotin as Crtp and the main phospholipase A_2 peak as PLA_2. This PLA_2 induced a moderate enzymatic activity that was strongly reduced by ρ-BPB, whereas the treatment with anhydrous acetic acid did not show a significant activity and the incubation of PLA_2 from the *Crotalus durissus collilineatus* with heparin induced a strong enzymatic activity. The ρ-BPB has been described as a strong alkylating agent that specifically used for the chemical modification of His48 that consequently eliminating the enzymatic activity. The His 48 is highly conserved in enzymatically active PLA_2 and play crucial role in the enzymatic catalysis (Verheij et al., 1980, Scott et al., 1992, Toyama et al., 1999). The enzymatic inhibition of *Crotalus durissus collilineatus* PLA_2 by anhydrous acetic acid was similar to observed for other sources of PLA_2. The effect of anhydrous acetic acid on the enzymatic activity of PLA_2 has been well described by Condrea et al., 1981 and Gowda et al., 1994 that observed a reduction of enzymatic power in around 25% after the treatment of different source PLA_2 with anhydrous acetic acid. The specific mode action of acetylation is not clear but there are some evidences that showed a

decreasing of ability of calcium to bind to catalytic site of PLA_2 and consequently decreasing or reducing the enzymatic activity of PLA_2 (Babu and Gowda, 1994 and Yang, 1997). The effect of heparin on the *Crotalus durissus collilineatus* PLA_2 was similar to described for other *Crotalus durissus terrificus* PLA_2 (Toyama et al., 2003). The precise mechanism of action of heparin is not still known, but probably involves the positive allosteric interaction between heparin and PLA_2 molecule.

The *Crotalus durissus collilineatus* PLA_2 is not a strong myotoxic compound and its myotoxic activity was significantly smaller than to native crotoxin. The discrepancy in these results founded probably is due the presence of other compound such as proteases that found in the native crotoxin, which will be increase the myonecrotic activity. Our results showed that myonecrotic activity induced by isolated PLA_2 from the *Crotalus durissus collilineatus* is due mainly the presence of the positively charged amino acid since the treatment of isolated PLA_2 with anhydrous acetic acid or heparin strongly reduced this effect. Díaz-Oreiro and Gutiérrez (1997), Soares et al. (2003) observed a drastic reduction of enzymatic or myonecrotic activities of the basic myotoxic PLA_2 treated with anhydrous acetic acid or incubated with heparin. Despite the PLA_2 from the *Crotalus durissus collilineatus* showed some structural and biological differences with to bothropic PLA_2, but both played the myonecrotic activity by similar way. The presence of the positively charged amino acid residues also will be important for the neurotoxic effect of this PLA_2 as shown in the figure 3b and the this effect is not strongly dependent of the enzymatic activity as suggest by the effect of treatment of this enzyme with ρ-BPB. The crotoxin has been described as the most potent neurotoxic compound found to *Crotalus durissus terrificus* venom and this compound it is composed by reversible association between a little neurotoxic basic PLA_2 and non neurotoxic and acid compound (crotapotin) (Habermann and Breithaupt, 1978 and Faure and Bon, 1988). Thus during years the neurotoxic effect of crotalic PLA_2 is mainly dependent of the presence of crotapotin. But in this paper we showed that *Crotalus durissus collilineatus* PLA_2 induced a strong neurotoxic effect independently the presence of crotapotin and this effect is strongly dependent of basic

amino acid residue located in the PLA_2. Thus the enzymatic activity is not so important for many biological effects induced by this PLA_2 in comparison to the Lys amino acid residue, but the enzymatic activity is very important for bactericidal effect of toxin whereas basic amino acid residues has little contribution for this. The electron microscopy also corroborated this observation and showed the bactericidal effect of this PLA_2 is dependent of the cell membrane destruction.

This protein showed the presence of 122 amino acid residues and this protein has high amino acid sequence identity with other crotalic snake venom such as Cdt F15, CROTOXB, MOJAVEB and MOJAVE (around 80%), but its amino acid sequence homology deep to around 55% for other PLA_2. The *Crotalus durissus collilineatus* PLA_2 showed conserved basic amino acid residues similarly to found in the bothropic PLA_2. This region probably is involved in the neurotoxic activity of this PLA_2. Several studies made with other PLA_2 also showed that C-terminal region play important role for the neurotoxic effect and the difference in the amino acid sequences of this region has a evident relationship to neurotoxic potency (Krizaj et al., 1989, Curin-Serbec et al., 1991, Lomonte et al., 2003 and Prijatelj et al., 2003). Thus these conserved basic amino acid residues seem to be involved in the neurotoxic effect of PLA_2 from the *Crotalus durissus collilineatus* but it is not the unique region responsible for this effect.

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Figure Captions

Figure 1.Chromatography profile of purification of whole venom by HPLC molecular exclusion (Figure 1a) and the purification of PLA_2 and crotapotin from the whole crotoxin by reverse phase HPLC (Figure 1b).

Figure 2. In figure 2a, it showed the enzymatic activity on the synthetic substrate and the effect of the some different treatments of this PLA_2 with ρ-BPB, anhydrous acetic acid and heparin. In figure 2b, it had shown the effects of these treatments on the PLA_2 on the myonecrosis.

Figure 3. In the figure 3a, we observed the neurotoxic effect of the isolated PLA_2 on the chick biventer cervicis, in presence or absence of crotapotin. In the figure 3b we observed the effect of some treatment on the neurotoxic effect of isolated PLA_2.

Figure 4. In the figure 4a, we showed the effect of the unmodified or modified PLA_2 against *Xanthomonas axonopodis pv passiflorae* (Figure 4a) and *Clavibacter michiganensis michiganensis* (Figure 4b).

Figure 5. The transmition electron microscopy scanning of the bacteria (Xanthomonas axonopodis pv passiflorae) in presence of native PLA_2 and treated with ρ-BPB, anhydrous acetic acid and heparin.

Figure 6. Determination of complete amino acid sequence of Cdcolli F6.

Figure 7. Amino acid alignment of Cdcolli F6 from the Crotalus durissus collilineatus with other crotalic and bothropic PLA_2 protein gift from the protein data bank (Pub Med).

Figure 1 Toyama et al.,

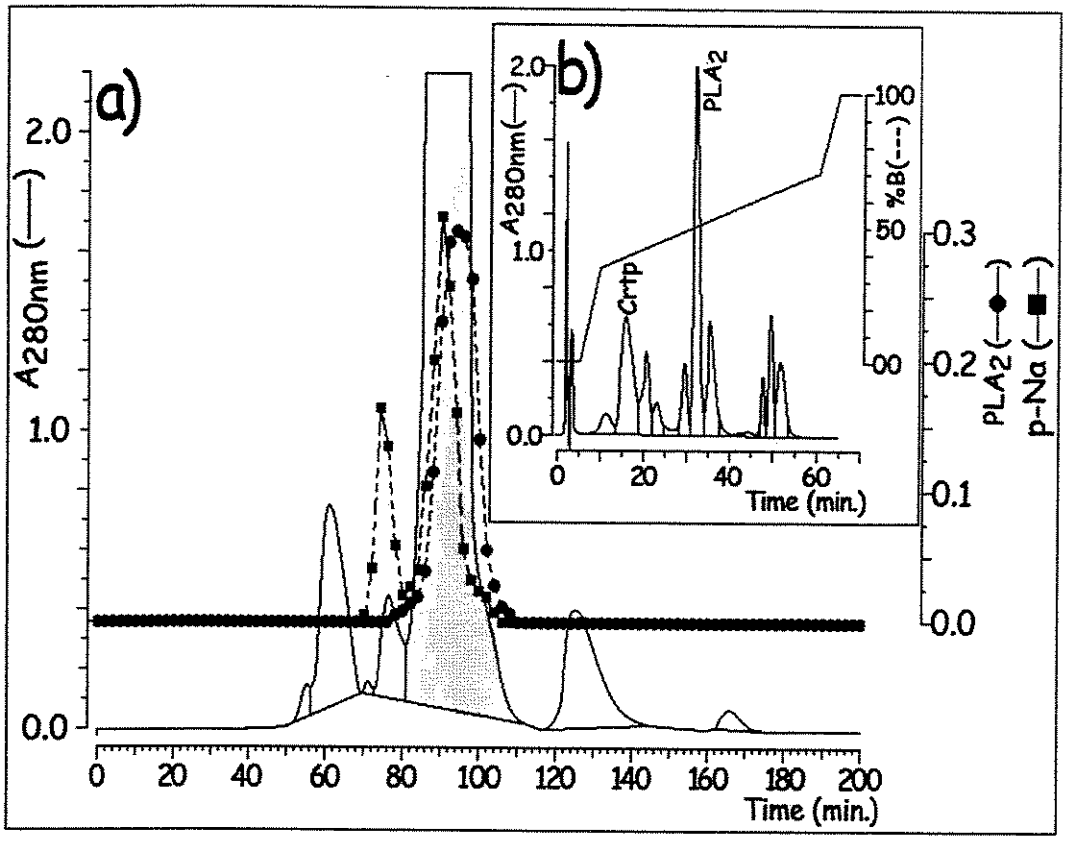


Figure 2

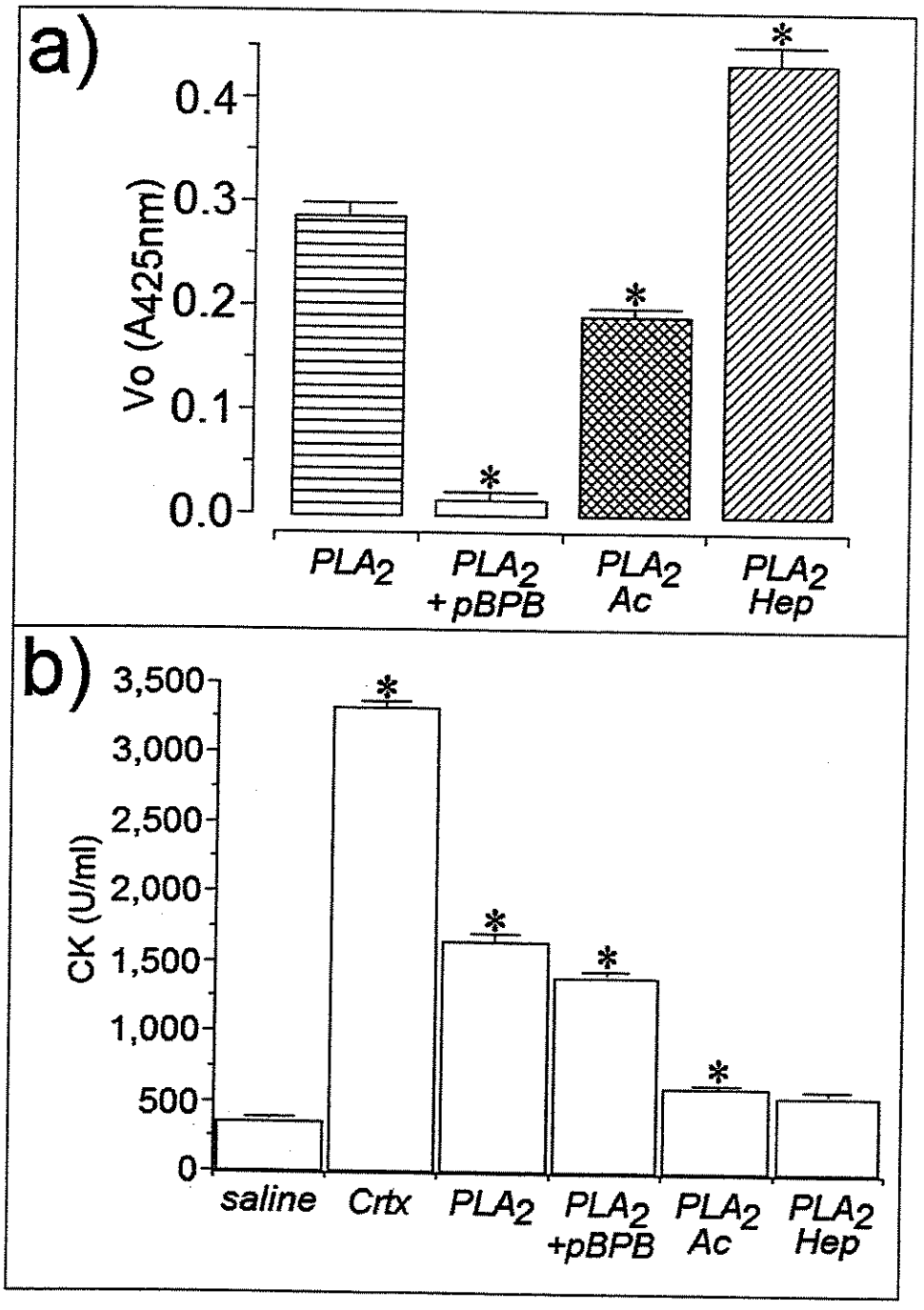

a)
Vo (A425nm)
0.0
0.1
0.2
0.3
0.4
*
PLA2
PLA2 +pBPB
PLA2 Ac
PLA2 Hep
b)
CK (U/ml)
0
500
1,000
1,500
2,000
2,500
3,000
3,500
saline
Crtx
PLA2
PLA2 +pBPB
PLA2 Ac
PLA2 Hep

Figure 3.

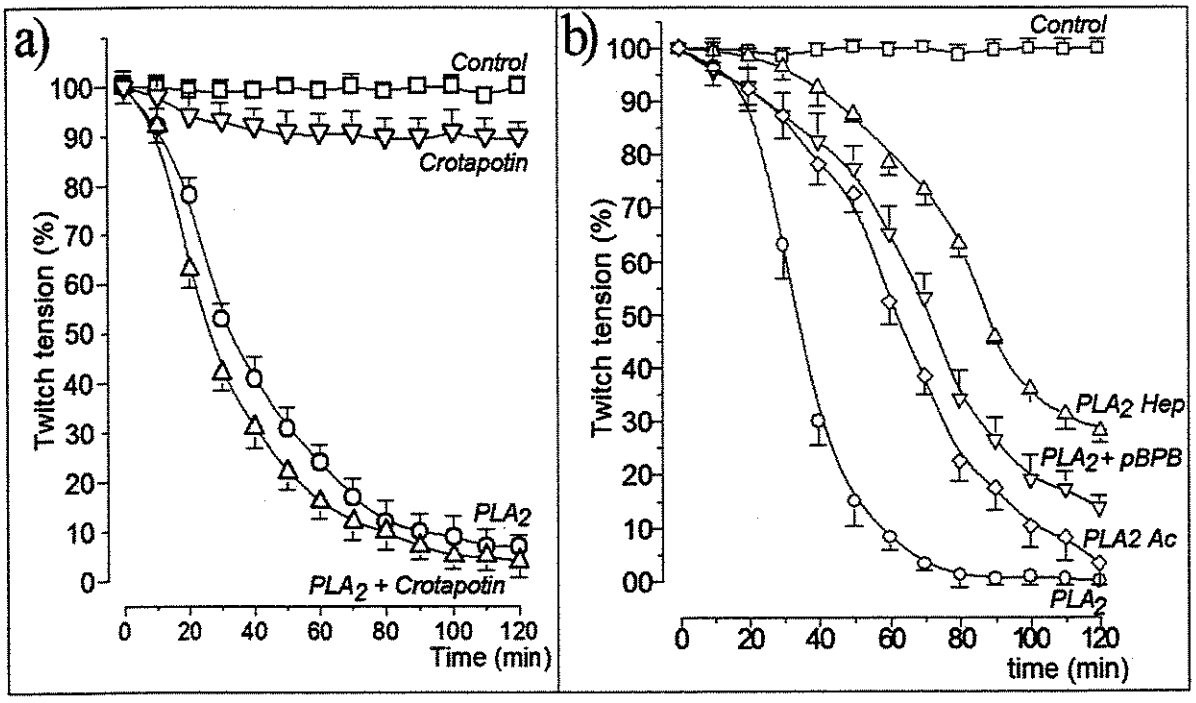

a)
Control
Crotapotin
PLA2
PLA2 + Crotapotin
Twitch tension (%)
Time (min)
b)
Control
PLA2 Hep
PLA2+ pBPB
PLA2 Ac
PLA2
Twitch tension (%)
time (min)

Figure 4.

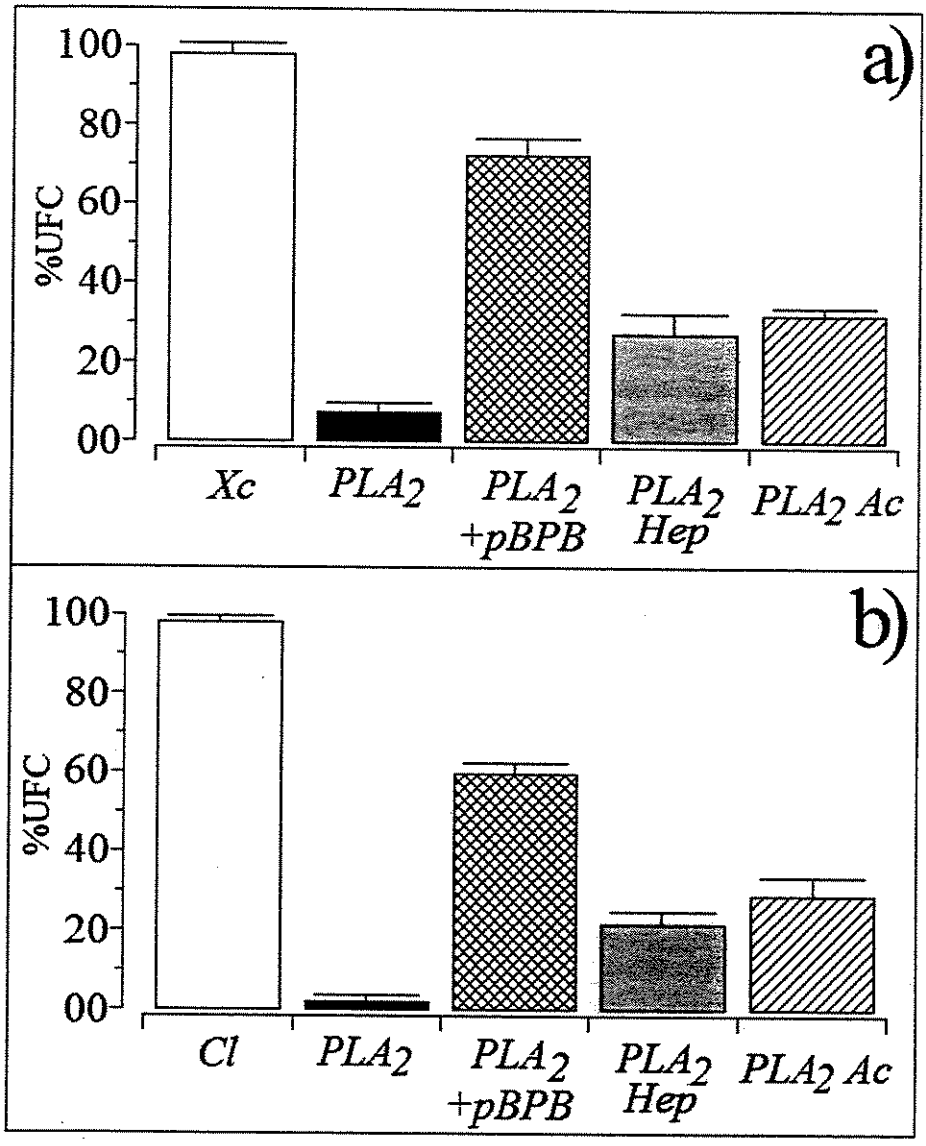

a)
100
80
60
40
20
00
%UFC
Xc
PLA2
PLA2 +pBPB
PLA2 Hep
PLA2 Ac
b)
100
80
60
40
20
00
%UFC
Cl
PLA2
PLA2 +pBPB
PLA2 Hep
PLA2 Ac

Figure 5.

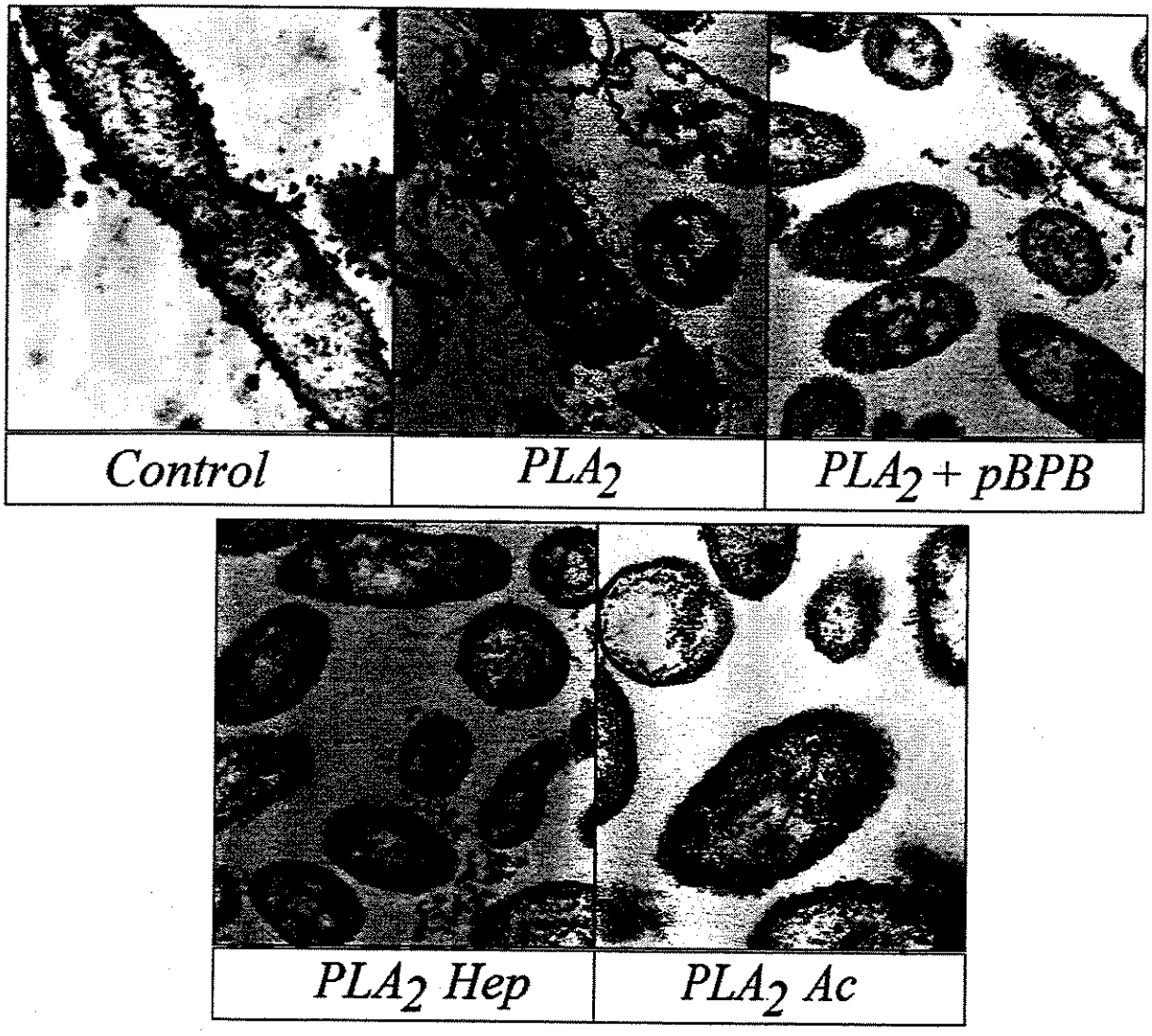
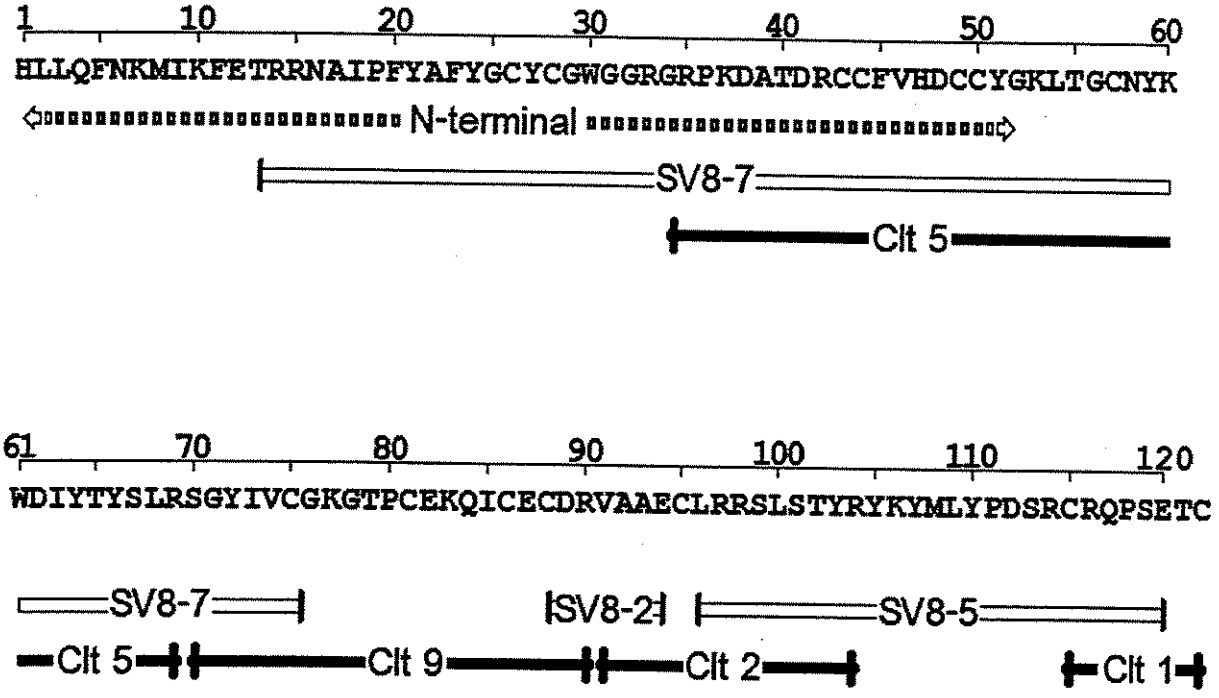


Figure 6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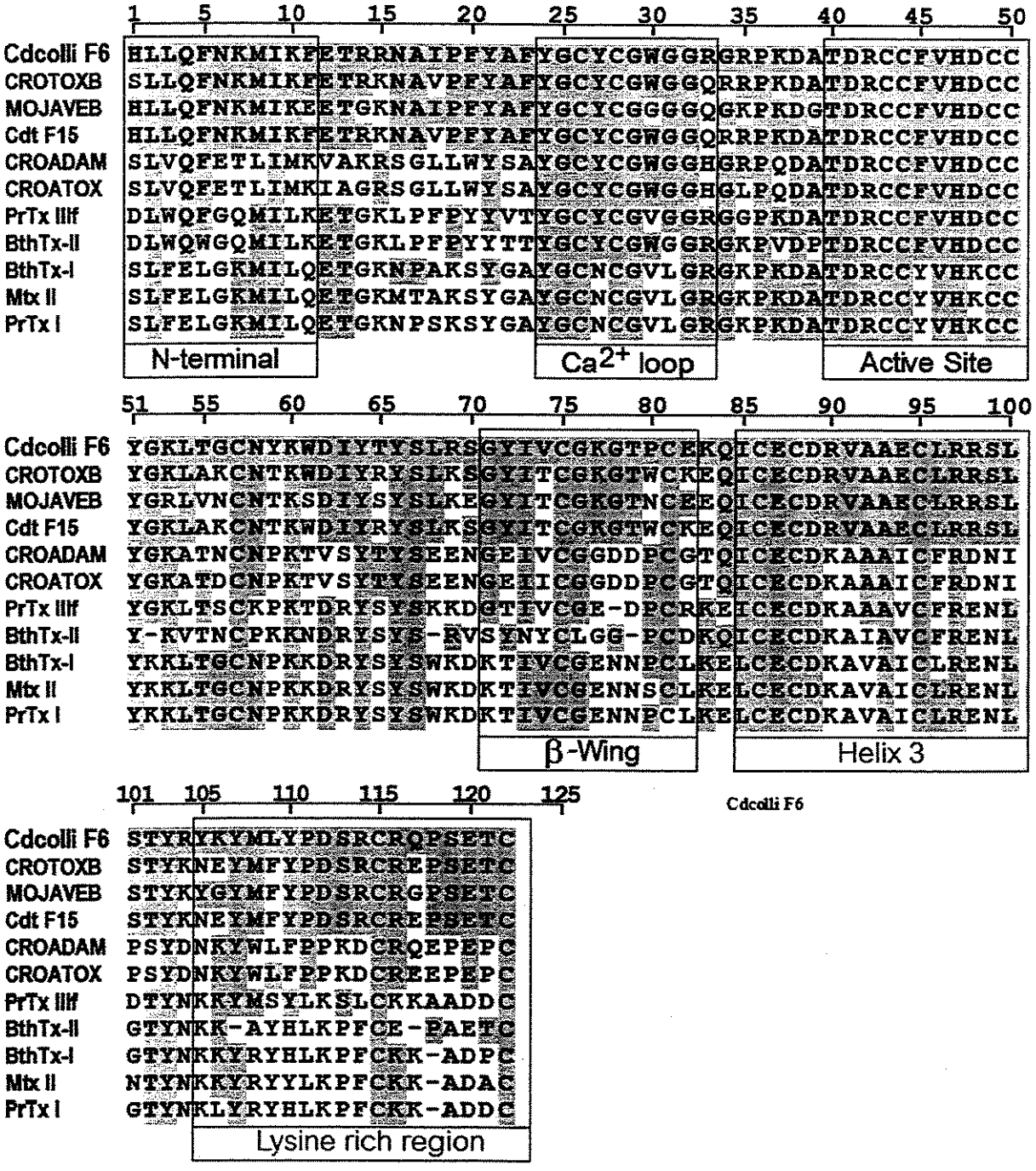
N-terminal
SV8-7
Clt 5
61 70 80 90 100 110 120
WDIYTYSLRSGYIVCGKGTPCEKQICECDRVAAECLRRSLSTYRYKYMLYPDSRCRQPSETC
SV8-7
SV8-2
SV8-5
Clt 5
Clt 9
Clt 2
Clt 1

Figure 7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Cdt F15 HLLQFNKMIKFETRKNAVPFYAFYGCYCGWGGQRRPKDATDRCCFVHDCC
CROADAM SLVQFETLIMKVAKRSGLLWYSAYGCYCGWGGHGRPQDATDRCCFVHDCC
CROATOX SLVQFETLIMKIAGRSGLLWYSAYGCYCGWGGHGLPQDATDRCCFVHDCC
PrTx IIIf DLWQFGQMILKETGKLPFPYYVTYGCYCGVGGRGGPKDATDRCCFVHDCC
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BthTx-I SLFELGKMILQETGKNPAKSYGAYGCNCGVLGRGKPKDATDRCCYVHKCC
Mtx II SLFELGKMILQETGKMTAKSYGAYGCNCGVLGRGKPKDATDRCCYVHKCC
PrTx I SLFELGKMILQETGKNPSKSYGAYGCNCGVLGRGKPKDATDRCCYVHKCC
N-terminal
Ca2+ loop
Active Site
51 55 60 65 70 75 80 85 90 95 100
Cdcolli F6 YGKLTGCNYKWDIYTYSLRSGYIVCGKGTPCEKQICECDRVAAECLRRSL
CROTOXB YGKLAKCNTKWDIYRYSLKSGYITCGKGTWCKEQICECDRVAAECLRRSL
MOJAVEB YGRLVNCNTKSDIYSYSLKEGYITCGKGTNCEEQICECDRVAAECLRRSL
Cdt F15 YGKLAKCNTKWDIYRYSLKSGYITCGKGTWCKEQICECDRVAAECLRRSL
CROADAM YGKATNCNPKTVSYTYSEENGEIVCGGDDPCGTQICECDKAAAICFRDNI
CROATOX YGKATDCNPKTVSYTYSEENGEIICGGDDPCGTQICECDKAAAICFRDNI
PrTx IIIf YGKLTSCKPKTDRYSYSKKDGTIVCGE-DPCRKEICECDKAAAVCFRENL
BthTx-II Y-KVTNCPKKNDRYSYS-RVSYNYCLGG-PCDKQICECDKAIAVCFRENL
BthTx-I YKKLTGCNPKKDRYSYSWKDKTIVCGENNPCLKELCECDKAVAICLRENL
Mtx II YKKLTGCNPKKDRYSYSWKDKTIVCGENNSCLKELCECDKAVAICLRENL
PrTx I YKKLTGCNPKKDRYSYSWKDKTIVCGENNPCLKELCECDKAVAICLRENL
β-Wing
Helix 3
101 105 110 115 120 125
Cdcolli F6
Cdcolli F6 STYRYKYMLYPDSRCRQPSETC
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CROATOX PSYDNKYWLFPPKDCREEPEPC
PrTx IIIf DTYNKKYMSYLKSLCKKAADDC
BthTx-II GTYNKK-AYHLKPFCE-PAETC
BthTx-I GTYNKKYRYHLKPFCKK-ADPC
Mtx II NTYNKKYRYYLKPFCKK-ADAC
PrTx I GTYNKLYRYHLKPFCKK-ADDC
Lysine rich region

Isolation of new L-amino acid oxidase from *Crotalus durissus cascavella*

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Toxicon

Isolation of new L-amino acid oxidase from *Crotalus durissus cascavella.*

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Abstract.

In this article we purified and characterized a new L-amino acid oxidase from the *Crotalus durissus cascavella* whole venom (Casca LAAO) by molecular exclusion and cation exchange HPLC. Casca LAAO is an acid glycoprotein with a pI value and molecular mass of 5.4 and 68 kDa, approximately, estimated by two dimension electrophoresis. Its N-terminal sequence was ADDRHPLAECFRETDYEEFLEIARNGLNATSNPKHVVIVGAFMSFL...This protein showed high content of acid charged and hydrophobic amino acid content. This enzyme induced platelet aggregation and its activity was dose dependent manner and was strongly inhibited by incubation of the platelet solution with aspirin (1^{-5}M) and indomethacin (1^{-5}M) whereas incubation with catalase abolished this effect. Casca LAAO also inhibited strongly the bacterial growth rate of Gram-negative (*Xanthomonas axonopodis pv passiflorae*) and Gram-positive (*Streptococcus mutans*). The antimicrobial effect also was strongly inhibited by addition of catalase in the incubation medium. Thus the peroxide hydrogen produced by Casca LAAO induces significantly modification of the membrane structure that will involve in the bacterial cell membrane lyses.

Key words.Crotalus durissus; platelet aggregation, LAAO, antimicrobial, catalase.

1.Introduction.

L-amino acid oxidase (LAAO, EC 1.4.3.2) is a flavoenzyme that catalyzes the oxidative deamination of L-amino acids substrate to an α-keto acid along with the production of ammonia and hydrogen peroxide and is widely distributed in snake venom. The snake LAAOs are much more active than the mammalian L-amino acid oxidase and has been widely used in studies to identify optical isomers of amino acids and laboratory preparation of α-keto acids from L-amino acids (Takatsuka et al., 2001 and Du et al., 2002). Because of its cofactor (flavin), this enzyme generally presents as yellow color. LAAOs from the bacterial, fungal and plant species are involved in the utilization of nitrogen sources but the function of snake venom LAAOs is still poorly understood, but the ability of this protein to induce or inhibit the platelet aggregation or induce apoptosis of different cells were well reported (Du et al., 2002). The purification of LAAOs from various snake venoms has been reported by several groups, LAAOs from different sources exhibit differences in specificity, stability, and other diverse biological activities as citocity, hemorrhage, hemolysis, edema, antibacterial and ant parasite activities was reported (Tempore et al., 2000, Ali et al., 2000).

Purified LAAOs from the snake venom are usually homodimeric, acid or polypeptide chain with pI values between 4.4 ~8.5, FMN- or FAD-binding (~2 mol/mo.l) glycol (~3,8 – 4%) protein with the approximately molecular mass of ~120 – 150 kDain the native and ~55-66 kDa in their monomeric forms (Ali et al., 2000). The snake venom LAAOs structure presents limited sequence homology with LAAOs from bacterial or fungal enzymes but these proteins probably share a similar topological architecture in addition to a preserved catalytic site (Ali et al., 2000). In this work we isolated a new LAAO from the *Crotalus durissus cascavella* whole venom and also investigate the role of H_2O_2 generated by this protein and involvement of some inflammatory mediator on the platelet aggregation and investigate the mode action of this protein against Gram-Negative and Gram-positive bacterium.

2. Material and Methods.

2.1. Reagents and Venom.

The venom was obtained from the Instituto Butantan and all chemical used here was analytical, HPLC and sequence grade from Sigma and Aldrich chemical, Waters, Applied Biosystens, Pierce and Bio Rad.

2.2. LAAO purification.

Molecular exclusion chromatography: Approximately 35 mg of whole venom from the *Crotalus durissus cascavella* venom was dissolved in 400µl ammonium bicarbonate buffer (0.2M; pH 8.0) and

homogenized at still complete dissolution, followed by clarification with high speed centrifugation (4500xg for 2min). The supernatant was recovered and injected on a molecular exclusion HPLC column (Superdex 75, 1x60cm, Pharmacia), previously equilibrated with same buffer used for dissolving the whole venom. The flow rate used for elution of the fraction was 0.2ml/ml; the chromatography was monitored at 280nm and the fractions isolated was immediately lyophilized and stored at -40°C. The LAAO activity fraction (Gyroxin) was stored for future repurification.

Ion exchange chromatography: Approximately 5 mg of gyroxin fraction purified fro the whole venom was dissolved in 250 µl ammonium bicarbonate buffer (0.05M; pH 7.9) and homogenized at still complete dissolution, followed by clarification with high speed centrifugation (4500xg for 2min). The supernatant was recovered and injected on a ion exchange HPLC column (Protein Pack DEAE 5PW, 0.75 x 10cm, Waters), previously equilibrated with same buffer used for dissolving the whole venom. For the elution of fraction was done using a discontinuous linear gradient of ammonium bicarbonate concentration of 1.0M at constant flow rate of 1.0ml/min, the chromatography run was monitored at 280nm and the fractions isolated was immediately lyophilized and stored at -40°C.

Purity degree of LAAO from Crotalus durissus cascavella: The LAAO fraction from molecular exclusion and ion exchange chromatography of *Crotalus durissus cascavella* venom was purified by reverse phase HPLC according to method described by Toyama et al. (2000). Briefly, three milligrams of whole LAAO was dissolved in 250µl of buffer A and centrifuged at 4500xg for 2 minutes and the supernatant was then applied on the analytical reverse phase HPLC, previously equilibrated with buffer A (0.1% trifluoroacetic acetic acid (TFA) for 15 minutes. The elution of the protein was then conducted using a linear gradient of buffer B (66.6% Acetonitrile in buffer A) and the chromatographic run was monitored at 214nm of absorbance. After elution the fraction was lyophilized and stored at -40°C. The purity degree of protein was assayed using two dimensional (2D) electrophoresis and MALDI TOF mass spectrometry. 2D electrophoresis was conducted as described by Anderson (1991). The spectrometry used proteins purified by HPLC, with the protein was spotted on a sample plate and introduced into the MALDI TOF mass spectrometer.

2.3. Microplate assay for LAAO

The assay was conducted in a 96-well microplate in triplicate; to 10 µl/ well of enzyme solution, 90 µl/ well of substrate solution was added to start the reaction. The standard reaction mixture contained 10mM L-Methionine, 2mM o-phenylenediamine (OPD), 0.81 U/ml horseradish peroxidase (HRP), and LAO in a total volume of 100ml in a total volume of 100µl/ well of 50mM Tris-HCl buffer (pH 8.5).

After incubation at 37°C for 60 min, the reaction was terminated by adding 50□l of 2M H_2SO_4. The absorbance of the reaction mixture was measured by a Spectra Max microplate reader (Molecular Devices, CA) at 492nm, using 630 nm as reference wavelength (492/ 630 nm) (Kishimoto e Takahashi, 2001). Hydrogen peroxide standards were used and the linear regression data calculated with the Origin 5.0 Software and LAO activity was expressed as nmoles H_2O_2/min.

2.4. N-terminal amino acid sequence determination

Three milligrams of purified protein were dissolved in 200 µl of 6M guanidine chloride (Merck, Darmstadt, Germany) containing 0.4mM Tris-HCl and 2 mM EDTA (final pH 8.15). Nitrogen was flushed over the top of the protein solution for 15 min, which was then reduced with DTT (6M, 200 µl) and carboxymethylated with ^{14}C–iodoacetic acid and cold iodoacetic acid. Nitrogen was again flushed over the surface of the solution and the reaction tube sealed. This solution was incubated in the dark at 37°C for 1 h and desalting was done on a Sephadex G 25 column (0.7 x 12 cm) in 1mM acetic acid buffer. The eluted reduced and carboxymethylated (RC) protein was then lyophilized and stored at -20°C. Analysis of the amino acid sequence of the RC-protein was performed with an Applied Biosystems model Procise f gas-liquid protein sequencer. The phenylthiohydantoin (PTH) derivatives of the amino acids were identified with an Applied Biosystems model 450 microgradient PTH-analyser.

2.5. Amino acid analysis

Approximately one nmol do the purified and RC-protein was treated hydrolyzed with 6N HCl (200µl) in presence of 10µl of phenol. The amino acid hydrolysis was performed at 106°C for 24 hour and after this time the excess of HCl was removed and hydrolyzed amino acid were redried with aqueous solution of (ethanol: water: triethylamine; 2:2:1 by vol). The pós-column derivatization was done with aqueous solution of phenylisothiocyanate (ethanol: water: triethylamine: phenylisothiocyanate; 7:1:1:1 by volume). Either sample or amino acid standard were derivatized using a PICO-TAG amino acid analyzer system. The analysis of the PTH-amino acid was made using a PICO-TAG amino acid analyzer (waters).

2.6. Antibacterial activity against Xanthomonas axonopodis. pv. passiflorae and Streptococcus mutans

Xanthomonas axonopodis pv. passiflorae (Gram-negative) bacterial strain and *Streptococcus mutans* (Gran-positive) were harvested from fresh agar plates and suspended in distilled sterilized water

(A_{600nm}=10^8 CFU/ml). Aliquots of bacterial suspension were diluted to a 10^3 CFU/ml and incubated with the isolated crotamine and CNBr peptide fragments peptides (150 μg/ml) for 1 h at 37 °C to *S. mutans* and 28°C to *X. a. pv. passiflorae* , after which the survival was assayed on blood agar and nutrient agar(Difco) respectively plates (n=5).

2.7. Electron microscopy

The transmission electron microscope of *Xanthomonas axonopodis. pv. passiflorae* and *Streptococcus mutans* assessments of morphological alterations were done with bacteria incubated with saline (control) and with LAAO incubated with bacteria . The bacterial samples were fixed with solution establish (2,5% glutaraldeide and cacodylate buffer 0.1M pH 7.4 and 0.1 M tanic acid) for 12 h at 4°C and refixed with 1% osmium tetroxide for 2h at 4°C. The sampes were dehydrated in increasing concentrations of ethanol and embedded in Epon resin. Polimeryzation was performed at 60°C for 48 h, and ultra-thin sections were prepared with a Sorvall MT-2 ultramicrotome. The sections were stained with 2%uranyl acetate for 20 min. fallowed by 2% lead citrate for 10 min. Samples were observed under a LEO-906 transmission electron microscope operating between 40 to 100 kV. The scanning electron microscopy (SEM) of *Xanthomonas axonopodis. pv. passiflorae and Streptococcus mutans* assessments of morphological alterations were done with bacteria incubated with saline (control) and with LAAO (treated). The samples were taken for examination after the incubation time requested for the antimicrobial activity (60 min). After centrifugation, pellets were fixed at 4°C in 0.1 M cacodylate buffer (pH 7.4) containing 2.5% (v/v) glutaraldehyde for 12 h. The bacterial samples were fixed again with 1% osmium tetraoxide for 2 h at 4°C. The samples were dehydrated in increasing concentration of ethanol. Specimens were coated with gold in vacuum using a Sputter Coater BALZERS SCD 050.

2.8. Platelet aggregation studies

Sample collection and aggregation studies: Venous blood was collected with informed consent from healthy volunteers who denied taking any medication in the previous 14 days Blood was collected by a two-syringe technique using polypropylene syringes and 19-gauge needles, and immediately transferred into polypropylene tubes containing 1/10th final volume of 3.8% trisodium citrate. For some experiments, blood was drawn using the same citrate concentration but with the addition of apyrase

(grade III, Sigma Chemical Co., St. Louis, MO) to achieve a final concentration of 5 U/ml (based on 5'-ATPase activity). Blood was taken into acid/citrate/dextrose anticoagulant and centrifuged for 20 min at 150 *g* to yield platelet-rich plasma (PRP), which was used within 3 h. For the preparation of WP, the PRP was recentrifuged in the presence of 50 nM prostacyclin at 800 *g* for 10 min to obtain a platelet pellet Vargas et al., (1982). This was suspended in physiological buffer (150 mM NaCl, 5 mM Hepes, 0.55 mM NaH_2PO_4, 7 mM $NaHCO_3$, 2.7 mM KCl, 0.5 mM $MgCl_2$, 5.6 mM glucose, pH 7.4) and diluted to a count of 3×10^8 platelets/ml. The platelets were left for 1 h at room temperature to recover their sensitivity to aggregating agents. In addition, platelets were prepared from PRP by lowering the pH to 6.5, centrifuging and restoring the pH to 7.4, as previously described (Naseem et al., 1995), to study these interactions on very fresh platelets. Platelet counts were performed on a Coulter S Plus (Coulter Electronics, Hialeah, FL) or by phase-contrast microscopy. Measurements of platelet aggregation were made in a Chrono-log Lumiaggregometer (Chrono-Log Corp., Havertown, PA), using 0.45 ml of patient platelet, which placed under the aggregometer cuvet that was maintained at 37°C and the suspension stirred at 1200 rpm with a magnetic stirrer.

2.9. Statistical analyze

The results were expressed as the mean ± SEM. The data were analyzed by analysis of variance (ANOVA) followed by a Bonferroni test. The level of significance was set at $p< 0.05$.

3.Results.

Crude venom from rattlesnake was first subjected to a Superdex 75 gel filtration HPLC column that gives four major fractions. The LAAO activity was found in the Gyroxin fraction that was collected and stored (Fig. 1a). Gyroxin was then applied on ion exchange chromatography where active LAAO peak was found in the initial of gradient (Fig. 1b). This fraction was eluted as single peak on the reverese phase HPLC that separated the FAD from the whole molecule (Fig. 2a). This high purified protein was then analyzed on the two dimensional electrophoresis where Casca LAAO was spoted as single protein with molecular mass of 68 kDa and pI value of 5.4 (Fig. 2b). The molecular mass of Casca LAAO determined by gel filtration HPLC column showed a molecular mass of 120kDa approximately (data not shown), suggesting that Casca LAAO has a dimeric conformation under natural condition. The purification yielded 0.28% of the crude venom protein. According to the LAAO activity (described below), the purified protein was concentrated 67-fold from the crude venom (Table I). The first 46 N-terminal amino acid residues of the intact enzyme were determined of the intact enzyme

reduced and carboxymethiled were determined by direct automated Edman degradation analysis to get additional evidence of purity and homology between different venom LAAOs. Comparison of the N-terminal sequence of Casca LAAO with the enzyme isolated from other snake venom reported so far, revealed a close sequence homology with *Agkistrodon hays blomhoffi* (~97.7%), *Agkistrodon hays hays* (~97.2%), *Crotalus adamanteus* (95.8%) and *Trimeresurus stejnegeri* (96.3%) (Fig. 3). The amino acid composition of the purified Casca LAAO after acid hydrolysis is shown in table II and systematically compared with other reported venom LAAOs. The amino acid composition of Casca LAAO does not differ substantially from those other reported venom LAAO. This protein also presented high contents of Asx (asparagines and aspartic acid) and Glx (glutamate and glutamic acid) and low content of basic charged amino acid, that confer a moderated acid character to molecule (Table II).

Casca LAAo induced platelet in human platelet-rich plasma (PRP) in a dose-dependent manner at 10, 5, 1 and 0.5μg/ml (Fig. 4). The ED50 for inducing platelet aggregation of Casca LAAO was estimated at 7μg/ml and this effect was dependent the production of H_2O_2 that is the end product of LAAO. The effect of presence of hydrogen peroxide in the reaction medium was well demonstrated by addition of Catalase together with Casca LAAO, which abolished this effect. Furthermore, we observed a strong platelet aggregation decreasing by addition of Indometacin (1×10^{-5}M) or Aspirin (1×10^{-5}M), which inhibitor of activation of endogenous PLA_2, suggest that the activation of endogenous phospholipase A_2 is also important for platelet aggregation induced by Casca LAAO (Fig. 4b).

The Casca LAAO was most effect against Gram-positive bacterial strain than to Gram-negative strain and its antibacterial activity were higher than to whole venom (Fig. 5a and 5b, respectively). For both bacterial strain, Casca LAAO antibacterial activity were strongly inhibited by addition of Catalase, 0.1mg/ml) in the bacteria and protein incubation medium. These results also showed that hydrogen peroxide generated by this enzyme was crucial for bacterial membrane destruction as observed in transmissition electron and scanning electron microscopy of bacterial treated by Casca LAAO and the bacterial growth inhibition curve (Fig 6, Fig. 7 and Fig. 5, respectively). The scanning electron microscopy reveled presence of membrane vesiculation presented on the both bacterial strain membranes (Fig. 6) and the transmission electron microscopy showed the presence of high content of darkness colored material on the membrane surface in both bacterial strain (Fig. 7), in some case we observed high vacuolization on the Gram-negative bacteria.

4.Discussion.

Purification and biochemical characterization: In the present work, we purified o novel L-amino acid oxidase (LAAO) from the *Crotalus durissus cascavella* whole venom, which purified at high molecular homogeneity after two chromatographic steps. This novel LAAO showed a high stable, moderate acid character molecule and has FAD as enzymatic cofactor, this compound represents around 0.28% of dried venom in case of *Crotalus durissus cascavella* but other venom has a large amount of LAAO (Torii et al., 1997). The biochemical properties as molecular mass, acid character and activity were consistent to found for other L-amino acid oxidase described in the literature (Du et al., 2002). This protein was eluted in the molecular exclusion HPLC appears with molecular mass of 115-125 kDa, but its molecular mass determined by two dimensional electrophoresis was a monomeric protein with molecular mass of 67kDa, these results suggest a formation of aggregate structure that commonly observed for glycoprotein. This aggregation or dimerization is commonly observed for LAAO (Stabeli et al., 2004). Its pI was observed around to 5-6 that is similar to the other enzymes described in the literature and the amino acid analysis corroborate this results because the presence of high content of Asp or Glu. Comparison of the N-terminal sequence of Casca LAAO with the enzyme isolated from other snakes venoms reported so far, revealed close sequence homology, in particular with *Agkistrodon contortrix laticinctus*, *Crotalus adamanteus* and *Trimeresurus stejnegeri*. In the N-terminal sequence, at least 46 amino acid residues showed the high content of acid amino acid residue (mainly glutamic acid) that found to be fully conserved in all sequences analyzed here and presence of methionine at 43st, thus suggesting the presence of a highly conserved glutaminic-rich motif.

Antibacterial activity: Skarnes (1970) first reported bactericidal effects elicited by LAAO from the *Crotalus adamanteus* venom. Sliels et al., (1991) also found that two LAAO from the venom of *Pseudechis australis* have power effect against Gram-positive and Gram-negative bacteria. In the present study, we showed that antibacterial activity of Casca LAAO is due to H_2O_2 generated by LAAO activity as confirmed by addition of catalase, a H_2O_2 scavenger that totally quenched the antibacterial activity. The hydrogen peroxide produced by Casca LAAO induces a bacterial membrane rupture and consequently promoted the membrane rupture and extravasation of plasmatic content to outside of cells that appear as oxidative elements (dark colored precipitate) in the outer of bacterial cell. The minimal concentration that inhibited the bacterial growth rate at 50% of UFC against *Xanthomonas axonopodis pv passiflorae* and *Streptococcus mutans* were: 35μg/ml and 12.3μg/ml, respectively, thus the amount of H_2O_2 generated was sufficient of inhibit bacterial growth. Our results suggest that ability of this protein

to binding on the bacterial membranes did not appeared to play an important role in the antibacterial activity such as described for achacin (Ehara et al., 2002), that isolated from the giant snail.

Platelet Aggregation: This protein induces a dose dependent platelet aggregation in platelet-rich plasma (PRP). The LAAO from the other venom play important role in the blood coagulation after the rattlesnake bites and is involved directly or indirectly for thrombosis or changes in the blood homeostasis because this protein for some species such as *Crotalus atrox*, Malayan pit viper (*Calloselasma rhodostoma*) (Torii et al., 1997). But in case of *Crotalus durissus cascavella,* the amount of LAAO is marginal if compared to other important toxins that also affect the blood homeostasis such as Convulxin (Francischetti et al., 2000) and Crotoxin (Landucci et al., 1994). At first time we might to conclude that Casca LAAO is not so important for general haemostatic alteration induced by venom, but its ability to induce the platelet aggregation is similar to other LAAO described in the literature and probably this compound will be acts synergistically with other proteins and in this case Casca LAAO was important for *Crotalus durissus cascavella* snake venom. Although it is not clear that the different biological and toxic effect induced by the venom LAAO are interrelated or/ and mediated by the same mechanism, the production of H_2O_2 an end product of the oxidation of L-amino acid. Also the real action of LAAO from the snake venom is controversy, because there is some report that characterized LAAO as potent inhibitor of platelet aggregation and other that showed ant agonic response, as potent inducer of platelet aggregation (Du et al., 2002). In case of Casca LAAO we characterized as good aggregating ability and this effect was completely abolished by addition of catalase. Indomethacin or aspirin that is general inhibitors of cyclooxygenase products were also able to strongly reduce this effect. The analysis of these results suggests that Casca LAAO induces platelet aggregation through the formation of H_2O_2, and subsequent thromboxane A_2 synthesis, which are previously described by other LAAO isolated from the *O. hannah* venom (Li et al., 1994).

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Table I The amino acid composition of L-amino acid oxidase from *Crotalus durissus cascavella* compare to other LAOs from the other sources.

Purification Step	Total protein (mg)	nmoles H_2O_2/min	Specific activity
Crude Venom	500	0,0562	0,0001124
Superdex 75	38	0,352	0,00926
SP 5PW	1,44	3,25	2,256

Table II The amino acid composition of L-amino acid oxidase from *Crotalus durissus cascavella.*

aa	Casca LAAO
Asp	10,75
Glu	9,56
Ser	6,78
Gly	8,05
His	2,75
Arg	4,68
Thr	5,85
Ala	7,28
Pro	5,22
Tyr	4,92
Val	6,42
Met	1,62
Cys	1,23
Ile	5,62
Leu	7,31
Phe	4,61
Lys	6,32
Trp	1,03
Total	100%

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Figure Caption.

Figure 1. Purification of whole venom on the molecular exclusion HPLC, the active LAAO fraction as eluted and identified as gyroxin (Fig. 1a) that repurified on the ion exchange HPLC that eluted a high purity protein (Fig. 1b).

Figure 2. Molecular homogeneity assessment of the Casca LAAO purified on molecular exclusion and ion exchange by reverse phase HPLC (Fig. 2a). The spot of the purified protein on two dimensional electrophoresis for estimation of molecular mass and pI value (Fig. 2b).

Figure 3. Sequence analysis of the N-terminal region of Casca LAAO with other LAAO from the other venons, LAAO Casca (LAAO from the *Crotalus durissus cascavella*), LAAO AGKRH (LAAO from the *Agkistrodon hays blomhoffi*), LAAO AGKRB (LAAO from the *Agkistrodon hays hays*), LAAO CROAD (LAAO from the *Crotalus adamanteus*) and LAAO TRISTJ (LAAO from the *Trimeresurus stejnegeri*).

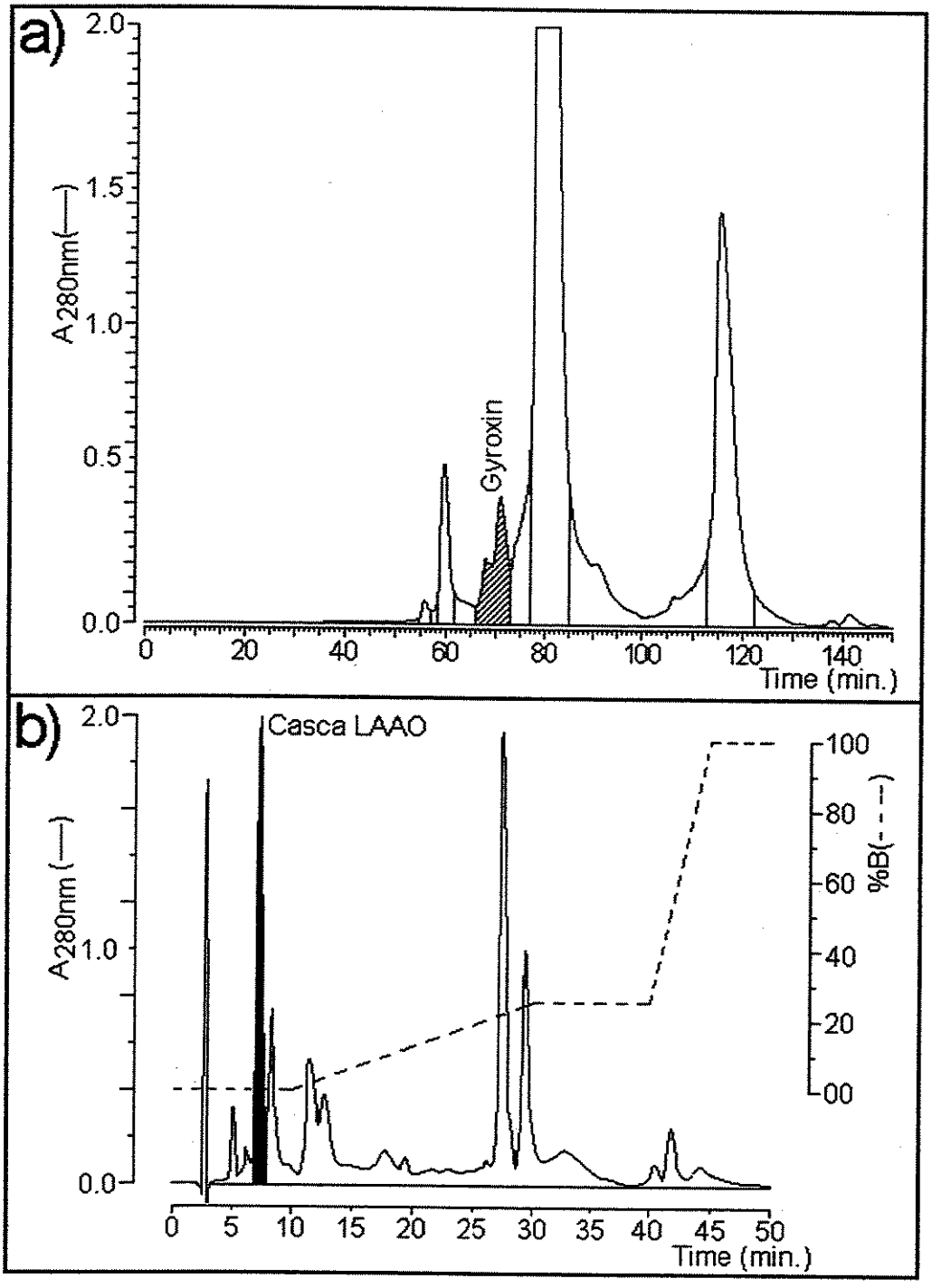
Figure 4. Effect of Casca LAAO on the PRP pallets preparation at different concentration (10, 5, 1 and 0.5μg/ml). In this protocol we used ADP as positive control of the aggregation (4a). In this protocols we evaluated the effect of Catalase (0.1mg/ml), indometacin (Indo, $1x10^{-5}$M) and Aspirin (Aspirin, $1x10^{-5}$M), in this protocol we use as control Casca LAAO (10μg/ml).

Figure 5. Antimicrobial effect of Casca LAAO incubated with *Xanthomonas axonopodis pv passiflorae* (5a) and *Streptococcus mutans* (5b), using a single dose of 250μg/ml in absence and presence of catalase (0.1mg/ml).

Figure 6. Scanning electron micrography of the *Xanthomonas axonopodis pv passiflorae* and *Streptococcus mutans* incubated with native Casca LAAO. In this protocol we used a single dose of 250μg/ml. The non treated bacteria was designated as control, native Casca LAAO treated as Xc+Casca LAAO (Casca LAAO plus *Xanthomonas axonopodis pv passiflorae*) or Sm+Casca LAAO (Casca LAAO plus *Streptococcus mutans*).

Figure 7. Transmission electron micrography of the *Xanthomonas axonopodis pv passiflorae* and *Streptococcus mutans* incubated with native Casca LAAO. In this protocol we used a single dose of 250μg/ml. The non treated bacteria was designated as control, native Casca LAAO treated as Xc+Casca LAAO (Casca LAAO plus *Xanthomonas axonopodis pv passiflorae*) or Sm+Casca LAAO (Casca LAAO plus *Streptococcus mutans*).

Figure 1.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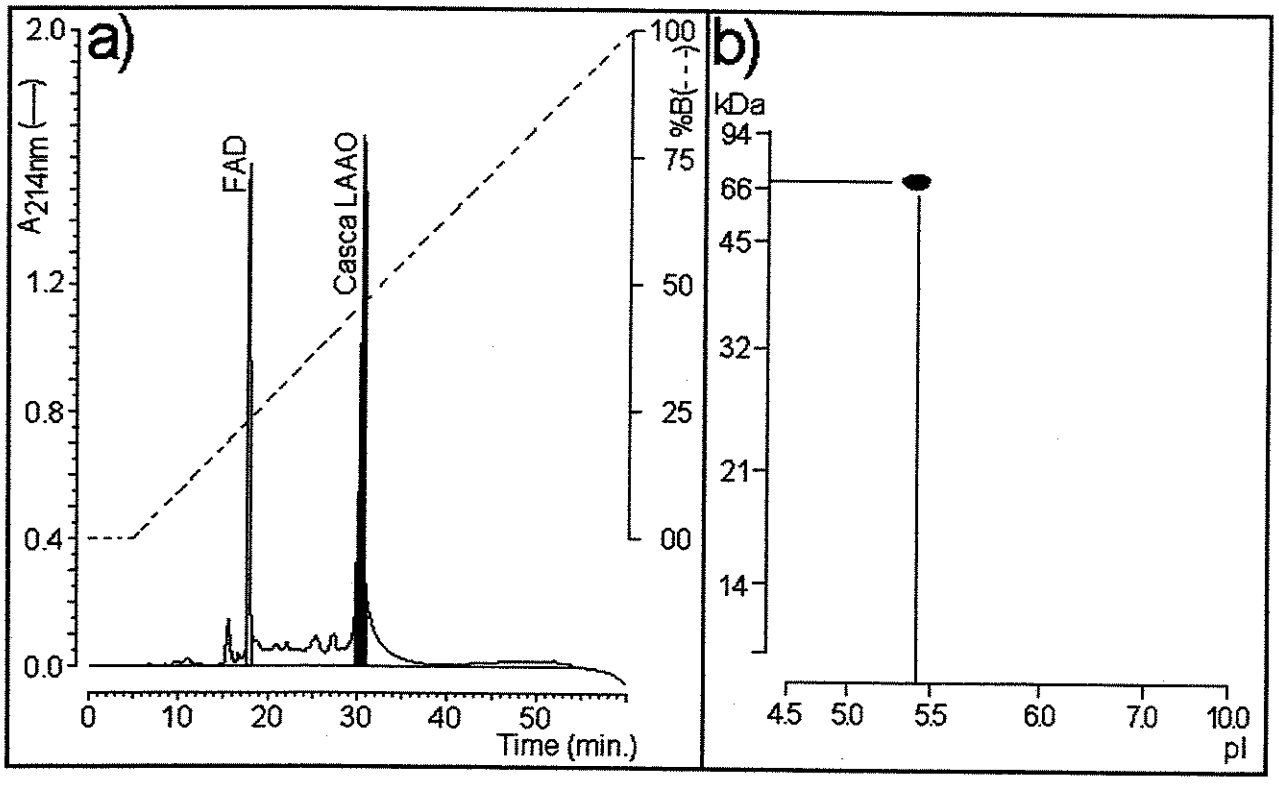
2.0
1.5
1.0
0.5
0.0
A280nm(—)
Gyroxin
0
20
40
60
80
100
120
140
Time (min.)
b)
2.0
1.0
0.0
A280nm (—)
Casca LAAO
100
80
60
40
20
00
%B(- - -)
0
5
10
15
20
25
30
35
40
45
50
Time (min.)

Figure 2.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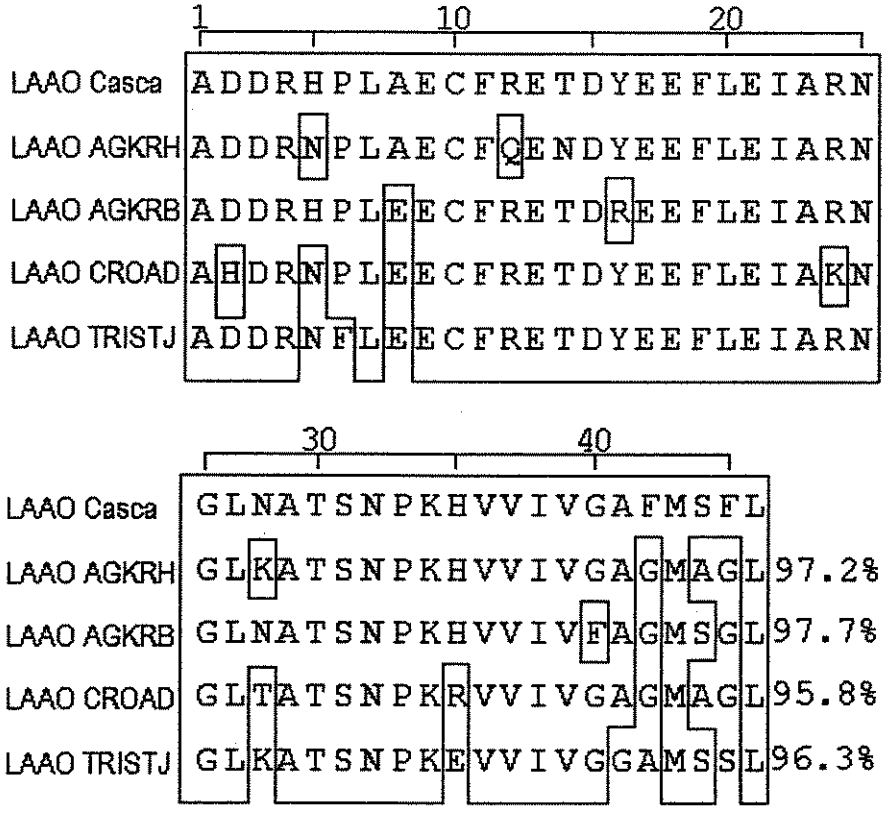
1.2
0.8
0.4
0.0
A214nm (—)
FAD
Casca LAAO
100
75
50
25
00
%B(- - -)
0
10
20
30
40
50
Time (min.)
b)
kDa
94
66
45
32
21
14
4.5
5.0
5.5
6.0
7.0
10.0
pI

Figure 3.


1
10
20
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LAAO AGKRH ADDRNPLAECFQENDYEEFLEIARN
LAAO AGKRB ADDRHPLEECFRETDREEFLEIARN
LAAO CROAD AHDRNPLEECFRETDYEEFLEIAKN
LAAO TRISTJ ADDRNFLEECFRETDYEEFLEIARN
30
40
LAAO Casca GLNATSNPKHVVIVGAFMSFL
LAAO AGKRH GLKATSNPKHVVIVGAGMAGL 97.2%
LAAO AGKRB GLNATSNPKHVVIVFAGMSGL 97.7%
LAAO CROAD GLTATSNPKRVVIVGAGMAGL 95.8%
LAAO TRISTJ GLKATSNPKEVVIVGGAMSSL 96.3%

Figure 4.

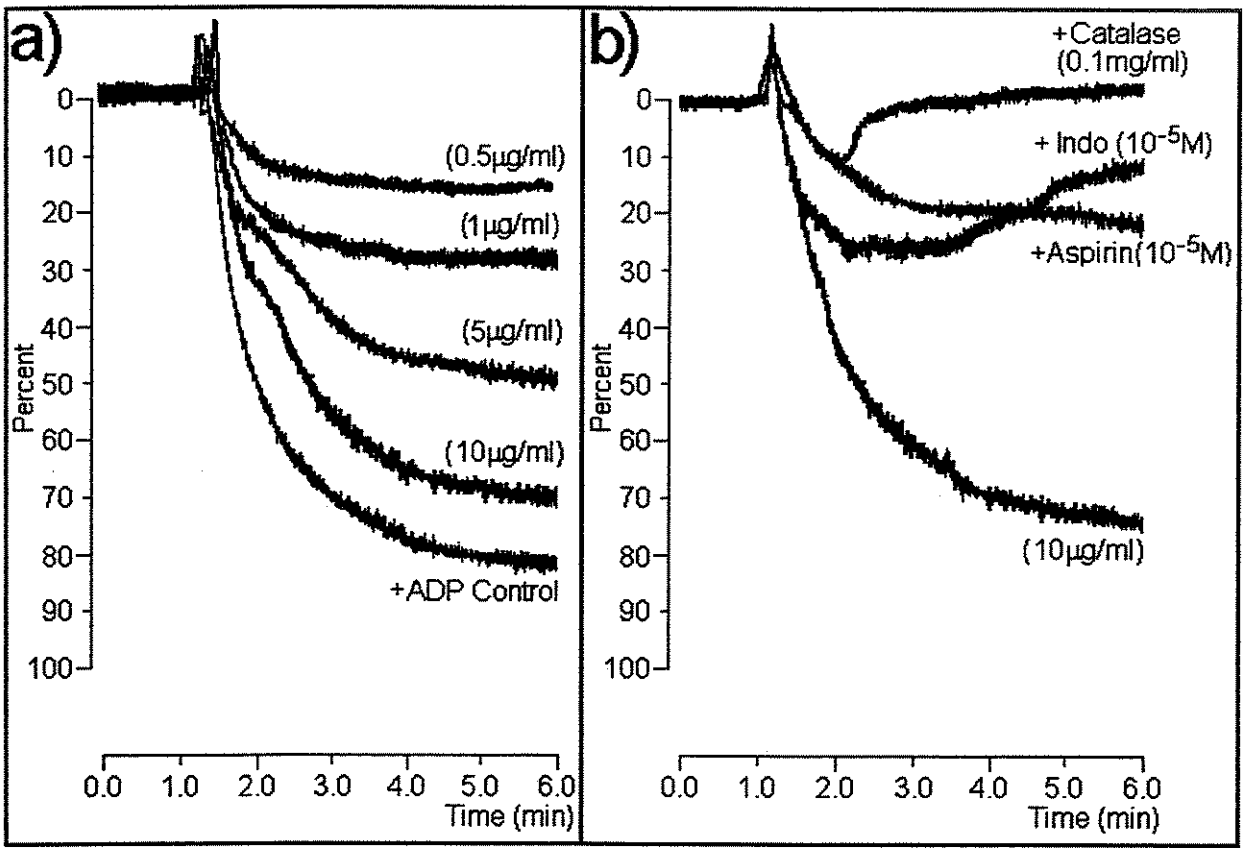

a)
0
10
20
30
40
50
60
70
80
90
100
Percent
(0.5µg/ml)
(1µg/ml)
(5µg/ml)
(10µg/ml)
+ADP Control
0.0
1.0
2.0
3.0
4.0
5.0
6.0
Time (min)
b)
+Catalase
(0.1mg/ml)
+ Indo (10^{-5}M)
+Aspirin(10^{-5}M)
(10µg/ml)
Percent
Time (min)

Figure 5.

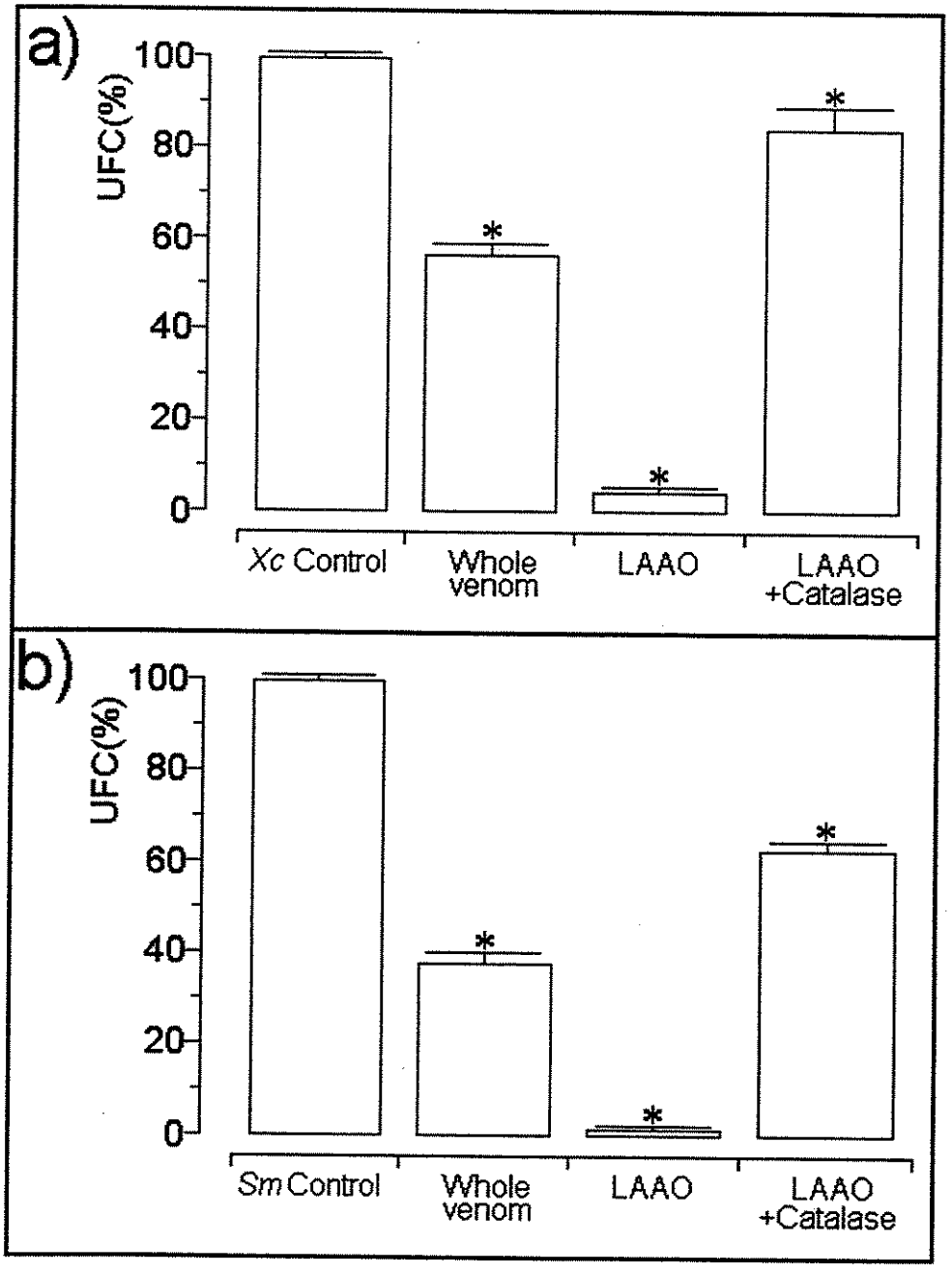
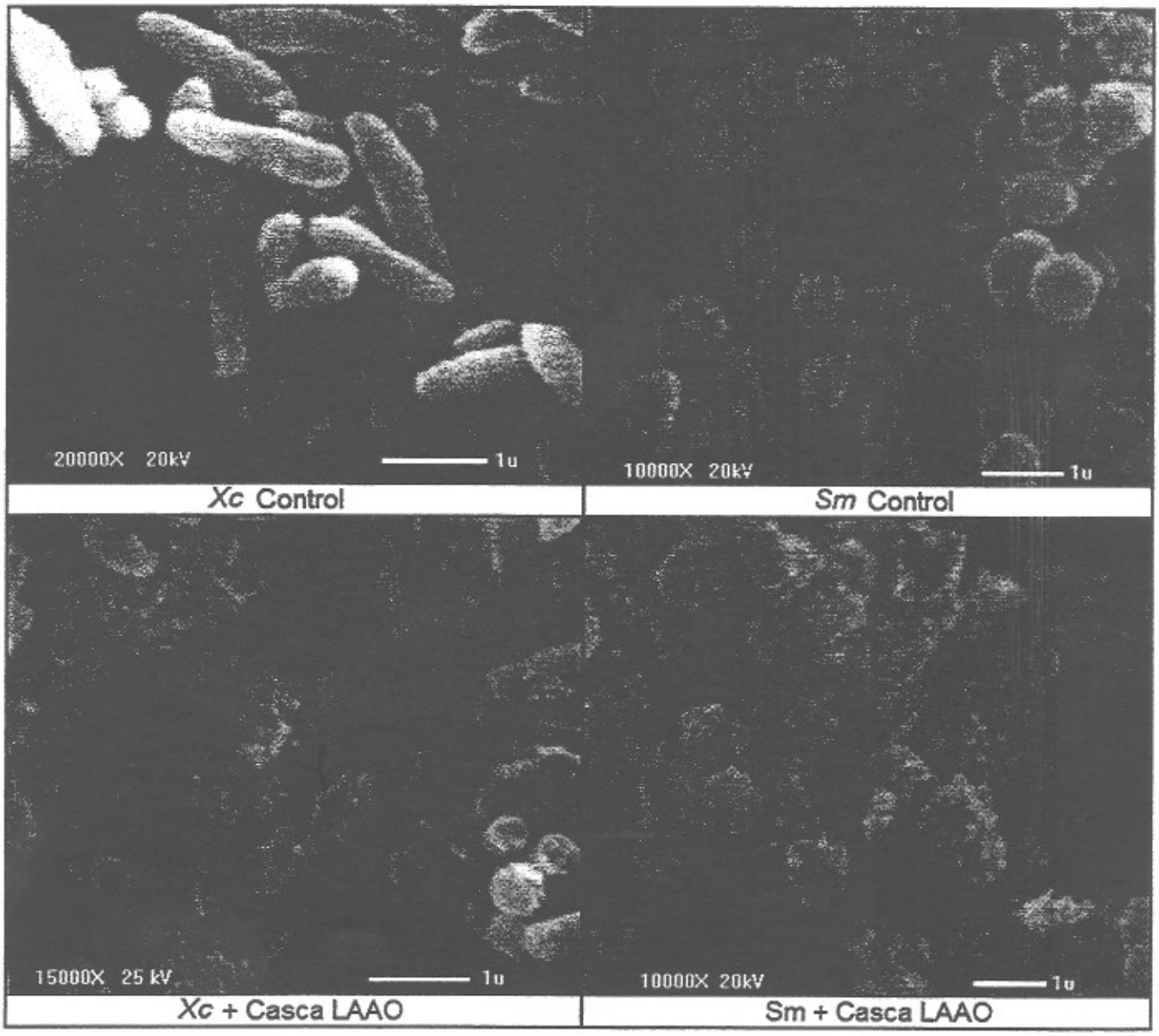

a)
UFC(%)
100
80
60
40
20
0
*
*
*
Xc Control
Whole venom
LAAO
LAAO +Catalase
b)
UFC(%)
100
80
60
40
20
0
*
*
*
Sm Control
Whole venom
LAAO
LAAO +Catalase

Figure 6.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1u
Xc Control
10000X 20kV
1u
Sm Control
15000X 25 kV
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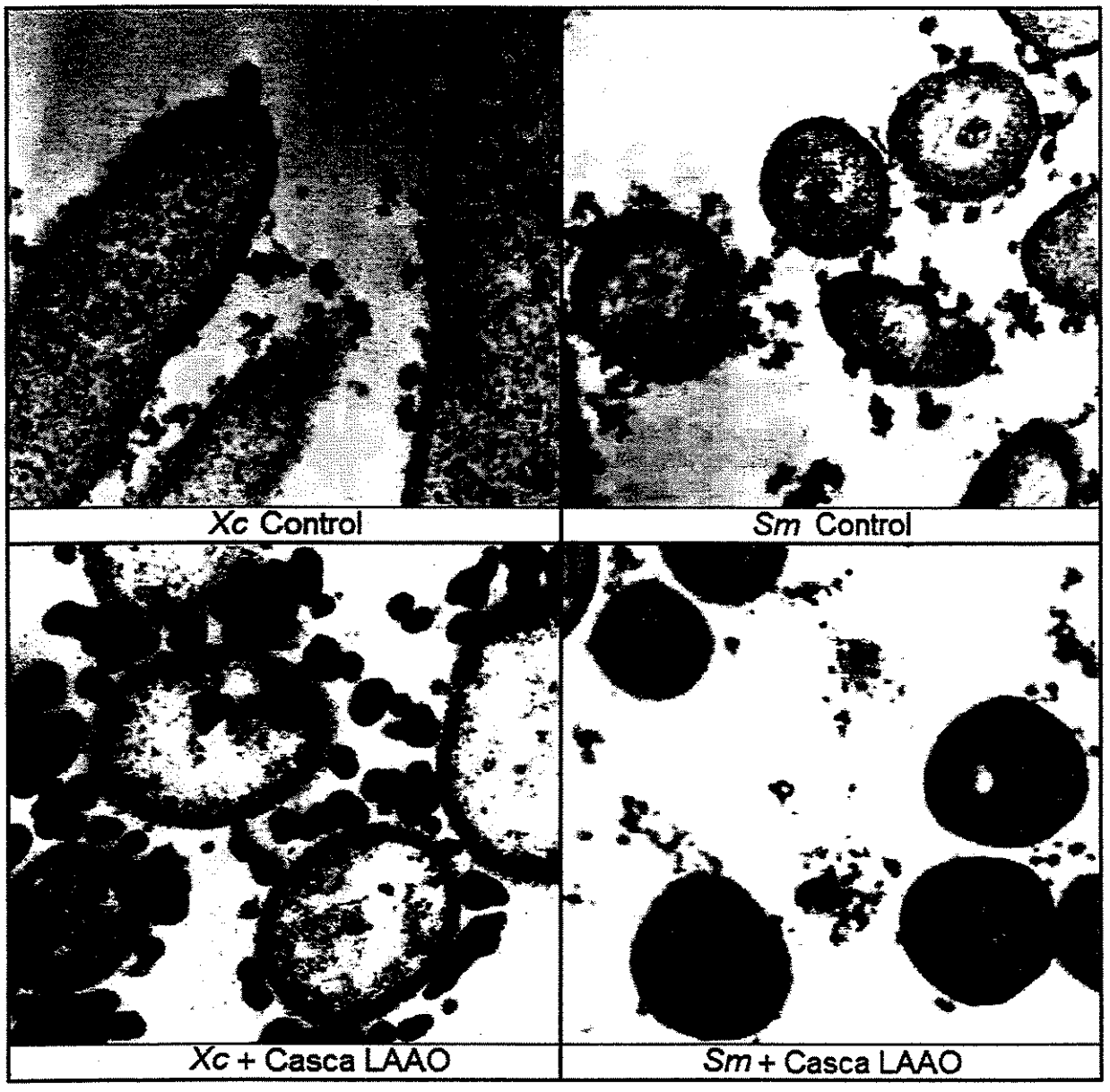
Xc + Casca LAAO
10000X 20kV
1u
Sm + Casca LAAO

Figure 7



5. CONCLUSÃO

5.1 Outras regiões moleculares da PLA_2, como a região do C-terminal, devido à presença de resíduos de aminoácidos carregados positivamente como os de lisina (Lys) e arginina (Arg), desempenham um importante papel na atividade antibacteriana.

5.2 A região C-terminal da crotamina, devido presença de resíduos de aminoácidos carregados positivamente (Arg e Lys) superficialmente posicionados na molécula, é responsável pela atividade antibacteriana.

5.3. A crotapotina, um polipetídeo ácido, possui moderada atividade antibacteriana, a qual acreditamos estar associada à hidrofobicidade da molécula;

5.4 A lectina do tipo C, além de desempenhar significativa atividade antibacteriana, é responsável pela nefrotoxicidade em rins isolados de ratos;

5.5. A LAO, dependente da produção de peróxido de hidrogênio que desestrutura a membrana bacteriana, desempenha sua atividade inibitória;

5.6 As análises ultra-estruturais e os dados bioquímicos indicaram que as frações isoladas do veneno de serpentes exercem atividade antibacteriana através de mecanismos de ação distintos: 1.interação das frações com a membrana bacteriana, induzindo a ruptura e desorganização com posterior lise celular; 2. desorganização citoplasmática através da entrada das frações por poros na membrana causando lise celular; 3.digestão da membrana com posterior extravasamento do conteúdo interno bacteriano com lise celular; 4.lise da membrana através dos radicais livres gerados pela reação enzimática do veneno com a membrana bacteriana.

As informações publicadas nos artigos contribuíram para aumentar o conhecimento das diferentes ferramentas moleculares oriundas dos venenos das serpentes que foram isoladas e caracterizadas nesta tese.

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