UNIVERSIDADE ESTADUAL PAULISTA
INSTITUTO DE BIOCIÊNCIAS
CAMPUS DE BOTUCATU

Estudos estruturais com a BthTX-II, uma Asp49-Fosfolipase A_2 miotóxica e com baixa atividade catalítica do veneno de *Bothrops jararacussu*

LUIZ CLÁUDIO CORRÊA

Dissertação apresentada ao Instituto de Biociências, Campus de Botucatu, UNESP, para obtenção do título de Mestre no Programa de PG em Biologia Geral e Aplicada.

Botucatu
2007

UNIVERSIDADE ESTADUAL PAULISTA
INSTITUTO DE BIOCIÊNCIAS
CAMPUS DE BOTUCATU

Estudos estruturais com a BthTX-II, uma Asp49-Fosfolipase A_2 miotóxica e com baixa atividade catalítica do veneno de *Bothrops jararacussu*

Luiz Cláudio Corrêa
Orientador: Prof. Adjunto Marcos Roberto de Mattos Fontes

Dissertação apresentada ao Instituto de Biociências, Campus de Botucatu, UNESP, para obtenção do título de Mestre no Programa de PG em Biologia Geral e Aplicada.

Botucatu
2007

Dedico este trabalho aos meus pais, Antônio e Nahir, que com tanta abnegação, se dedicaram à formação e à dignidade dos filhos.

Agradecimentos

- *Ao meu orientador, o Professor Adjunto Marcos Roberto de Mattos Fontes, pela oportunidade e pela amizade que me propiciou nesse período tão enriquecedor.*

- *À Fundação de Auxílio à Pesquisa do Estado de São Paulo (FAPESP) e ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) que financiaram este projeto de pesquisa.*

- *Ao Laboratório Nacional de Luz Síncrotron (LNLS), que disponibilizou uso de equipamento multi-usuário para a realização deste trabalho.*

- *Aos colegas de curso, pela amizade, companheirismo e disposição para dividir comigo, o conhecimento necessário para iniciar e concluir os estudos.*

- *Sobretudo, a Deus, pois sem Ele, nada se realiza.*

LISTA DE ABREVIATURAS

Acidic-Ag	Asp49-PLA_2 ácida de *Agkistrodon halys pallas*
Asp49-PLA_2	Fosfolipase A_2 com Aspartato na posição 49
Basic-Ag	Asp49-PLA_2 básica de *Agkistrodon halys pallas*
BnSP-6 e BnSP-7	Miotoxina I e Miotoxina II de *Bothrops neuwiedi pauloensis*
BPB	Brometo de *p*-bromofenacila
BthA-I	Asp49-PLA_2 ácida de *Bothrops jararacussu*
BthTX-I	Miotoxina I de *Bothrops jararacussu*
BthTX-II	Bothropstoxina I de *Bothrops jararacussu*
Lys49-PLA_2	Fosfolipase A_2 com Lisina na posição 49 (homóloga)
MjTX-I	Miotoxina I de *Bothrops moojeni*
NBSF	Fluoreto de 2-nitrobenzenosulfonil
NPSC	Cloreto de *o*-nitrofenilsulfenil
PLA_2	Fosfolipase A_2 (E. C. 3. 1. 1. 4.)
PrTX-II	Miotoxina II de *Bothrops pirajai*
PrTX-III	Piratoxina III de *Bothrops pirajai*

RESUMO

Fosfolipases A_2 (PLA_2s) são os componentes responsáveis, nos venenos botrópicos, pela destruição da membrana celular, através de um mecanismo de hidrólise de fosfolipídios. Uma classe de PLA_2s homólogas, que sofreu uma mutação natural no resíduo Asp49 para Lys49, não apresenta atividade catalítica, porém, estas enzimas podem exercer diversas atividades farmacológicas, como por exemplo, a miotoxicidade. Várias PLA_2s têm sido purificadas de venenos botrópicos. Três enzimas foram purificadas do veneno de *Botrrops jararacussu*, uma Lys49-PLA_2 (BthTX-I), com atividade miotóxica, e duas Asp49-PLA_2s, sendo uma ácida (BthA-I) com alta atividade catalítica, mas não miotóxica, e outra básica (BthTX-II), que, embora seja uma Asp49-PLA_2, possui baixa atividade catalítica, mostrando-se porém, miotóxica. Isso coloca BthTX-II numa posição intermediária entre BthTX-I e BthA-I. Este trabalho apresenta a determinação da estrutura tridimensional da botropstoxina II (BthTX-II), realizada pelo método da cristalografia de proteínas, assim como um estudo comparativo dessa estrutura com as de outras PLA_2s (Asp49 e Lys49) purificadas de venenos de serpentes dos gêneros *Bothrops* e *Agkistrodon.* Os estudos revelam uma grande modificação na posição do *loop* de ligação do cálcio, região de resíduos que coordenam este íon, fundamental para a atividade catalítica, em BthTX-II, quando comparada com outras Asp49-PLA_2s que apresentam essa atividade. A cadeia lateral do resíduo Tyr28, presente nessa região, encontra-se em posição oposta à mesma nas cataliticamente ativas Asp49-PLA_2s, o que a leva a se ligar ao átomo Oδ2 do resíduo Asp49, que deveria fazer parte da coordenação do íon cálcio. Estas modificações impedem a ligação do íon cálcio e assim, podem ser responsáveis pela baixa atividade catalítica encontrada em BthTX-II, a qual também não apresenta a Lys122, que é associada com a atividade miotóxica nas Lys49-PLA_2. Em contrapartida, esta possui um resíduo ácido (Asp) nesta posição, o que indica um processo alternativo para esta atividade na BthTX-II.

Palavras chave: Cristalografia de Raio-X; Asp49-fosfolipase A_2; Veneno de *Bothrops jararacussu*; Miotoxicidade; Baixa atividade catalítica.

ABSTRACT

Phospholipases A_2 are components of *Bothrops* venoms responsible for disruption of cell membrane integrity via hydrolysis of its phospholipids. A class of homologous PLA_2, that underwent a natural mutation in the residue Asp49 for a Lys49, doesn't show catalytic activity, however, these enzymes can perform different pharmacology activities, such as myotoxicity. Several PLA_2s have been purified from *bothropic* venoms. Three enzymes were purified from *Bothrops jararacussu* venom, a Lys49-PLA_2 (BthTX-I), with myotoxic activity, and two Asp49-PLA2s; an acidic (BthA-I) with high catalytic activity, but no myotoxic, and a basic (BthTX-II), that, although being an Asp49-PLA_2, presents low catalytic activity, however displaing myotoxicity. This put BthTX-II in an intermediate position between BthTX-I and BthA-I. This work presents the three-dimensional structure determination of bothropstoxin II (BthTX-II) performed by protein crystallography method and, a comparative study of this protein with other PLA_2 (Asp49 and Lys49) purified from venom of *Bothrops* and A*gkistrodon* genus. The studies showed a severe distortion of calcium binding loop - fundamental region for the catalytic activity - in the BthTX-II when it is compared with other Asp49-PLA_2s that show this activity. The side chain of the residue Tyr28, present in this region, is in an opposite position in relation to the same residue in the catalytic activity Asp49-PLA_2s, making bond with the atom Oδ2 of the residue Asp49, which should coordinate the calcium. BthTX-II does not present Lys122, responsible for the polarization of the residue Cys29, which causes the direction of the amine group (NH) for the Gly30 for the solvent in the hydrophobic channel. These modifications prevent the binding of the calcium ion, so, they are responsible for the low catalytic activity found in BthTX-II. Additionally, BthTX-II does not present a Lys residue in the position 122, which is associated with myotoxic activity. In contrast, it has an acidic residue in this position (Asp), indicating an alternative process for this activity in the BthTX-II.

Keyboards: X-ray crystallography; Asp49-phospholipase A_2; *Bothrops jararacussu* venom; Myotoxicity; low catalytic activity.

SUMÁRIO

No Brasil, existem 24 espécies de serpentes pertencentes ao gênero *Bothrops* (Melgarejo, 2003), que habitam, preferencialmente, ambientes úmidos como matas, áreas cultivadas e locais de proliferação de roedores (zonas rurais e periferia das grandes cidades). Estas serpentes são responsáveis por cerca de 85-90% do total de acidentes ofídicos que ocorrem no país (Rosenfeld, 1971; Ferreira *et al.*, 1992 e Ribeiro *et al.*, 1993), o que as tornam, assunto de grande interesse científico, médico e social. Venenos de serpentes são compostos por uma complexa mistura de proteínas, enzimas, peptídeos e compostos inorgânicos que podem apresentar diversas atividades farmacológicas e/ou biológicas, sendo seu estudo, uma área bastante promissora na busca e desenvolvimento de novos medicamentos.

A natureza e as propriedades biológicas dos componentes dos venenos de serpentes são peculiares para cada espécie animal e a concentração destes componentes pode variar intra-especificamente, devido a diversos fatores como variações climáticas, geográficas, sexuais, etárias, alimentares, tempo decorrido entre as extrações de venenos, heranças genéticas e outros (Lomonte & Carmona, 1992; Valiente *et al*., 1992; Monteiro *et al*., 1998 e Soares *et al.*, 2004).

Dentre as diversas toxinas que compõem os venenos botrópicos, estão as fosfolipases (PLA_2 - E.C.3.1.1.4), enzimas largamente distribuídas na natureza e extensivamente estudadas. (Dennis, 1983; Waite, 1987 e Dennis, 1994). A primeira vez que se estudou a atividade enzimática, hoje denominada atividade PLA_2, foi no século XIX, utilizando-se venenos de serpentes (Stephens *et al.*, 1898).

PLA_2s são proteínas multifuncionais capazes de participar como mediadoras de vários processos inflamatórios, podendo ser aplicadas em diversas áreas da medicina, por exemplo, na detecção de pré-eclampsia severa, ações gerais de anestésicos, tratamento de artrites reumatóides, atuando como agentes bacteriológicos em glândulas lacrimais e outros tecidos, bloqueando a entrada do vírus dentro da célula hospedeira e destacando-se como um potencial agente antimalárica (Uhl *et al*., 1997; Moreira *et al*., 2002 e Soares *et al*., 2004).

Diversas PLA_2(s) têm sido descobertas em secreções ($sPLA_2$ ou extracelulares) e no citosol ($cPLA_2$ ou intracelulares). As PLA_2s intracelulares apresentam alta massa molecular (85 kDa), geralmente estão associadas a membranas, são cálcio-dependentes e estão envolvidas no metabolismo de fosfolipídios (perturbações das membranas) na sinalização celular e no remodelamento de fosfolipídios (Mukherjee *et al*., 1994; Arni & Ward, 1996 e Six & Dennis, 2000). Já as extracelulares são as encontradas em fluidos

biológicos, particularmente em secreções pancreáticas, exudados inflamatórios e em venenos de répteis (serpentes e lagartos) e insetos (Rosenberg, 1990; Arni & Ward, 1996 e Ownby, 1999). Trata-se de enzimas pequenas (119 a 143 aminoácidos), de baixa massa molecular (12 a 15 kDa), estáveis e cálcio-dependentes e que atuam sobre substratos lipídicos (principalmente fosfolipídios) hidrolisando a ligação éster *sn*-2 ("sequentially numbered") (Figura 01).

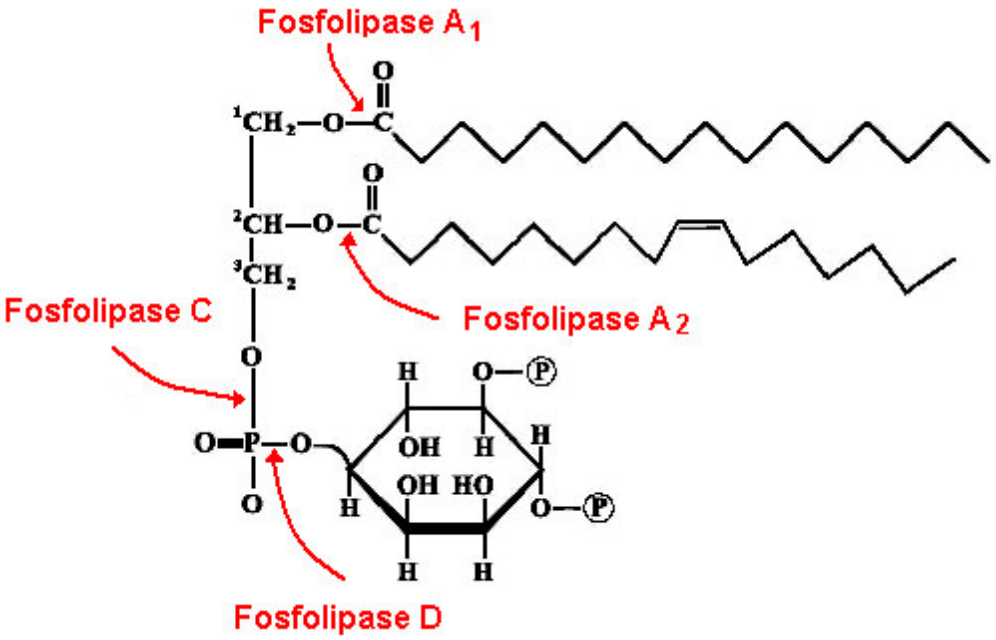


Figura 01: Representação esquemática de um fosfolipídeo e os locais de atuação das diferentes fosfolipases (Lehninger *et* al., 1993).

Os produtos liberados na atividade catalítica das PLA_2s são lisofosfolipídios, importantes na sinalização celular e perturbação de membranas (Moolenaar *et al.*,1997), e ácidos graxos (Waite, 1987 e Arni & Ward, 1996) tais como o ácido oléico (reserva energética) e o ácido aracdônico (precursor de eicosanóides, os quais atuam como mediadores de inflamações).Inicialmente, as PLA_2(s) foram divididas em dois grupos, baseado nas posições das pontes dissulfeto (Heinrikson *et al.*, 1977; Dufton & Hider,1983), mas, com o avanço das técnicas de seqüenciamento e caracterização estrutural, tem havido um constante aumento dos seus grupos e subgrupos. Atualmente, a superfamília das PLA_2s é composta por um conjunto de quinze grupos (Schaloske *et al*., 2006) que apresentam um alto grau de homologia seqüencial e estrutural, diferenciando-se somente pela localização e quantidade de pontes dissulfeto e pelos comprimentos de seus loops (Dennis, 1994). PLA_2s

dos grupos I e II são os componentes principais dos venenos de serpentes (Rosenberg, 1990), sendo que aquelas do segundo grupo são os compostos predominantes dos venenos de serpentes da família Viperidae, à qual pertencem às espécies do gênero *Bothrops*.

Apesar dessa pequena variação seqüencial e estrutural, as PLA_2s destacam-se por seu grande espectro de atividades biológicas, como: neurotoxicidade pré e/ou pós-sináptica, miotoxicidade, cardiotoxicidade, mionecrose, anticoagulante (inibição de agregação plaquetária), efeitos convulsivos, indução de edema, atividades hipotensora, hemolítica, hemorrágica, entre outros efeitos (Kini & Evans, 1989; Rosenberg, 1990; Evans & Kini, 1997; Kini, 1997; Gutiérrez & Lomonte, 1997; Gerrard *et al.*, 1993; Ownby, 1999; Braud *et al.*, 2000; Valentin & Lambeau, 2000; Condrea *et al.*, 1981; Lloret & Moreno, 1993; Andrião-Escarso *et al.*, 2000; Gutiérrez *et al.*, 1980 e Andrião-Escarso *et al.*, 2002).

Os principais elementos da estrutura secundária das PLA_2s (Figura 02) são a região N-terminal, constituída por uma α-hélice I, denominada "h1" (resíduos 1-12, os quais formam o canal hidrofóbico das PLA_2s; região altamente conservada envolvida na ligação e orientação de substratos à proteína), em seguida tem-se a hélice curta ("short helix") constituída pelos resíduos 18-23. Entre os resíduos 25-34, está localizada a região de ligação do íon Ca^{+2}, que é coordenado por duas ligações com átomos de oxigênio carboxílicos do Asp49, três ligações com átomos de oxigênio dos resíduos Tyr28, Gly 30, Gly32, além de duas moléculas de água. Na seqüência, vem a α-hélice II ("h2") constituída pelos resíduos 40-55. Esta se liga a uma curta folha β antiparalela ("β-wing") (resíduos 75-77 e 82-84). Estes dois últimos seguimentos, "h2" e "β-wing", alocam resíduos que constituem o sítio catalítico das PLA_2s. Após a região de "β-wing", os resíduos 90-107 formam α-hélice III ("h3") que se liga a uma região bastante flexível, denominada C-terminal (resíduos 108-133), (Arni & Ward, 1996; Ward *et* al.,1998; de Azevedo, 1999 e Magro *et al*, 2003). Este modelo segue o sistema de numeração proposto por Renetseder *et al*., 1985.

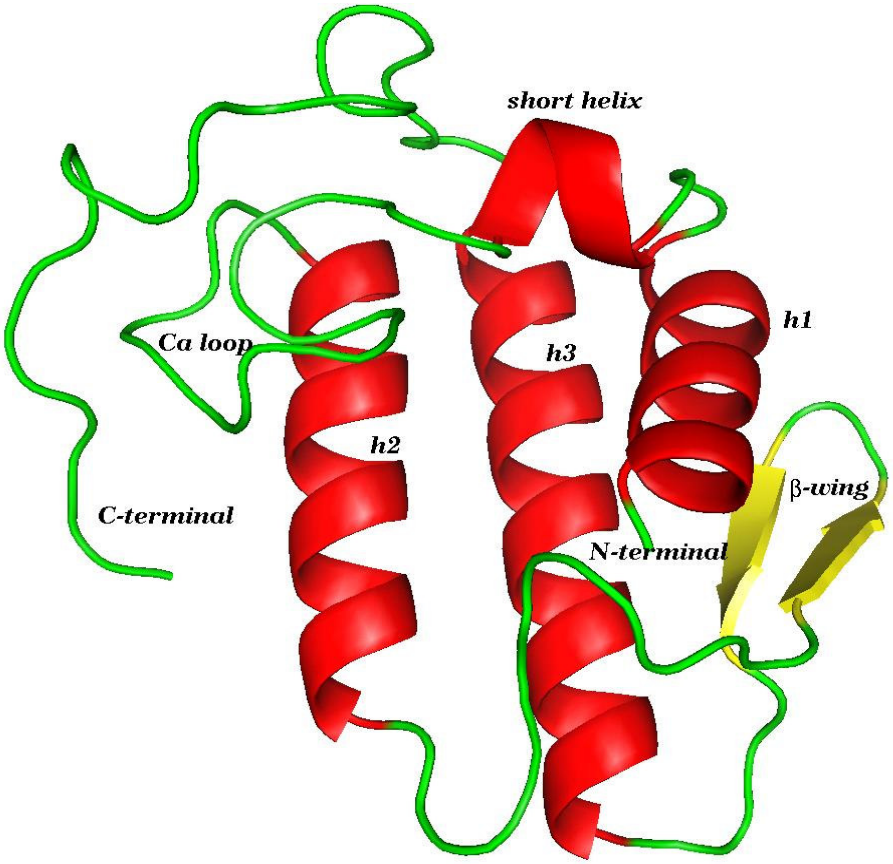


Figura 02: Representação esquemática do enovelamento de uma PLA_2. Figura feita pelo programa "*Pymol*" (Delano, 2002).

Para uma melhor compreensão dos mecanismos de ação das PLA_2s, muitos estudos têm sido realizados, tanto com proteínas em sua forma nativa como utilizando complexos destas com diversos ligantes, que podem inibir de forma parcial ou completa as propriedades farmacológicas das proteínas, ou simplesmente revelar as interações que determinam a ligação destas substâncias aos seus sítios protéicos específicos, além da análise de variedades mutantes, uma vez que a mutação de resíduos específicos tem como objetivo estabelecer a importância de determinados aminoácidos e/ou segmentos protéicos para a atividade da proteína como um todo.

Estudos com modificações químicas têm mostrado efeitos importantes nas atividades farmacológicas das PLA_2s. A alquilação da His48, resíduo altamente conservado do sítio catalítico das PLA_2s, pelo brometo de *p*-bromofenacil (BPB) induz à perda da atividade hidrolítica de fosfolipídios e à diminuição dos efeitos tóxicos e farmacológicos destas proteínas (Rodrigues *et al.*, 1998; Zhao *et al.*, 1998; Soares *et al.*,

2000 a,b e Soares *et al.*, 2001a). Outras modificações químicas como a sulfonação de resíduos de Tyr pelo fluoreto de *p*-nitrobenzenosulfonil (NBSF) e a sulfonação de resíduos Trp pelo cloreto de *o*-nitrofenilsulfenil (NPSC) também levam à redução de efeitos tóxicos dessas enzimas (Yang *et al*., 1985; Soares *et al.*, 2000 a,b; Soares *et al.*, 2001b; Soares & Giglio, 2003).

Outro ponto interessante no estudo das relações estruturais e funcionais das PLA_2s diz respeito à ação de inibidores naturais, que atuam de forma a minimizar os efeitos destas enzimas durante o processo inflamatório. Entre as substâncias naturais que comprovadamente apresentam capacidade de inibir o processo inflamatório está a vitamina E (α-tocoferol). Esta substância atua inibindo tanto a atividade das PLA_2s quanto o ciclo da cicloxigenase (Pentland *et al.*, 1992; Traber & Packer, 1995) e por isso, tem sido utilizada nos tratamentos das doenças de Alzheimer e Parkinson e especula-se que bloqueie a progressão da doença de Alzheimer inibindo a atividade PLA_2 e estabilizando as membranas neurais (Ebadi *et al.,* 1996; Sano *et al*., 1997).

Em 2002, Chandra *et al.* co-cristalizaram uma PLA_2 dimérica do veneno de *Daboia russeli pulchella* complexada com α-tocoferol e observaram a presença de somente uma molécula de α-tocoferol ligada a um dos monômeros. Os testes bioquímicos mostraram uma diminuição de 50% em sua atividade catalítica ao serem tratadas com α-tocoferol, mesmo em alta concentração do inibidor.

Uma mutação natural do resíduo Asp49, fundamental para a atividade catalítica das PLA_2s, por uma Lys deu origem a um outro grupo de enzimas denominadas Lys49-PLA_2 ou PLA_2s homólogas (Francis *et al.*, 1991; Homsi-Brandenburgo *et al.*, 1988; Ward *et al.*, 1995). Estas não apresentam atividade catalítica, embora mantenham diversas outras funções farmacológicas (Lomonte *et al.*, 1994a). A mutação em questão ocorre numa conhecida região de ligação do íon Ca^{+2} das PLA_2s cataliticamente ativas (Asp49-PLA_2s) (Figura 03). A presença da Lys no lugar do Asp na posição 49 torna impossível a coordenação do íon Ca^{+2} (Scott *et al.* 1990). O Nε da cadeia lateral do resíduo Lys49 ocupa a posição do íon, o que inviabiliza a ocorrência da reação catalítica (Lee *et al*., 2001), uma vez que este íon, coordenado pelo Asp, é responsável, conjuntamente com outros aminoácidos, pela fixação do substrato ao sítio enzimático das PLA_2s.

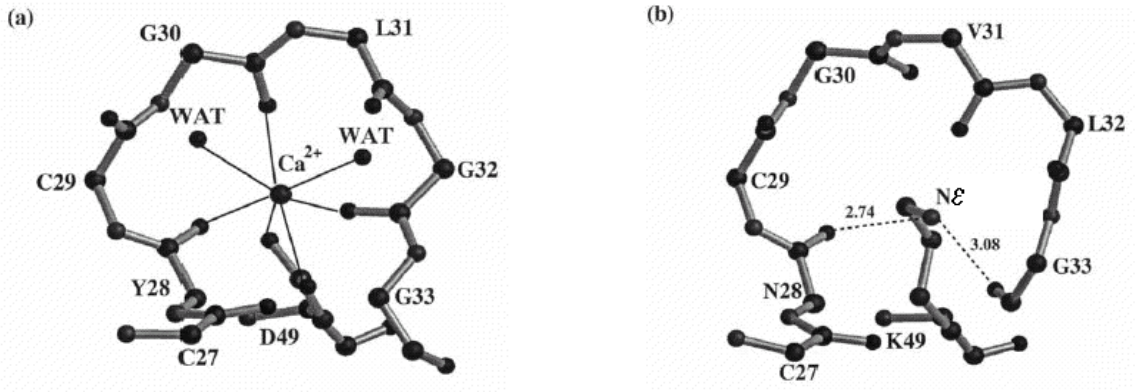


Figura 03: *a.* Região de ligação do íon Ca^{+2} ("calcium binding loop") de uma PLA_2 cataliticamente ativa de *Naja naja naja* (Asp49). *b.* Região análoga à região de ligação do Ca^{+2} de uma Miotoxina II (PLA_2 homóloga) cataliticamente inativa de *Bothrops asper* (Lys49) (Arni & Ward, 1996 e Ward *et al*., 1998).

Estudos de mutagênese sítio-dirigida com a substituição do resíduo Lys49 por um Asp em BthTX-I (Lys49-PLA_2 – cataliticamente inativa) (Ward *et al*., 2002) demonstraram que esta proteína continuou não apresentando atividade catalítica, sugerindo que a perda dessa atividade não está somente relacionada à mutação de Asp49 por Lys49, mas a um conjunto de outros fatores estruturais, como a interação da região do C-terminal (Lys122) com a Gly30. O Nε da cadeia lateral do resíduo Lys122 forma ponte de hidrogênio com o resíduo Cys29, direcionando o grupo amina (NH) da Gly30 para a exposição do solvente no canal hidrofóbico (Ward *et al*., 2002) e o Nε da cadeia lateral do resíduo Lys49 forma pontes de hidrogênio com átomos de oxigênio das cadeias principais dos resíduos Asn28 e Gly30. Este arranjo de pontes de hidrogênio está conservado na maioria das estruturas das Lys49-PLA_2s. A posição e orientação dos resíduos His48, Tyr52, Asp99 e demais resíduos que participam da atividade catalítica estão altamente conservadas tanto nas PLA_2s, como nas PLA_2s homólogas.

Apesar da incapacidade de promover a hidrólise de substratos lipídicos, as Lys49-PLA_2s apresentam um pronunciado efeito miotóxico tanto *in vivo* quanto *in vitro* (Gutierrez & Lomonte, 1997). Esta atividade pode ocorrer independentemente da substituição da Asp49 por Lys, sugerindo a independência da ligação do íon Ca^{2+} ao sítio ativo, ao contrário do verificado no processo normal de catálise de lipídios (Rufini *et al.*, 1992; Díaz *et al.*, 1991; Díaz *et al.*, 1992 e Ward *et al*., 1998).

Lomonte *et al.* (1994b) e Gutiérrez & Lomonte (1995) propõem que a região C-terminal seja responsável pela miotoxicidade, por ser rica em resíduos hidrofóbicos e

básicos. A região de C-terminal, por ser bastante flexível, se insinuaria através das membranas (celulares ou artificiais), promovendo a desestabilização destas, ou atuaria como "âncora", possibilitando o contato de sítios protéicos reativos desconhecidos.

Experimentos de mutação sítio-dirigida comprovaram o envolvimento da região C-terminal nas atividades miotóxica e de rompimento de membranas das Lys49-PLA_2s (Chioato *et al*., 2002 e Ward *et al*., 2002). A substituição dos resíduos Lys115, 116 e 122 por alaninas levou à redução da atividade de dano à membrana, porém, não houve alteração da atividade miotóxica. Substituições dos resíduos Tyr117 → Trp117, Arg118 → Ala118, Tyr119 → Trp119 e Lys122 → Ala122 eliminaram as cargas positivas de suas cadeias laterais, reduzindo significante a atividade miotóxica, porém a atividade de dano à membrana não foi alterada. Por fim, quando apenas Lys122 foi substituída por Ala, foram reduzidas, tanto a atividade miotóxica (40%) como a de dano à membrana, além de não ocorrer nenhuma atividade hidrolítica, indicando que a lisina da posição 122 é um suporte evidente para a ativação da interrupção do ciclo catalítico. Assim, os experimentos sugerem que estes resíduos da região C-terminal estão envolvidos no *motif* estrutural que determina as atividades de miotoxicidade e dano à membrana e ainda indicam que estas são independentes.

A análise de PLA_2s de venenos de serpentes mostra que as mutações ocorrem mais freqüentemente na região dos éxons, o que sugere a duplicação genética e uma evolução acelerada dos éxons como responsáveis pela multiplicidade de isoformas encontradas em um único veneno.

Acredita-se que substituições naturais ocorridas principalmente na superfície da molécula destas proteínas possam ser produtos de mudanças de especificidade frente a tecidos específicos (Soares *et al*., 2004).

Características regionais também podem influenciar na ampla variedade da composição das toxinas do veneno de serpentes. O veneno da espécie *Bothrops neuwiedi pauloensis* pode apresentar, dependendo do local, somente a toxina BnSP-7; somente sua isoforma BnSP-6, além de haver um terceiro grupo que abriga as duas toxinas (BnSP-7 e BnSP-6) (Rodrigues *et al*., 1998, Soares *et al*., 1998 e Soares *et al*., 2000b).

Estudos comparativos entre as Lys49-PLA_2s e as Asp49-PLA_2s mostram que os resíduos que compõem o sítio catalítico (His48, Tyr52 e Asp99) e a região de ligação do lipídio (Leu5, Val102 e Leu106) destacam-se como os mais relevantes e altamente conservados.

O grande leque de ações farmacológicas e bioquímicas, a despeito da significativa uniformidade estrutural, é a base do amplo interesse despertado por estes dois grupos protéicos na comunidade científica. A identificação de prováveis relações estruturais e funcionais torna-se mais fácil devido às pequenas variações existentes entre estas moléculas.

O conhecimento de estruturas tridimensionais e respectivos estudos das relações estrutura/função, tanto das PLA_2s e PLA_2s homólogas nas formas nativas como complexadas e co-cristalizadas são de extrema importância devido ao interesse médico, social e econômico, sendo uma informação indispensável para o desenvolvimento racional de medicamentos e/ou derivados de toxinas menos agressivos a serem utilizados na produção de soros antiofídicos.

A cristalografia de raios-X é o método mais utilizado para a determinação tridimensional de estruturas macromoleculares. Determinar estruturas de cristais de proteína requer monocristais, os quais são analisados por experimentos de difração de raios-X. A formação de monocristais de proteínas só ocorre em condições limitadas determinadas empiricamente (Kobe *et al.*, 1999). Dentre os principais parâmetros que afetam a solubilidade e conseqüentemente o processo de cristalização podemos destacar:

- ✓ <u>Parâmetros físico-químicos intrínsecos:</u>
  - Supersaturação (volume e concentração de proteína e precipitante);
  - Variação da temperatura;
  - pH da solução de cristalização (uso de soluções tampão, junto à solução protéica);
  - Força iônica;
  - Pureza das substâncias químicas utilizadas;
  - Efeitos de densidade, pressão, viscosidade e gravidade;
  - Partículas sólidas (solubilização incompleta da proteína ou do precipitante).
- ✓ <u>Parâmetros bioquímicos e biofísicos:</u>
  - Presença de ligantes (ligação de substratos, co-fatores e íons metálicos);
  - Aditivos específicos (agentes redutores, detergentes não-iônicos e poliaminas);
  - Degradação e/ou desnaturação da amostra.
- ✓ <u>Amostra protéica:</u>
  - Contaminantes macromoleculares (falhas durante o processo de purificação; poeira);
  - Heterogeneidade conformacional ou da seqüência dos aminoácidos;

- Concentração da amostra.

Assim, a obtenção de cristais com qualidade cristalográfica não é uma atividade trivial, podendo levar meses para se alcançar indícios da formação de cristais e, ainda assim, pode ser necessário mais um tempo significativo para melhorar a qualidade dos mesmos. Há ainda casos em que cristais, simplesmente não são obtidos.

Grande esforço tem sido empreendido e, nos últimos anos, diversas Asp49-PLA_2s e Lys49-PLA_2s de veneno de serpentes têm sido cristalizadas e elucidadas estruturalmente com o intuito de obterem-se tais correlações (Holland *et al.*, 1990; Arni *et al.*, 1995; de Azevedo Jr. *et al.*, 1997; da Silva-Giotto *et al.*, 1998; de Azevedo Jr. *et al*, 1999; Arni *et al*., 1999; Lee *et al*, 2001; Magro *et al.*, 2003; Magro *et al.*, 2004).

Três frações identificadas como PLA_2s foram purificadas do veneno de *B. jararacussu* (Fig. 04). Esta serpente, de nome popular jararacussu, se distribui desde o sul da Bahia até o noroeste do Rio Grande do Sul, pode atingir 1,80 m de comprimento, sendo talvez, a mais imponente do gênero. É a espécie que maior quantidade de veneno produz (Melgarejo, 2003).

A primeira enzima é uma Lys49-PLA_2 básica denominada BthTX-I (Homsi-Brandeburgo *et al*., 1988). Trata-se de uma enzima básica (pI= 8.2) com 121 resíduos de aminoácidos e peso molecular de 13,667 KDa (Cintra *et al*, 1993; Ward *et al*., 1995) . Apesar de não possuir atividade catalítica, estudos mostram que essa enzima apresenta alto potencial miotóxico (Cintra *et al*., 1993). A segunda e a terceira foram caracterizadas como Asp49-PLA_2, sendo uma ácida (BthA-I) e outra básica (BthTX-II).

BthA-I é uma enzima que apresenta 122 resíduos de aminoácidos, peso molecular de aproximadamente 14 KDa e pI= 4,5. Estudos de caracterização mostraram que esta apresenta uma alta atividade catalítica e também, efeitos hipotensivo, de indução de edema e inibição de agregação plaquetária, porém, não foram detectadas, atividades miotóxica, neurotóxica, citotóxica (Andrião-Escarso *et al*., 2002).

BthTX-II foi purificada por Homsi-Brandeburgo *et al* (1988) e, desde então, tem sido alvo de vários estudos. Pereira *et al.* (1998) realizaram seu sequenciamento pelo método de Edman (Edman & Begg, 1967) e encontraram 120 resíduos de aminoácidos, sendo apenas uma metionina. A enzima apresentou atividade PLA_2 residual. Um outro estudo de sequenciamento, dessa vez por cDNA, foi realizado por Kashima *et al.* (2004), que encontraram 122 resíduos, sendo 2 metioninas. Outras diferenças foram encontradas nas seqüências, como a presença, no primeiro sequenciamento, de um Trp e uma Arg nas

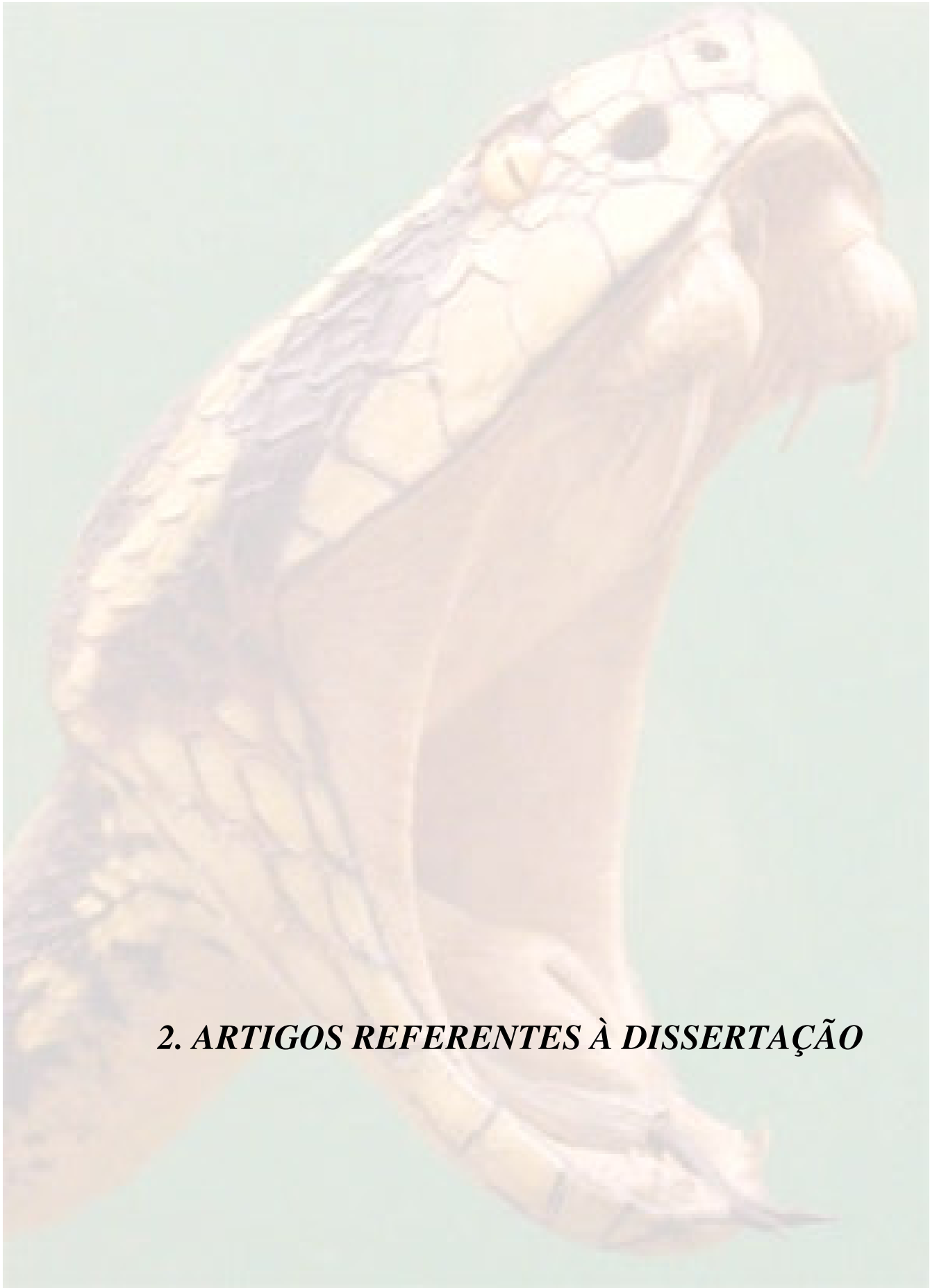
posições 5 e 34, respectivamente, enquanto no segundo, tratava-se de uma Phe na posição 5 e uma Gln na posição 34.

Um estudo de purificação e caracterização enzimática de uma nova PLA_2 básica do veneno de *B. jararacussu* utilizando-se três passos de HPLC (troca iônica, fase reversa e exclusão molecular) separou oito frações, sendo que a fração sete (Bj VII) correspondeu a BthTX-II (Bonfim *et al*., 2001). Esta apresentou alta atividade miotóxica e significativa atividade neuromuscular em nervo frênico de camundongos; no entanto, não apresentou atividade PLA_2.

Figura 04: Espécime adulto de *Bothrops jararacussu.* (Foto: Leonardo, 2006).

Essa variação funcional encontrada em três enzimas extraídas do veneno da mesma espécie, com estruturas e seqüências de aminoácidos tão similares, faz com que o estudo estrutural destas se torne objeto de grande interesse para os ramos das ciências da saúde, uma vez que pequenas modificações são responsáveis pela redução, ou até mesmo perda da(s) atividade(s) tóxica(s). As estruturas de BthTX-I e BThA-I foram elucidadas recentemente e estudos bioquímicos estruturais destas com ligantes e/ou inibidores têm sido realizados (da Silva-Giotto *et al*., 1998; Soares & Giglio, 2003; Magro *et al*., 2004; Takeda *et al*., 2004; Murakami *et al*., 2006). Dessa forma, o objetivo deste trabalho foi

determinar a estrutura tridimensional de BthTX-II e assim, realizar um estudo comparativo com PLA_2s extraídas do veneno de *B. jararacussu*, bem como de outras espécies da família Viperidae, a fim de tentar elucidar as bases moleculares da ampla gama de efeitos farmacológicos apresentados por essas enzimas.



2. ARTIGOS REFERENTES À DISSERTAÇÃO

2.1. O artigo: “Preliminary X-ray crystallographic studies of BthTX-II, a myotoxic Asp49-phospholipase A_2 with low catalytic activity from *Bothrops jararacussu* venom”, referente aos estudos de cristalização de BthTX-II, foi publicado pelo periódico *Acta Crystallographica section F- Structural Biology and Crystallization communications* (ver apêndice).

2.2. O artigo: “Crystal structure of BthTX-II, a myotoxic Asp49-phospholipase A_2 with low catalytic activity from *Bothrops jararacussu* venom”, referente aos estudos estruturais de BthTX-II, será submetido para publicação.

Preliminary X-ray crystallographic studies of BthTX-II, a myotoxic Asp49-phospholipase A2 with low catalytic activity from *Bothrops jararacussu* venom

L. C. Corrêa,[a] D. P. Marchi- Salvador,[a] A. C. O. Cintra,[b] A. M. Soares[b] and M. R. M. Fontes[a]*

[a]Departamento de Física e Biofísica, Instituto de Biociências, UNESP, CP 510, CEP 18618-000, Botucatu-SP, Brazil, and [b]Departamento de Análises Clínicas, Toxicológicas e Bromatoloógicas, FCFRP, USP, Ribeirão Preto/SP, Brazil

*Correspondence e-mail: fontes@ibb.unesp.br

Abstract

For the first time, a complete X-ray diffraction data set has been collected from a myotoxic Asp49-phospholipase A_2 (Asp49-PLA_2) with low catalytic activity (BthTX-II from *Bothrops jararacussu* venom) and a molecular-replacement solution has been obtained with a dimer in the asymmetric unit. The quaternary structure of BthTX-II resembles the myotoxin Asp49-PLA_2 PrTX-III (piratoxin III from *B. pirajai* venom) and all non-catalytic and myotoxic dimeric Lys49- PLA_2s. In contrast, the oligomeric structure of BthTX-II is different from the highly catalytic and non-myotoxic BthA-I (acidic PLA_2 from *B. jararacussu*). Thus, comparison between these structures should add insight into the catalytic and myotoxic activities of bothropic PLA_2s.

1. Introduction

Phospholipases A_2 (PLA_2s; EC 3.1.1.4) belong to a superfamily of proteins which hydrolyze the *sn*-2 acyl groups of membrane phospholipids to release fatty acids, arachidonic acid and lysophospholipids (van Deenen & de Haas, 1963). The coordination of the Ca^{2+} ion in the PLA_2 calcium-binding loop includes an Asp at position 49 which plays a crucial role in the stabilization of the tetrahedral transition-state intermediate in catalytically active PLA_2s (Scott *et al.*, 1992). In the genus *Bothrops*, PLA_2s are the main components of the venoms produced by species classified into this animal group. In

addition to their primary catalytic role, snake-venom PLA_2s show other important toxic/pharmacological effects, including myonecrosis, neurotoxicity, cardiotoxicity and haemolytic, haemorrhagic, hypotensive, anticoagulant, platelet-aggregation inhibition and oedemainducing activities (Gutiérrez & Lomonte, 1997; Ownby, 1998; Andrião-Escarso *et al*., 2002). Some of these activities are correlated with the enzymatic activity, but others are completely independent (Kini & Evans, 1989; Soares & Giglio, 2004). It has been suggested that some specific sites of these molecules have biochemical properties that are responsible for the pharmacological and toxic actions, including the anticoagulant and platelet-inhibition activities (Kini & Evans, 1989). PLA_2s are also one of the enzymes involved in the production of eicosanoids. These molecules have physiological effects at very low concentrations; however, increases in their concentration can lead to inflammation (Needleman *et al*., 1986). Thus, the study of specific PLA_2 inhibitors is important in the production of structure based anti-inflammatory agents. Many non-catalytic homologous PLA_2s (Lys49-PLA_2s) have been purified from *Bothrops* snake venoms and have been structurally and functionally characterized (Marchi-Salvador *et al*., 2005, 2006; Watanabe *et al*., 2005; Soares *et al*., 2004; Magro *et al*., 2003; Lee *et al*., 2001; Arni *et al*., 1995, 1999; da Silva-Giotto *et al*., 1998). However, little is known about the bothropic catalytic Asp49-PLA_2 (Magro *et al*., 2004, 2005; Rigden *et al*., 2003; Serrano *et al*., 1999; Pereira *et al*., 1998; Daniele *et al*., 1995; Homsi-Brandeburgo *et al*., 1988). Despite the structures of a large number of PLA_2s having been solved by crystallography to date, many questions still need to be clarified. For example, there are PLA_2s with high, moderate and no catalytic activity (Magro *et al*., 2004; Rigden *et al*., 2003; da Silva- Giotto *et al*., 1998). However, for all these 'classes' of PLA_2s the majority of residues of the catalytic machinery are conserved. Similarly, toxic (e.g. myotoxicity, cytotoxicity) and pharmacological effects (e.g. anticoagulant, hypotensive and platelet-aggregation activities) are far from being completely understood. An acidic catalytic PLA_2 (BthA-I) has been isolated from B. jararacussu venom and characterized (Andrião-Escarso *et al*., 2002; Roberto *et al*., 2004). BthA-I is three to four times more catalytically active than BthTX-II (bothropstoxin-II from B. jararacussu) and other basic Asp49-PLA_2s from Bothrops venoms, but is not myotoxic, cytotoxic or lethal (Andrião-Escarso *et al*., 2002). Other activities demonstrated by this enzyme are time-independent oedema induction, hypotensive response in rats and platelet-aggregation inhibition (Andrião-Escarso *et al*., 2002). The crystal structure of BthA-I has been recently described in two conformational states: monomeric and dimeric (Magro *et al*., 2004). Additionally, Magro *et al*. (2005)

solved the structure of BthA-I chemically modified with BPB (*p*-bromophenacyl bromide) and showed important tertiary and quaternary structural changes in this enzyme. This novel oligomeric structure is more energetically and conformationally stable than the native structure and the abolition of pharmacological activities (including anticoagulant, hypotensive effect and platelet-aggregation inhibition) by the ligand may be related to the oligomeric structural changes. The isolation, biochemical/pharmacological characterization and amino-acid sequence of bothropstoxin II from *B. jararacussu* (BthTX-II) have been reported (Homsi-Brandeburgo *et al*., 1988; Gutiérrez *et al*., 1991; Pereira *et al*., 1998). Protein sequencing indicated that BthTX-II is an Asp49-PLA_2 and consists of 120 amino acids (MW = 13 976 Da). The protein shows myotoxic, oedematogenic and haemolytic effects and low phospholipase activity (Homsi- Brandeburgo et al., 1988; Gutiérrez *et al*., 1991). Recently, it has been shown that BthTX-II induces platelet aggregation and secretion through multiple signal transduction pathways (Fuly *et al*., 2004). Despite BthTX-II having been crystallized more than ten years ago (Bortoleto *et al*., 1996), the structure has not been solved to date, probably owing to the low completeness of the data set (50–60% completeness). The crystals belonged to the tetragonal crystal system and preliminary analysis indicated the presence of three molecules in the asymmetric unit (Bortoleto *et al*., 1996). However, a careful analysis of the Matthews coefficient indicated that a tetrameric conformation is also possible (VM = 2.4 $Å^3$ Da^{-1}), which also occurs in the Lys49-PLA_2 MjTX-I (myotoxin I from *B. moojeni* venom) structure formed of two Lys49-PLA_2 dimers (Marchi-Salvador *et al*., 2005; personal communication). In the present paper, we describe the crystallization of BthTX-II (bothropstoxin-II) from *B. jararacussu* venom in the monoclinic system, the collection of a complete X-ray diffraction data set and molecular-replacement solution. This study should improve the understanding of the relation of the myotoxic and low catalytic activity mechanisms to the structural features of this protein when compared with BthTX-I (Lys49-PLA2 from *B. jararacussu* venom) and BthA-I, which possess no and high catalytic activity, respectively.

2. Experimental procedures

2.1. Purification

BthTX-II was isolated from *B. jararacussu* snake venom by gel filtration and ion-exchange chromatography as previously described (Homsi-Brandeburgo *et al*., 1988).

2.2. Crystallization

A lyophilized sample of BthTX-II was dissolved in ultrapure water at a concentration of 12 mg ml^{-1}. The sparse-matrix method (Jancarik & Kim, 1991) was used to perform initial screening of the crystallization conditions (Crystal Screens I and II; Hampton Research). Large crystals of BthTX-II were obtained by the conventional hanging-drop vapour-diffusion method (MacPherson, 1982), in which 1 µl protein solution and 1 µl reservoir solution were mixed and equilibrated against 500 µl of the same precipitant solution. The BthTX-II was crystallized using a solution containing 20% (v/v) 2-propanol, 13% (w/v) polyethylene glycol 4000 and 0.1 M sodium citrate pH 5.6. The best crystals measured approximately 0.4 x 0.2 x 0.1 mm after two months at 291 K (Fig. 1).

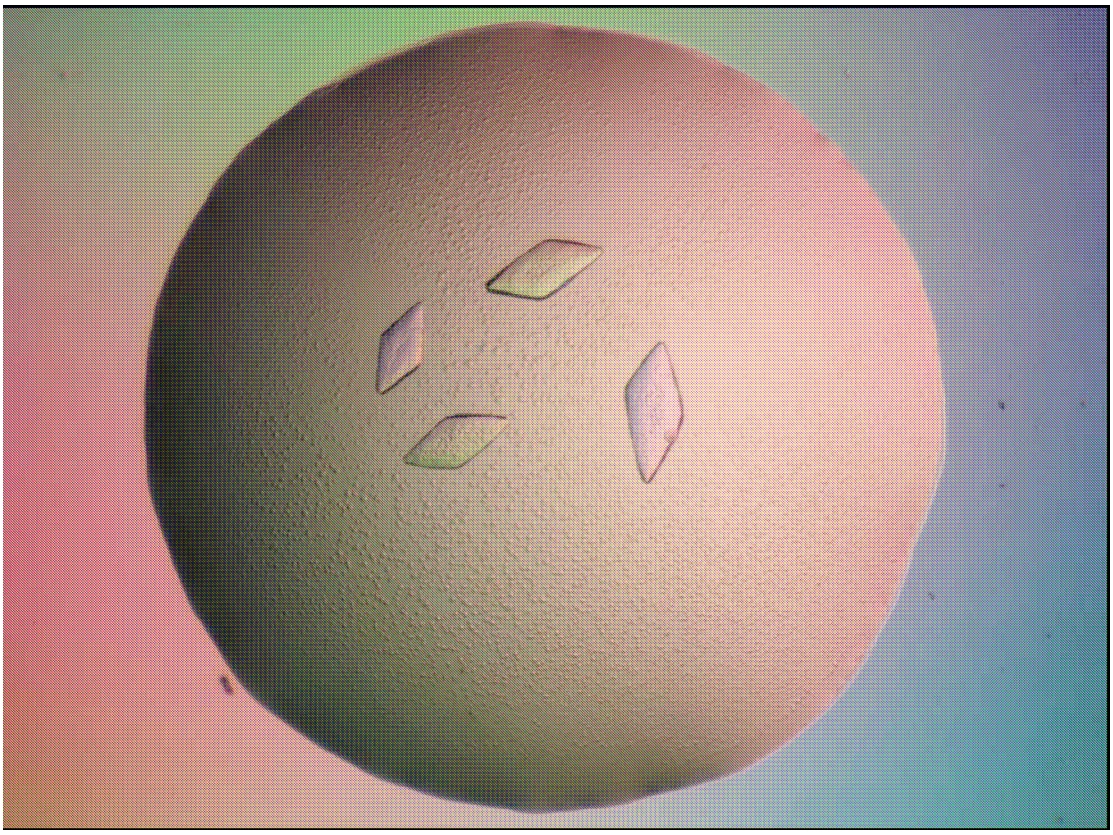


Figure 1: Crystals of BthTX-II from *B. jararacussu* venom.

2.3. X-ray data collection and processing

X-ray diffraction data from BthTX-II crystals were collected using a wavelength of 1.427 Å at a synchrotron-radiation source (Laboratório Nacional de Luz Síncrotron, LNLS, Campinas, Brazil) with a MAR CCD imaging-plate detector (MAR Research). A crystal was mounted in a nylon loop and flash-cooled in a stream of nitrogen at 100 K using no

cryoprotectant. The crystal-to-detector distance was 100 mm and an oscillation range of 1° was used; 149 images were collected. The data were processed to 2.13 Å resolution using the HKL program package (Otwinowski & Minor, 1997).

3. Results and discussion

The data-collection statistics are shown in Table 1. The data set is 96.1% complete at 2.13 Å resolution, with R_{merge} = 9.1%. The crystals belong to space group C2, with unit-cell parameters a = 58.9, b = 98.5, c = 46.7 Å and = 125.9°. Packing parameter calculations based on the protein molecular weight indicate the presence of a dimer in the asymmetric unit. This corresponds to a Matthews coefficient (Matthews, 1968) of 2.0 $Å^3$ Da^{-1} with a calculated solvent content of 37.4%, which are within the expected range for typical protein crystals (assuming a value of 0.74 cm^3 g^{-1} for the protein partial specific volume). The crystal structure was determined by molecular-replacement techniques implemented in the program AMoRe (Navaza, 1994) using the coordinates of a monomer of PrTX-III (PDB code 1gmz). The quaternary structure of BthTX-II is very similar to those of PrTX-III and all dimeric bothropic Lys49-PLA_2s (Rigden *et al*., 2003; Soares *et al*., 2004) and totally different from those of native dimeric BthA-I and BthA-I–bromophenacyl bromide and BthA-I-α-tocopherol complexes (Magro *et al*., 2004, 2005; Takeda et al., 2004). In conclusion, a complete X-ray diffraction data set has been collected from a low catalytic activity Asp49-PLA_2 for the first time (to 2.13 Å) and a molecular-replacement structure solution has been obtained. The quaternary structure of BthTX-II resembles those of the myotoxin PrTX-III (which does not bind Ca^{2+} ions) and all noncatalytic and myotoxic dimeric Lys49-PLA_2s (Rigden *et al*., 2003; Soares *et al*., 2004). In contrast, the oligomeric structure of BthTX-II is different from that of the high catalytic activity and non-myotoxic BthA-I (Magro *et al*., 2004). Thus, comparison between these structures should add insight into the catalytic and myotoxic activities of bothropic PLA_2s.

Table 1

X-ray diffraction data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

Unit Cell (Å)	a= 58.9, b= 98.5, c= 46.7, β= 125.9
Space Group	C2
Resolution (Å)	40-2.13 (2.21-2.13)[a]
Unique reflections	11560 (1127)[a]
Completeness (%)	96,1(94,2)[a]
R_{merge}† (%)	9.1 (26.4)[a]
Radiation source	Synchrotron (LNLS-MX1)
Data collection temperature (K)	100
Sigma cutoff (I) for data processing‡	-3
I/σ (I)	10.6 (3.7)[a]
Redundancy	3.0 (2.9)[a]
Matthews Coefficient (V_M Å^3 Da^{-1})	2.0
Molecules in the ASU	2
Solvent content (%)	37,4

†$R_{merge} = \Sigma_{hkl}(\Sigma_i(|I_{hkl,i}-\langle I_{hkl}\rangle|))/\Sigma_{hkl,i}\langle I_{hkl}\rangle$, where $I_{hkl,i}$ is the intensity of an individual measurement of the reflection with Miller indices *hkl*, and $\langle I_{hkl}\rangle$ is the mean intensity of that reflection. Calculated for $I>-3\sigma(I)$. ‡ Data processing used the HKL suite (Otwinowski & Minor, 1997).

The authors gratefully acknowledge financial support from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação para o Desenvolvimento da UNESP (FUNDUNESP) and Laboratório Nacional de Luz Síncrontron (LNLS, Campinas-SP).

References

Andrião-Escarso, S. H., Soares, A. M., Fontes, M. R. M., Fuly, A. L., Corrêa, F. M. A., Rosa, J. C., Greene, L. J. & Giglio, J. R. (2002). Biochem. Pharmacol. **64**, 723–732.

Arni, R. K., Fontes, M. R. M., Barberato, C., Gutiérrez, J. M., Díaz-Oreiro, C. & Ward, R. J. (1999). Arch. Biochem. Biophys. **366**, 177–182.

Arni, R. K., Ward, R. J. & Gutiérrez, J. M. (1995). Acta Cryst. D**51**, 311–317.

Bortoleto, R. K., Ward, R. J., Giglio, J. R., Cintra, A. C. O. & Arni, R. K. (1996). Toxicon, **34**, 614–617.

Deenen, L. L. M. van & de Haas, G. H. (1963). Biochem. Biophys. Acta, **70**, 538–553.

Daniele, J. J., Bianco, I. D. & Fidelio, G. D. (1995). Arch. Biochem. Biophys. **318**, 65–70.

da Silva-Giotto,M. T., Garratt, R. C.,Oliva,G.,Mascarenhas, Y. P., Giglio, J. R., Cintra, A. C. O., de Azevedo, W. F. Jr, Arni, R. K. & Ward, R. J. (1998). Proteins, **30**, 442–454.

Fuly, A. L., Soares, A. M., Marcussi, S., Giglio, J. R. & Guimarães, J. A. (2004). Biochimie, **86**, 731–739.

Gutiérrez, J. M. & Lomonte, B. (1997). Venom Phospholipase A2 Enzymes: Structure, Function and Mechanism, edited by R. M. Kini, pp. 321–352. Chichester: Wiley & Sons.

Gutiérrez, J. M., Nunez, J.,Diaz, C., Cintra, A. C.O.,Homsi-Brandeburgo,M. I. & Giglio, J. R. (1991). Exp. Mol. Pathol. **55**, 217–229.

Homsi-Brandeburgo, M. I., Queiroz, L. S., Santo-Neto, H., Rodrigues-Simioni, L. & Giglio, J. R. (1988). Toxicon, **26**, 615–627.

Jancarik, J. & Kim, S.-H. (1991). J. Appl. Cryst. **24**, 409–411.

Kini, R. M. & Evans, H. J. (1989). Toxicon, **27**, 613–635.

Lee,W. H., da Silva-Giotto,M. T., Marangoni, S., Toyama, M. H., Polikarpov, I. & Garratt, R. C. (2001). Biochemistry, **40**, 28–36.

MacPherson, A. (1982). Preparation and Analysis of Protein Crystals. New York: Wiley.

Magro, A. J., Soares, A. M., Giglio, J. R. & Fontes, M. R. M. (2003). Biochem. Biophys. Res. Commun. **311**, 713–720.

Magro, A. J., Murakami, M. T., Marcussi, S., Soares, A. M., Arni, R. K. & Fontes, M. R. M. (2004). Biochem. Biophys. Res. Commun. **323**, 24–31.

Magro, A. J., Takeda, A. A. S., Soares, A. M. & Fontes, M. R. M. (2005). Acta Cryst. D**61**, 1670–1677.

Marchi-Salvador, D. P., Fernandes, C. A. H., Amui, S. F., Soares, A. M. & Fontes, M. R. M. (2006). Acta Cryst. F**62**, 600–603.

Marchi-Salvador, D. P., Silveira, L. B., Soares, A.M. & Fontes,M. R.M. (2005). Acta Cryst. F**61**, 882–884.

Matthews, B. W. (1968) J. Mol. Biol. **33**, 491–497.

Navaza, J. (1994). Acta Cryst. A**50**, 157–163.

Needleman, P., Turk, J., Jakschik, B. A., Morrison, A. R. & Lefkowith, J. B. (1986). Annu. Rev. Biochem. **55**, 69–102.

Otwinowski, Z. & Minor, W. (1997). Methods Enzymol. **276**, 307–326.

Ownby, C. L. (1998). J. Toxicol. Toxin Rev. **17**, 1003–1009.

Pereira, M. F., Novello, J. C., Cintra, A. C. O., Giglio, J. R., Landucci, E. T., Oliveira, B. & Marangoni, S. (1998). J. Protein Chem. **17**, 381– 386.

Rigden, D. J., Hwa, L. W., Marangoni, S., Toyama, M. H. & Polikarpov, I. (2003). Acta Cryst. D**59**, 255–262.

Roberto, P. G., Kashima, S., Marcussi, S., Pereira, J. O., Astolfi-Filho, S., Nomizo, A., Giglio, J. R., Fontes, M. R. M., Soares, A. M. & Franc¸a, S. C. (2004). Protein J. **23**, 273–285.

Scott, D. L., Achari, A., Vidal, J. C. & Sigler, P. B. (1992). J. Biol. Chem. **267**, 22645–22657.

Serrano, S. M. T., Reichl, A. P., Mentele, R., Auerswald, E. A., Santoro, M. L., Sampaio, C. A. M., Camargo, A. C. M. & Assakura, M. T. (1999). Arch. Biochem. Biophys. **367**, 26–32.

Soares, A. M., Fontes, M. R. M. & Giglio, J. R. (2004). Curr. Org. Chem. **8**, 1677–1690.

Soares, A. M. & Giglio, J. R. (2004). Toxicon, **42**, 855–868.

Takeda, A. A. S., dos Santos, J. I., Marcussi, S., Silveira, L. B., Soares, A. M. & Fontes, M. R. M. (2004). Biochim. Biophys. Acta, **1699**, 281–284.

Watanabe, L., Soares, A. M., Ward, R. J., Fontes, M. R. M. & Arni, R. K. (2005). Biochimie, **87**, 161–167.

Crystal structure of a myotoxic Asp49-phospholipase A_2 with low catalytic activity, insights into Ca^{2+} independent catalytic mechanism

Corrêa, L.C.[a], Marchi-Salvador, D.P.[a], Cintra, A.C.O.[b], Soares, A.M.[b], Fontes, M.R.M.[a]

[a] Departamento de Física e Biofísica, Instituto de Biociências, UNESP, Botucatu-SP, Brazil

[b] Departamento de Análises Clínicas, Toxicológicas e Bromatológicas, FCFRP, USP, Ribeirão Preto/SP, Brazil

Abstract

Phospholipases A_2 belong to the superfamily of proteins which hydrolyze the *sn*-2 acyl groups of membrane phospholipids to release arachidonic acid and lysophospholipids. A myotoxic Asp49-phospholipase A_2 (Asp49-PLA_2) with low catalytic activity (BthTX-II from *Bothrops jararacussu* venom) was crystallized and the molecular-replacement solution has been obtained with a dimer in the asymmetric unit. The quaternary structure of BthTX-II resembles the myotoxin Asp49-PLA_2 PrTX-III (piratoxin III from B. pirajai venom) and all non-catalytic and myotoxic dimeric Lys49- PLA_2s, however it is different from the highly catalytic and non-myotoxic BthA-I (acidic PLA_2 from *B. jararacussu*) and other Asp49-PLA_2s. BthTX-II structure showed a severe distortion of calcium binding loop leading to displacement of C-terminal region. Tyr28 side chain, present in this region (calcium binding loop), is in an opposite position in relation to the same residue in the catalytic activity Asp49-PLA_2s, making hydrogen bond with the atom Oδ2 of the catalytic residue Asp49, which should coordinate the calcium. Additionally, BthTX-II was crystallized in presence of calcium ions, and the resulting showed Ca^{2+}-binding loop adopts very similar conformation and any ion is present in this region. These facts lead to the conclusion that the BthTX-II is not able to bind calcium ions, consequently, we suggest that its low catalytic function is based in an alternative way compared with other phospholipases A_2.

Keywords: X-ray crystallography; Asp49-phospholipase A_2; *Bothrops jararacussu* venom; Myotoxicit; low catalytic activity.

1. Introduction

Phospholipases A_2 (PLA_2s; EC 3.1.1.4) belong to a superfamily of proteins which hydrolyze the sn-2 acyl groups of membrane phospholipids to release fatty acids, arachidonic acid and lysophospholipids (van Deenen & de Haas, 1963). The coordination of the Ca^{2+} ion in the PLA_2 calcium-binding loop includes an Asp at position 49 which plays a crucial role in the stabilization of the tetrahedral transition-state intermediate in catalytically active PLA_2s (Scott *et al.*, 1992). In the genus Bothrops, PLA_2s are the main components of the venoms produced by species classified into this animal group. In addition to their primary catalytic role, snake-venom PLA_2s show other important toxic/pharmacological effects, including myonecrosis, neurotoxicity, cardiotoxicity and haemolytic, haemorrhagic, hypotensive, anticoagulant, platelet-aggregation inhibition and oedemainducing activities (Gutiérrez & Lomonte, 1997; Ownby, 1998; Andrião-Escarso *et al.*, 2002). Some of these activities are correlated with the enzymatic activity, but others are completely independent (Kini & Evans, 1989; Soares & Giglio, 2003). It has been suggested that some specific sites of these molecules have biochemical properties that are responsible for the pharmacological and toxic actions, including the anticoagulant and platelet-inhibition activities (Kini & Evans, 1989). PLA_2s are also one of the enzymes involved in the production of eicosanoids. These molecules have physiological effects at very low concentrations; however, increases in their concentration can lead to inflammation (Needleman *et al.*, 1986). Thus, the study of specific PLA_2 inhibitors is important in the production of structure based anti-inflammatory agents. Many non-catalytic homologous PLA_2s (Lys49-PLA_2s) have been purified from *Bothrops* snake venoms and have been structurally and functionally characterized (Marchi-Salvador *et al.*, 2005, 2006; Watanabe *et al.*, 2005; Soares *et al.*, 2004; Magro *et al.*, 2003; Lee *et al.*, 2001; Arni *et al.*, 1995, 1999; da Silva-Giotto *et al.*, 1998). However, little is known about the bothropic catalytic Asp49-PLA_2s (Magro *et al.*, 2004, 2005; Rigden *et al.*, 2003; Serrano *et al.*, 1999; Pereira *et al.*, 1998; Daniele *et al.*, 1995; Homsi-Brandeburgo *et al.*, 1988). Despite the structures of a large number of PLA_2s having been solved by crystallography to date, many questions still need to be clarified. For example, there are PLA_2s with high, moderate and no catalytic activity (Magro *et al.*, 2004; Rigden *et al.*, 2003; da Silva- Giotto *et al.*, 1998). However, for all these 'classes' of PLA_2s the majority of residues of the catalytic machinery are conserved. Similarly, toxic (e.g. myotoxicity, cytotoxicity) and pharmacological effects (e.g. anticoagulant, hypotensive and platelet-

aggregation activities) are far from being completely understood. An acidic catalytic PLA_2 (BthA-I) has been isolated from *B. jararacussu* venom and characterized (Andrião-Escarso *et al*., 2002; Roberto *et al*., 2004). BthA-I is three to four times more catalytically active than BthTX-II (bothropstoxin-II from B. jararacussu) and other basic Asp49-PLA_2s from Bothrops venoms, but is not myotoxic, cytotoxic or lethal (Andrião-Escarso *et al*., 2002). Other activities demonstrated by this enzyme are time-independent oedema induction, hypotensive response in rats and platelet-aggregation inhibition (Andrião-Escarso *et al*., 2002). The crystal structure of BthA-I has been recently described in two conformational states: monomeric and dimeric (Magro *et al*., 2004). Additionally, Magro *et al*. (2005) solved the structure of BthA-I chemically modified with BPB (*p*-bromophenacyl bromide) and showed important tertiary and quaternary structural changes in this enzyme. This novel oligomeric structure is more energetically and conformationally stable than the native structure and the abolition of pharmacological activities (including anticoagulant, hypotensive effect and platelet-aggregation inhibition) by the ligand may be related to the oligomeric structural changes. The isolation, biochemical/pharmacological characterization and amino-acid sequence of bothropstoxin II from *B. jararacussu* (BthTX-II) have been reported (Homsi-Brandeburgo *et al*., 1988; Gutiérrez *et al*., 1991; Kashima *et al*., 2004). Protein sequencing indicated that BthTX-II is an Asp49-PLA_2 and consists of 122 amino acids (MW = 13 976 Da). The protein shows myotoxic, oedematogenic and haemolytic effects and low phospholipase activity (Homsi- Brandeburgo *et al*., 1988; Gutiérrez *et al*., 1991). Recently, it has been shown that BthTX-II induces platelet aggregation and secretion through multiple signal transduction pathways (Fuly *et al*., 2004). BthTX-II has been crystallized and a complete data set has been collected (Corrêa *et al*., 2006).

In the present paper, we describe crystal structure of basic Asp49-PLA_2-BthTX-II isolated from the venom of *Bothrops jararacussu* and a comparative analysis with other Asp49 and Lys49 phospholipases A_2 from snake venoms. This study provides deeper understanding of structural basis of low catalytic and high myotoxic activity of this protein and may introduce to a totally new Ca^{2+}-independent catalytic mechanism for PLA_2s.

2. Material and methods

2.1. Purification

BthTX-II was isolated from *B. jararacussu* snake venom by gel filtration and ion-exchange chromatography as previously described (Homsi-Brandeburgo *et al*., 1988).

2.2. Crystallization

A lyophilized sample of BthTX-II was dissolved in ultra pure water at a concentration of 12 mg ml^{-1}. Crystals of BthTX-II were obtained by the conventional hanging-drop vapour-diffusion method (MacPherson, 1982) using Crystal Screens I (by Hampton Research) with the sparse-matrix method (Jancarik & Kim, 1991). Better crystals were obtained using a solution containing 20%(v/v) 2-propanol, 13%(w/v) polyethylene glycol 4000 and 0.1 M sodium citrate pH 5.6 (Corrêa *et al*., 2006) in which 1 µl protein solution and 1 µl reservoir solution were mixed and equilibrated against 500 µl of the same precipitant solution. The crystals measured approximately 0.4 x 0.2 x 0.1 mm after two months at 291 K.

2.3. X-ray data collection and processing

X-ray diffraction data from BthTX-II crystals were collected using a wavelength of 1.427 Å at a synchrotron-radiation source (Laboratório Nacional de Luz Sincrotron, LNLS, Campinas, Brazil) with a MAR CCD imaging-plate detector (MAR Research). A crystal was mounted in a nylon loop and flash-cooled in a stream of nitrogen at 100 K using no cryoprotectant. The crystal-to-detector distance was 100 mm and an oscillation range of 1^{o} was used; 149 images were collected. The data were processed to 2.19 Å of resolution (Corrêa *et al*., 2006), using the HKL program package (Otwinowski & Minor, 1997).

2.4. Structure determination and refinement

The crystal structure of BthTX-II was solved by the Molecular Replacement Method using the program AMoRe (Navaza, 1994) and coordinates of PrTX-III (Rigden *et al*., 2003). The model choice was based on the best results of correlation and R-factor from the AMoRe program. After a cycle of simulated annealing refinement using the CNS program (Brunger *et al*., 1998), the electron densities were inspected and the amino acid sequence as obtained from the cDNA of BthTX-II (Kashima *et al*., 2004) was inserted. The modeling process was always performed by manually rebuilding with the program "O" (Jones *et al*., 1990). Electron density maps calculated with coefficients 3|Fobs|-2|Fcalc| and simulated annealing omit maps calculated with analogous coefficients were generally used. The model was improved, as judged by the free R-factor (Brunger, 1992), through rounds of crystallographic refinement (positional and restrained isotropic individual B-factor refinement, with an overall anisotropic temperature factor and bulk solvent correction) using the CNS program (Brunger *et al*., 1998), and manual rebuilding with the program

"O" (Jones *et al.*, 1990). Solvent molecules were added and refined also with the program CNS (Brunger *et al.*, 1998). The refinement converged to R_{free} and R_{cryst} of 22.7% and 20.7% respectively. The final models comprise 1891 protein atoms, 229 water molecules and 2 sodium ions. The refinement statistics are shown in Table 1. The quality of the model was checked with the program Procheck (Laskowski *et al.*, 1993). The contacts were analyzed with the program Dimplot (Wallace et al., 1995) and the buried surface areas were calculated using the program CNS (Brunger *et al.*, 1998). The coordinates have been deposited in the RCSB Protein Data Bank with ID codes 2OQD.

2.5. Comparative analyzes

For molecular comparisons of the Asp49-PLA_2 and Lys49-PLA_2 structures, the program "O" (Jones *et al.*, 1990) was used with only the Cα coordinates. The comparative analysis of PLA_2s has been performed with BthTX-II, BthTX-I and BthA-I (from *B. jararacussu*), PrTX-II and PrTX-III (from *B. pirajai*), apart of a basic and an acid Asp49-PLA_2s (from *A. h. Pallas*). An alignment of the class IIA Asp49 and Lys49 PLA_2s from different species (*Bothrops jararacussu*, *Bothrops pirajai* and *Agkistrodon halys pallas*) was produced using only the secondary structure residues using ClustalW program ((Higgins *et al.*, 1994).

3. Results

3.1. Overall structure of the BthTX-II

The crystals of complex BthTX-II diffract to 2.19 Å and are monoclinic, space group C2 with unit cell constants of a=58.9, b=98.5, c=46.7 Å and β=125.9°. The refinement converged to a crystallographic residual of 20.7 % (R_{free}=22.7 %) for all data between 30.0 Å and 2.19 Å (Table 1).

The structure shows excellent overall stereochemistry with no residues found in the disallowed or generously allowed regions of the Ramachandran plot. The overall Procheck G-factor is -0.1 (Laskowski *et al.*, 1993).

Table 1. X-ray data collection and refinement statistics

Unit cell (Å)	a= 58.912; b= 98.458; c= 46.720 β= 125.89°
Space group	C2
Resolution (Å)	30.01-2.19 (2.33-2.19)[a]
Unique reflections	10,687 (1619)[a]
Completeness (%)	95.9 (92.9)[a]
R_{merge}[b] (%)	9.1 (26.4)[a]
I/σ (I)	10.6 (3.7)[a]
Redundancy	3.0 (2.9)[a]
R_{cryst}[c] (%)	20.7 (35.9)[a]
R_{free}[d] (%)	22.7 (40.4)[a]
Number of non-hydrogen atoms:	
Protein	1891
Water	229
Na^+ ion	2
Mean B factor (Å^2)[e]	
Overall	35.1
Na^+ ion	31.6
R.m.s deviations from ideal values[e]	
Bond lengths (Å)	0.023
Bond angles (°)	2.3
Ramachandram plot[f] (%)	
Residues in most favored region	88.9
Residues in additional allowed region	11.1
Coordinate error (Å)[e]	
Luzzati plot (cross-validated Luzzati plot)	0.27 (0.31)
SIGMAA (cross-validated SIGMAA)	0.34 (0.35)

[a] Numbers in parenthesis are for the highest resolution shell.

[b] $R_{merge} = \Sigma_{hkl}(\Sigma_i(|I_{hkl,i}-\langle I_{hkl}\rangle|))/\Sigma_{hkl,i}\langle I_{hkl}\rangle$, where $I_{hkl,i}$ is the intensity of an individual measurement of the reflection with Miller indices h, k and l, and $\langle I_{hkl}\rangle$ is the mean intensity of that reflection. Calculated for $I>-3\sigma$ (I).

[c] $R_{cryst} = \sum_{hkl}(||Fobs_{hkl}|-|Fcalc_{hkl}||)/|Fobs_{hkl}|$, where $|Fobs_{hkl}|$ and $|Fcalc_{hkl}|$ are the observed and calculated structure factor amplitudes.

[d] R_{free} is equivalent to R_{cryst} but calculated with reflections (5 %) omitted from the refinement process.

[e] Calculated with the program CNS (Brünger *et al.*, 1998).

[f] Calculated with the program PROCHECK (Laskowski *et al.*, 1993).

BthTX-II is a dimeric structure with seven disulfide bridges in each monomer and like other class II PLA_2s, has the following structural features: (i) an N-terminal α-helix;

(ii) a "short" helix, (iii) a Ca^{2+} binding loop; (iv) two anti-parallel α-helices (2 and 3); (v) two short strands of anti-parallel β-sheet (β-wing); and (vi) a C-terminal loop (Figure 1). The catalytic network for class II PLA_2s, formed by His48, Tyr52, Tyr73 and Asp99, is fully conserved.

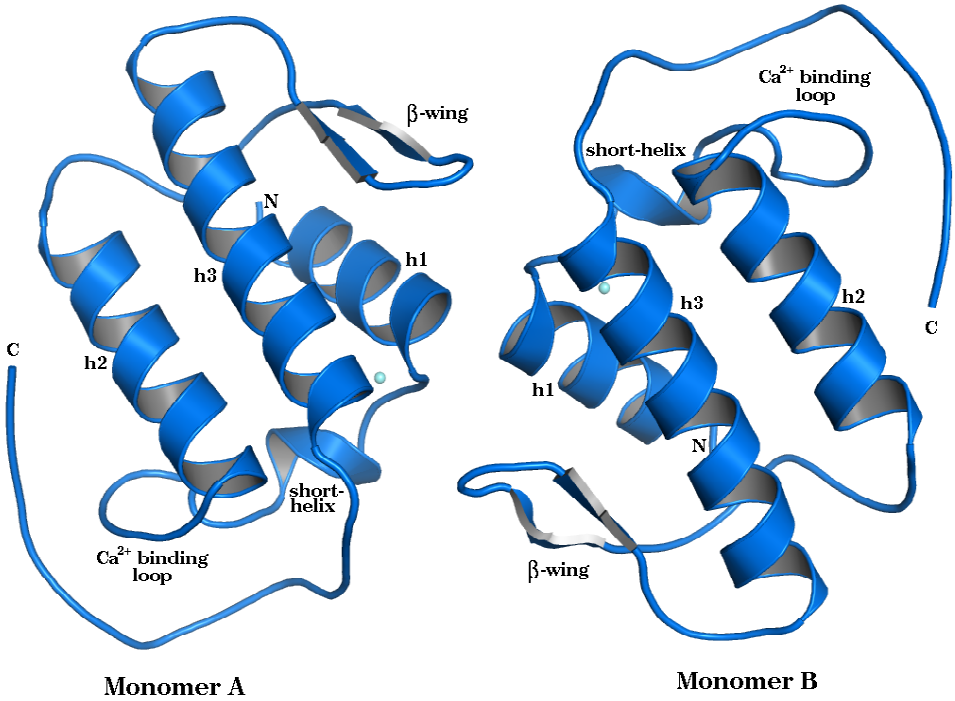
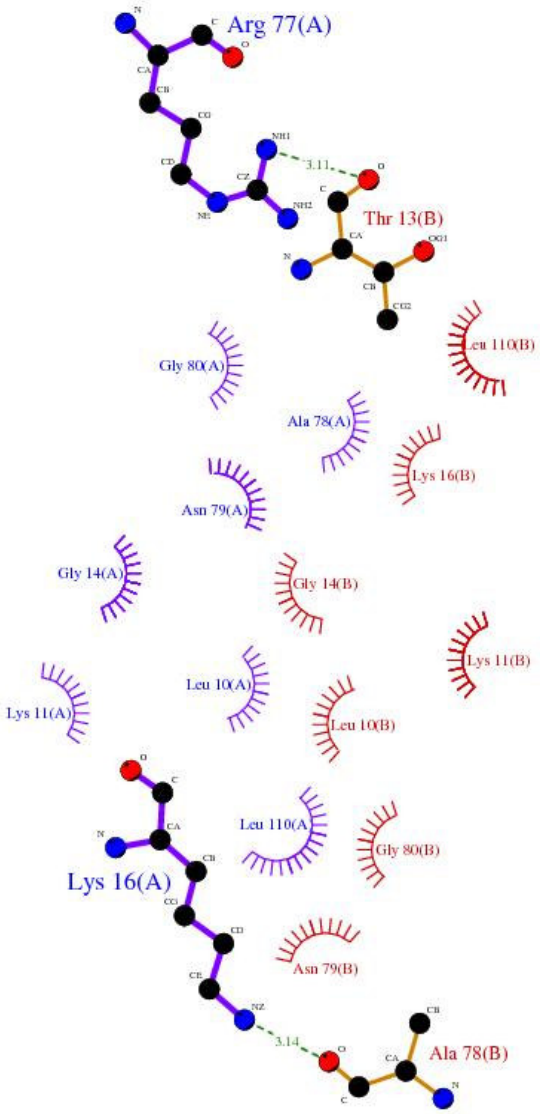


Figure 1- Dimeric structure of BthTX-II is showed as a ribbon diagram. The figure was drawn using *Pymol program* (DeLano, 2002).

The monomers of BthTX-II are related by an approximate two-fold axis perpendicular to the β-wing (Figure 1). Hydrophobic contacts and two intermolecular hydrogen bonds (Arg77 (mon.A)-Thr13 (mon. B) and Lys16 (mon. A)-Ala78 (mon. B) contribute to the stabilization of the dimer. The majority of contacts involve residues of h1 α-helix (residues 10-14), β-wing (residues 78-80) and Trp110 from C-terminal (Figure 2a). It has been found an alternative dimeric conformation analyzing the crystal packing of protein (Figure 2b) (see discussion section).



a

Key

Residues of first surface

Residues of second surface

Hydrogen bond and its length

His 53 Residues involved in hydrophobic contact(s)

Corresponding atoms involved in hydrophobic contact(s)

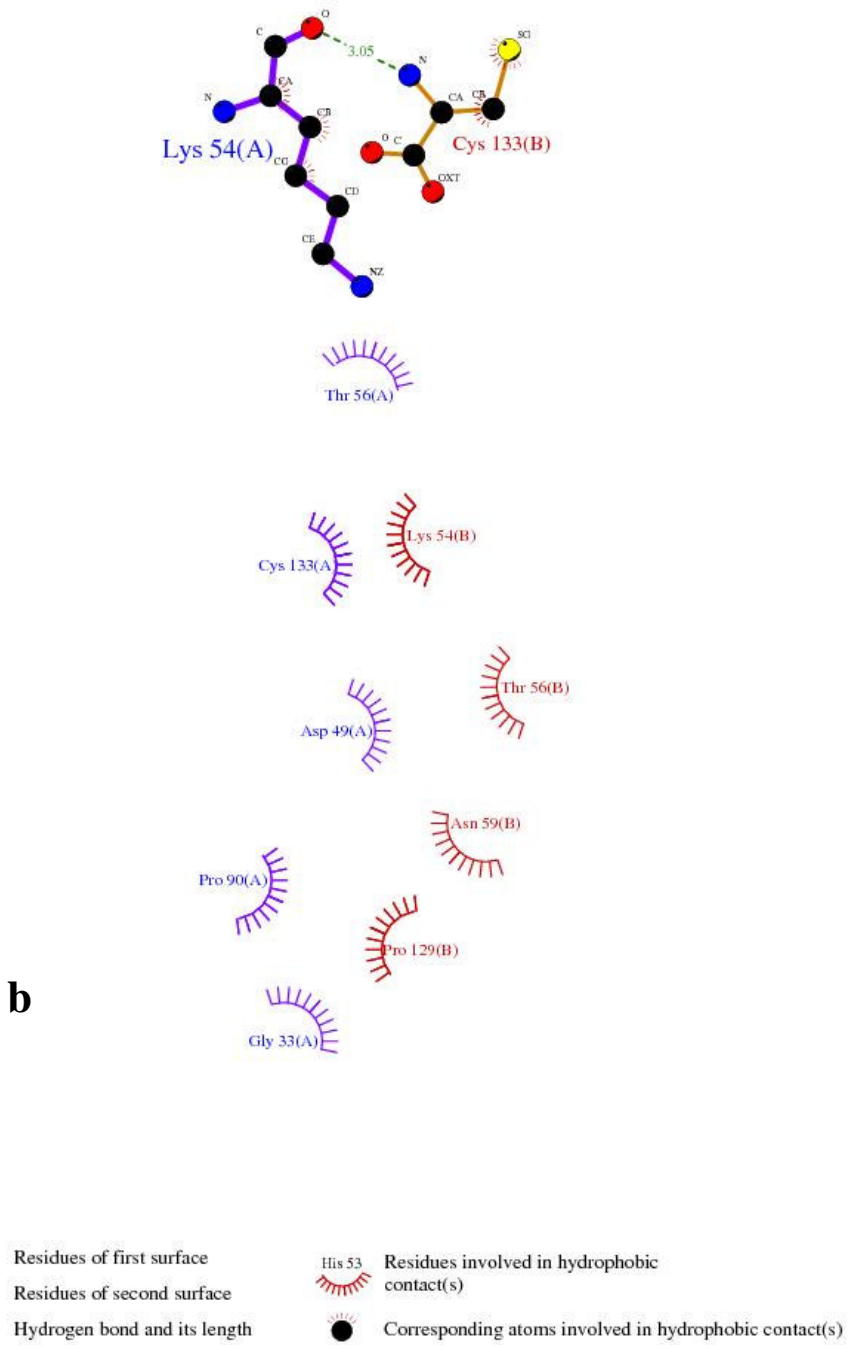


Figure 2- Contacts in the dimeric interface of BthTX-II. a) Conformation used in the structure of BthTX-II; b) Alternative dimer. By Dimplot (Wallace *et al*., 1995).

The monomers are very similar, where the r.m.s. deviation of Cα atoms is 0.73 Å after the superposition between them. The average B-factor for both m-BthA-I and d-BthA-I is 35.1 $Å^2$. The two Na^+ ions in the final model, one bound to each subunit, are well defined by density, with B-factor of 31.6 $Å^2$, which is similar of entire structure (35.1

Å^2). These ions are sited in the hydrophobic channel of catalytic site bound to the Phe19 residue, for both monomers.

The Figure 3 shows the BthTX-II structure according with B-factor value of each residue. It can be observed C-terminal regions of both monomers and β-wing region of monomer B have high B-factors values and are the more flexible regions of protein.

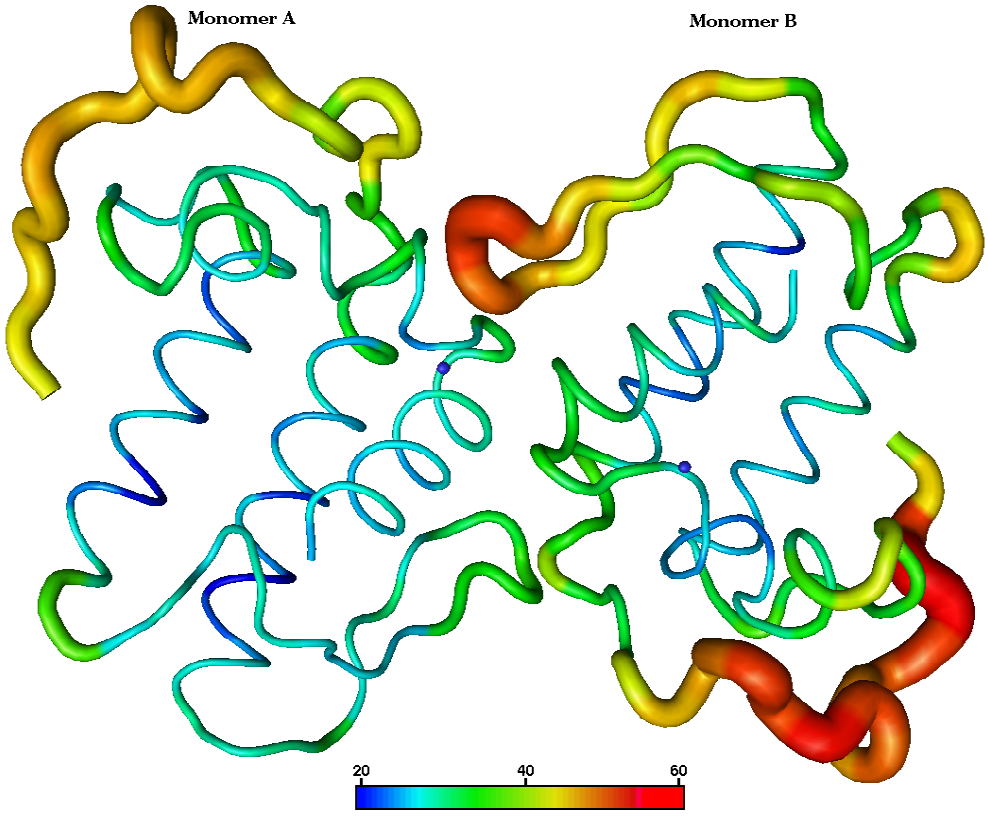


Figure 3- Diagram of B-factor values from BthTX-II. Regions with higher B-factor values are show in yellow and red. By *Pymol program* (DeLano, 2002).

The B-factors in C-terminal are 41 Å^2 and 46 Å^2 of the monomers A and B, respectively, while their values are 31 Å^2 and 44 Å^2 in the β-wing regions of monomers A and B, respectively. In contrast, the three main helixes (h1, h2 and h3) in the core region of protein are very stable. The Ca^{2+}-binding loop of both monomers, despite their unusual conformation compared to other PLA_2s, have B-factors values comparable to entire structure (32 Å^2 and 34 Å^2, respectively for monomers A and B).

3.2. Comparison between PLA$_2$s

Figure 4 shows the alignment of the class IIA Asp49 and Lys49 PLA$_2$s from different species: *Bothrops jararacussu* (Kashima *et al*., 2003; Magro *et al*., 2004, Cintra *et al*., 1993), *Bothrops pirajai* (Rigden et al., 2003; Lee *et al*., 2001) and *Agkistrodon halys pallas* (Zhao *et al*., 1997; Chen *et al*., 1987) produced using only the secondary structure residues. The sequence identity related to BthTX-II varies from 80.0 % (basic Asp49-PLA$_2$-PrTX-III) to 56% (Lys49-PLA$_2$-BthTX-I) and 57 % (acidic Asp49-PLA$_2$-BthA-I).

```
                    10         20         30         40         50         60          Identity
BthTX-II   DLWQFGQMI-LKETGKLPFPYYTTYGCYCGWGGQGQPKDATDRCCFVHDCCYG---KLTNCK-----P  100%
PrTX-III   DLWQFGKMI-LKETGKLPFPYYVTYGCYCGVGGRGGPKDATDRCCFVHDCCYG---KLTSCK-----P   80%
Basic-Ag   HLLQFRKMI-KKMTGKEPVVSYAFYGCYCGSGGRGKPKDATDRCCFVHDCCYE---KVTGCD-----P   67%
PrTX-II    SLFELGKMI-LQETGKNPAKSYGAYGCNCGVLGRGKPKDATDRCCYVHKCCYK---KLTGCN-----P   59%
Acidic-Ag  SLIQFETLI-MKVAKKSGMFWYSNYGCYCGWGGQGRPQDATDRCCFVHDCCYG---KVTGCD-----P   58%
BthA-I     SLWQFGKMI-NYVMGESGVLQYLSYGCYCGLGGQGQPTDATDRCCFVHDCCYG---KVTGCD-----P   57%
BthTX-I    SLFELGKMI-LQETGKNPAKSHGAYGCNCGVLGRGKPKDATDRCCYVHKCCYK---KLTGCD-----P   56%

           70         80         90         100        110        120         130
BthTX-II   KTDRYSYSRENGVIICG-EGTPCEKQICECDKAAAVCFRENLRTYK-KRYMAYPDVLCKKP-AEKC    100%
PrTX-III   KTDRYSYSRKDGTIVCG-ENDPCRKEICECDKAAAVCFRENLDTYN-KKYMSYLKSLCKKX-ADDC     80%
Basic-Ag   KWDDYTYSWKNGTIVCG-GDDPCKKEVCECDKAAAICFRDNLKTYK-KRYMAYPDILCSSK-SEKC     67%
PrTX-II    KKDRYSYSWKDKTIVCG-ENNPCLKELCECDKAVAICLRENLGTYN-KKYRYHLKPFCKKA--DKC     59%
Acidic-Ag  KMDVYSFSEENGDIVCG-GDDPCKKEICECDRAAAICFRDNLTLYNDKKYWAFGAKNCPQEESEPC     58%
BthA-I     KIDSYTYSKKNGDVVCG-GDDPCKKQICECDRVATTCFRDNKDTYD-IKYWFYGAKNCQEK-SEPC     57%
BthTX-I    KKDRYSYSWKDKTIVCG--ENNCLKELCECDKAVAICLRENLGTYN-KKYRYHLKPFCKKA--DAC     56%
```

Figure 4- Amino acid sequence alignments of Asp49 and Lys49-PLA$_2$s: BthTX-I, BthTX-II and BthA-I (from *B. jararacussu*); PrTX-II and PrTX-III (from *B. pirajai*); Basic-Ag and Acidic-Ag (Basic and Acidic Asp49-PLA2 from *A. h. pallas*). Produced by the ClustalW program (Higgins *et al*., 1994).

Table 2 catalogs r.m.s. deviations after superposition between monomer A from BthTX-II and other monomers A of PLA$_2$s: BthA-I (Murakami *et al*., 2006) and BthTX-I from *B. jararacussu*, acidic and basic Asp49-PLA$_2$ form *A. h. pallas* (Wang *et al*., 1996; Zhao *et al*., 1998), Lys49-PLA$_2$ PrTX-II (Lee *et al*., 2001) and low catalytic basic Asp49-PLA$_2$ PrTX-III (Rigden *et al*., 2003). This comparison shows the effect caused mainly by Ca^{2+} binding loop distortion on the tertiary structure of BthTX-II and PrTX-III. The BthTX-II and PrTX-III monomers are very similar, with an r.m.s. deviation of about 0.5 Å between Cα atoms. By contrast, the comparisons of the BthTX-II with other PLA$_2$s have r.m.s. deviations higher than 1.5 Å.

Table 2. Superposition of monomer A from BthTX-II with monomer A from other PLA_2s (r.m.s. deviation of Cα atoms).

Protein	r.m.s.d (Å)
PrTX-III (*B. pirajai*)	0.490
Basic-Ag (*A. h. pallas*)	1.527
Acidic-Ag (*A. h. pallas*)	1.835
BthA-I (*B. jararacussu*)	1.924
PrTX-II (*B. pirajai*)	1.711
BthTX-I (*B. jararacussu*)	1.508

Figure 5a shows the superposition between the three main helices (h1, h2, and h3) of the monomers A of the BthTX-II, BthTX-I, BthA-I, acidic and basic Asp49 from *Agkistrodon halys pallas*, PrTX-II and PrTX-III. This comparison indicates that there are two main regions with significant structural differences of the BthTX-II in comparison with other PLA_2s: the Ca^{2+}-binding loop and the C-terminal region. Both structural differences seem to be generate by extreme distortion of Ca^{2+}-binding loop (Figure 5b) which starts in the different configuration adopted by Tyr28 side chain and, mainly by important diversion taken by the main chain dihedral angles of Cys29. The Gly30, Trp31 and Gly32 backbones are approximately perpendicular to the all other PLA_2s, with exception of PrTX-III, which adopts very similar conformation. In the present structure, the catalytic residue Asp49, which is in a similar position that other PLA_2s, is hydrogen bound to the Tyr28, residue from Ca^{2+}-binding loop (Figure 6a). The Trp31 side chain adopts a conformation totally opposite to the Leu31 in BthA-I (Figure 6b) pointing to the C-terminal region.

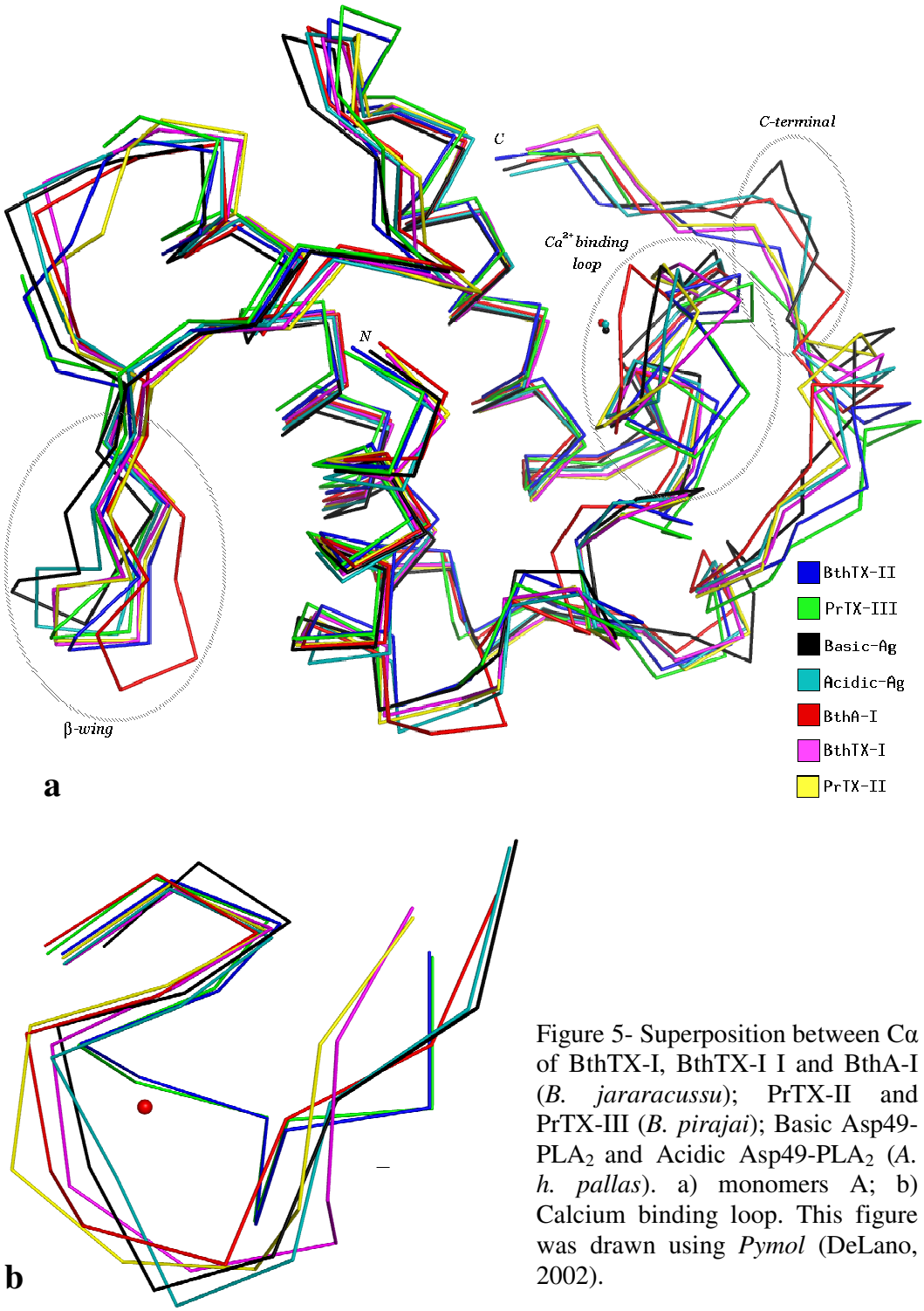


Figure 5- Superposition between Cα of BthTX-I, BthTX-I I and BthA-I (*B. jararacussu*); PrTX-II and PrTX-III (*B. pirajai*); Basic Asp49-PLA_2 and Acidic Asp49-PLA_2 (*A. h. pallas*). a) monomers A; b) Calcium binding loop. This figure was drawn using *Pymol* (DeLano, 2002).

Superposition between Cα atoms of the BthTX-II monomers resulted in an r.m.s. deviation of 0.73 Å while the same superposition between native BthA-I monomers

resulted in a value of 0.54 Å (Magro *et al*., 2004). These results are similar to those obtained for other dimeric PLA_2s (Magro *et al.*, 2003).

The superposition of Ca^{2+} binding loop of these proteins (Figure 5b) resulted in a large difference between r.m.s deviations of BthTX-II in comparison with other PLA_2s (table 3).

Table 3. Superposition between Calcium binding loop of the monomers A from BthTX-II and other PLA_2s (r.m.s. deviation of Cα atoms).

Protein	r.m.s.d (Å)
PrTX-III (*B. pirajai*)	0.208
Basic-Ag (*A. h. pallas*)	2.074
Acidic-Ag (*A. h. pallas*)	2.249
BthA-I (*B. jararacussu*)	2.299
PrTX-II (*B. pirajai*)	2.111
BthTX-I (*B. jararacussu*)	2.097

The quaternary structure of BthTX-II resembles the Lys49-PLA_2s (Soares *et al*., 2004) and one of two possible dimers of PrTX-III (Rigden *et al*., 2003 - these authors found two possible biological dimers for PrTX-III) structures. Fig 7 shows the superposition of the monomers A from BthTX-II, BthTX-I and PrTX-III (Cα atoms of α-helices h1, h2 and h3 were used in the superposition) indicating similar quaternary structure. However, as previously demonstrated (Magro *et al*., 2003), the monomers of these dimeric PLA_2s may adopt different relative aperture between them.

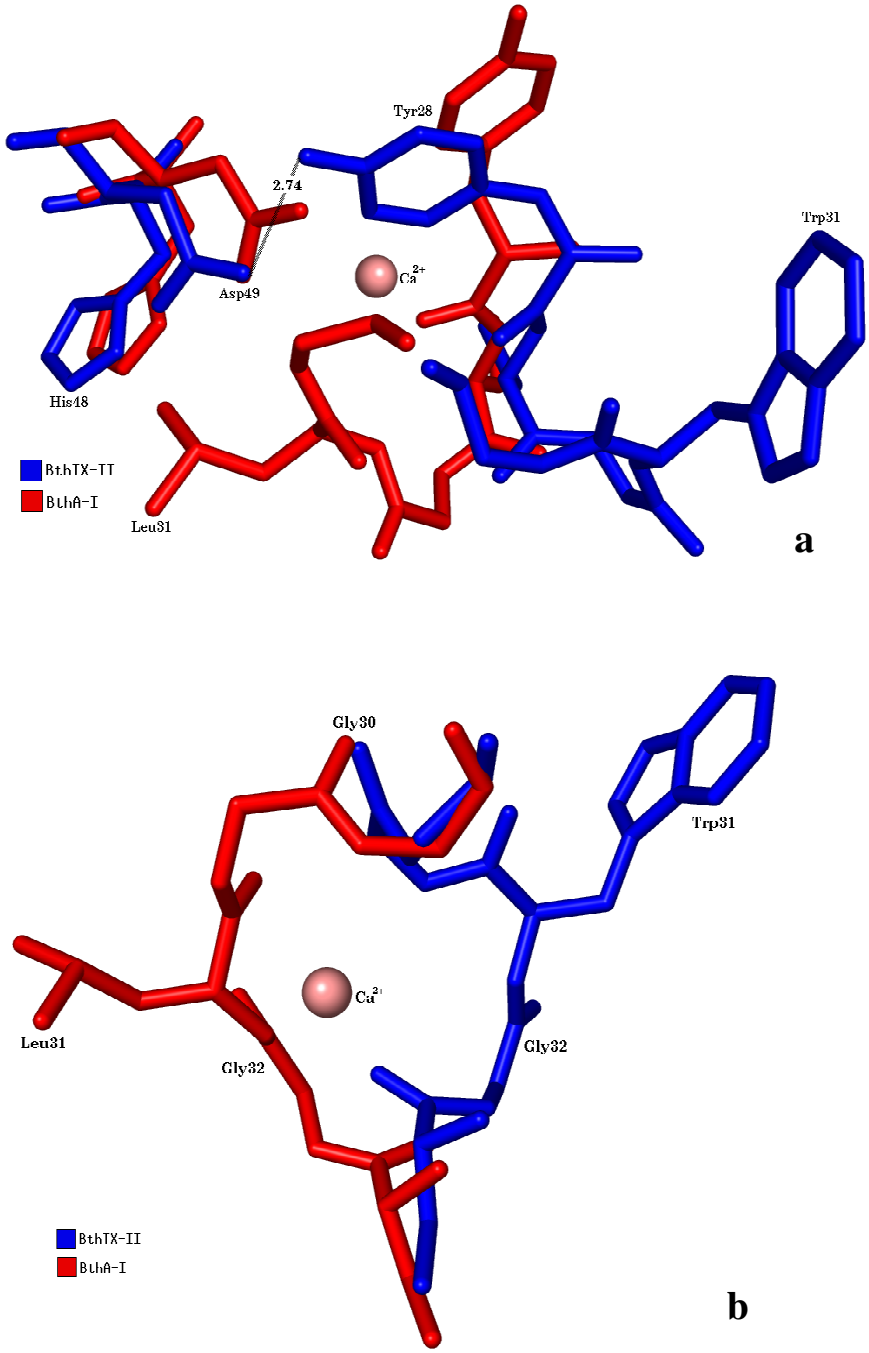


Figure 6- a) Configuration adopted by Tyr28 in BthTX-II in comparison with BthA-I. b) Perpendicular position between residues 30 and 31 of BthTX-II and BthA-I. Magenta sphere is an ion Ca^{2+} present in the structure of BthA-I.

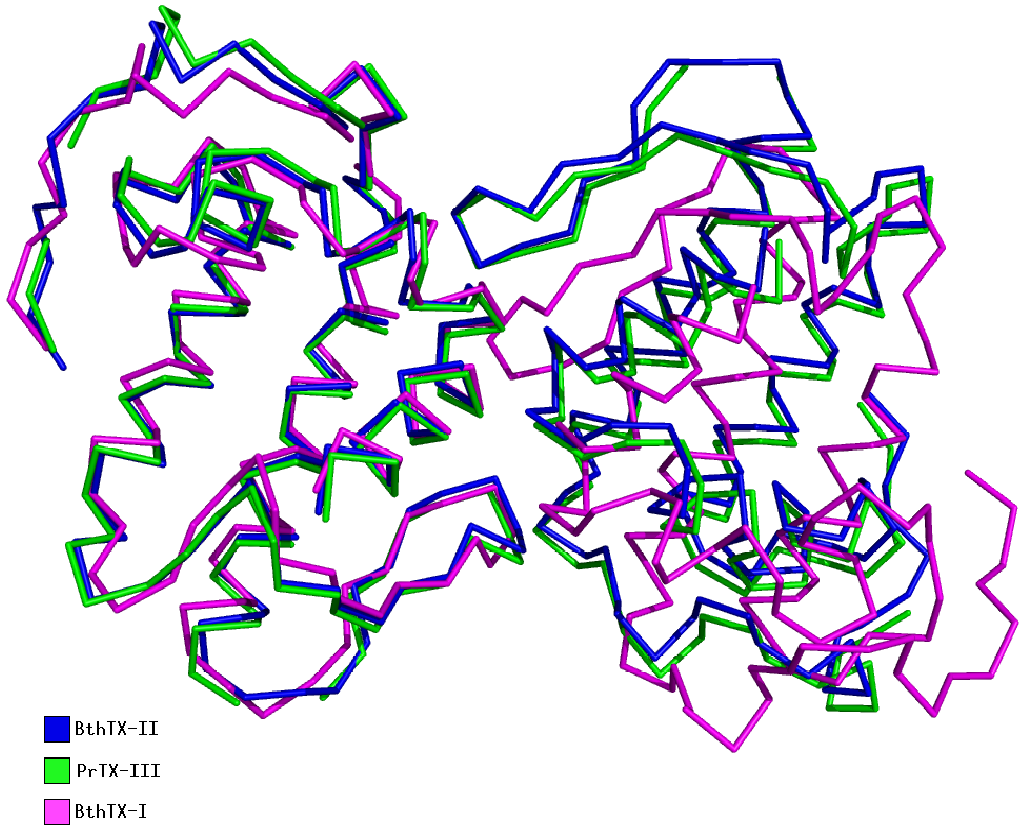


Figure 7- Superposition between dimers of BthTX-II, PrTX-III and BthTX-I.

4. Discussion

4.1. Quaternary structure

BthTX-II and PrTX-III are unique proteins among class I/II PLA_2s studied to this date. They have actions of both PLA_2s classes Asp49-PLA_2s (e.g catalytic activity) and Lys49-PLA_2s (myotoxic and cytotoxic actions), however its catalytic activity is three to four times less than BthA-I. BthTX-II also is able to induce platelet aggregation in a concentration-dependent manner (Fuly *et al*., 2004), in contrast with BthA-I which caused a hypotensive response in rats and inhibited platelet aggregation (Andrião-Escarso *et al*., 2002).

The oligomeric state is an important and enigmatic issue for many of phospholipase A_2 structures solved to this date date (Magro *et al*., 2003; Pan *et al*., 2001; de Oliveira *et al*., 2001; Dekker, 2000; Snijder *et al*, 1999; Sanchez *et al*., 2001). It has been shown the majority of Lys49-PLA_2s are dimeric is solution using eletrophoretic and spectroscopic techniques (da Silva-Giotto *et al*., 1998; Arni *et al*., 1999 and Soares *et al*., - BnSP-7-ABB

2000). However, GodMT-II crystal structure is a monomer (Arni et al., 1999) while MjTX-I has a tetrameric conformation (Marchi-Salvador *et al*., 2005). For GodMT-II, it is likely its monomeric conformation is due to physicochemical conditions used in the crystallization experiments (low pH value). It has been shown oligomeric conformation is essential for myotoxic, cytotoxic and Ca^{2+}-independent membrane damaging effects (Angulo *et al*., 2005; de Oliveira *et al*., 2001 and Ruller *et al*., 2005). These authors also showed that this oligomeric conformation is pH dependent, which confirms the hypothesis GodMT-II is monomeric just because it was crystallized at low pH. Recent site-directed mutagenesis associated with spectroscopic experiments also demonstrated the importance of dimeric conformation of Lys49-PLA_2s in its biological function (Ruller *et al*, 2005).

For the acidic PLA_2s from B. jararacussu (BthA-I), in contrast with the Lys49-PLA_2s, there is no consensus regarding its oligomeric conformation. BthA-I native structure was solved in monomeric and dimeric conformations (Magro *et al*., 2004). Furthermore, it has been solved BthA-I complexed with *p*-bromophenacyl bromide inhibitor in an alternative dimeric and more stable conformation compared to native BthA-I (Magro *et al*., 2005a). BthA-I was also crystallized in presence of α-tocopherol inhibitor showing monomeric and dimeric conformations (Magro et al., 2005b).

Figure 7 shows that the BthTX-II structure resembles those of Lys49-PLA_2s, however an alternative crystal lattice can be observed for BthTX-II. In this assembly, the dimeric conformation is kept only by one hydrogen bond and a few hydrophobic interactions (Fig. 2) from C-terminal and short helix regions. Similar conformations were found for PrTX-III structure (Rigden *et al*., 2003).

We suggest dimeric Lys49-PLA_2 assembly is the conformation for the BthTX-II based on three main reasons. (i) This conformation is stabilized by larger number hydrophobic contacts (seven interactions) and two intermolecular hydrogen bonds. In contrast, the alternative conformation is stabilized by six hydrophobic contacts and one intermolecular hydrogen bond (Fig. 2a, b). (ii) The higher *B* factors values are found in the C-terminus which is in the outer part of structure (Fig. 3), while for the alternative assembly, this region is in the interface of the monomers. In contrast, the β-wing *B* factors, which are 31 and 44 in subunits *A* and *B*, respectively, are close to the overall *B* factor of 35.1 $Å^2$ and should stay in the inner part of structure. It has been show for the monomeric Lys49-PLA_2 (Arni *et al*., 1999) high values of *B* factors compared to the structure. (iii) The Lys49-PLA_2 conformation is found in more than ten crystallographic structures which

have at least five different space groups suggesting no influence of crystal packing of molecules.

Recently, it has been solved the crystal structure of complex Basp-II bound to suramin (Murakami *et al*., 2005). This structure revealed that one molecule suramin binds both monomers of protein; this configuration is only permitted for an alternative dimer of Lys49-PLA_2s where the hydrophobic surfaces surrounding the entrance to the active sites form the dimer interface. This "alternative dimer" found in this structure is not the same alternative assembly found for BthTX-II structure whose interfacial area is much smaller than those of Basp-II/suramin complex.

4.2. The distorted Ca^{2+} binding loop – possible relation with low catalytic activity

BthTX-II and PrTX-III structures revealed Ca^{2+}-binding loops extremely distorted. The binding of Ca^{2+}, required for phospholipase activity in Asp49-PLA_2s (Scott & Sigler, 1994), involves the main-chain carbonyl groups of residues 28, 30 and 32. In the BthA-I and the Acidic Asp49-PLA_2 from *A. h. pallas*, both monomerics, crystallized in the presence of Ca^{2+}, the distance between this ion and the Gly30 O were 4.92 Å and 2.24 Å, respectively, while the same measurements in PrTX-III was 8.6 Å in subunit A. In BthTX-II, this distance was 8.8 $Å^2$. These values show clearly, a distortion of the Ca^{2+}-binding loop in the present structure beyond that expected from the simple absence of Ca^{2+} ion.

Asp49-PLA_2s have been solved in the native form and in the presence of Na^+ or Ca^{2+} ions. The crystal structure of native BthA-I was recently described in two conformational states: monomeric (m-BthA-I – in the presence of Na^+ ions) and dimeric (d-BthA-I) (Magro *et al*., 2004). Subsequently, high resolution structures of m-BthA-I have been described, both in the *apo* form and in the presence of Ca^{2+} ions (Murakami et *al*., 2006). Interesting, for all these BthA-I structures (five monomers in the total), the Ca^{2+}-binding loops have approximately the same conformation. The Na^+ and Ca^{2+} ions or a water molecule, for native protein, bond in the same position in the Ca^{2+}-binding loop for all structures. Consequently, we can conclude that Ca^{2+}-binding loop does not suffer substantial conformational alterations for the Asp49-PLA_2s solved in the presence of Ca^{2+} when compared to native ones.

BthTX-II was crystallized in the presence of Na^+ ions. These ions are bound to Phe19 in both monomers, in contrast with native monomeric BthA-I structure whose ions are bound to the Ca^{2+} binding loop. Similarly, other Asp49-PLA_2s which are able to bind

Ca^{2+} ions are also able to bind Na^{+} ions at approximately the same position in the Ca^{2+}-binding loop (Singh *et al*., 2001).

Magro *et al*. (2005) solved the structure of BthA-I chemically modified with BPB (*p*-bromophenacyl bromide) and showed important tertiary and quaternary structural changes in this enzyme. The comparison between the BPB inhibited and native BthA-I leads to the observation of three main regions with significant structural differences: the Ca^{2+}-binding loop, the C-terminal, and the β-wing. The presence of the inhibitor in the active site displaces the Ca^{2+} binding loop which interacts with C-terminal region, also displacing it. Residues Gly30, Leu31, and Leu32 are displaced by BPB group, yielding a different conformation in the Ca^{2+} binding loop (Magro *et al*., 2005). Additionally, it has been suggested that divalent cations like Ca^{2+} protect PLA_2 against inactivation by BPB and that Ca^{2+} does not bind to the inactivated PLA_2 (Volwerk *et al*., 1974). Similarly, acidic PLA_2 from *A. h.* Pallas in the presence of Ca^{2+} prevents BPB modification (Zhang *et al.*, 1994). This fact is probable due to the absence of a water molecule that coordinates the Ca^{2+} ion generated by distorted geometry of Ca^{2+} binding loop (Renetseder *et al.*, 1988; Zhao *et al.*, 1998). These examples of PLA_2/ligands complexes shows that distorted Ca^{2+} binding loops cannot bind bivalent ions.

Then, these two examples above denotes that PLA_2s, which binds Ca^{2+} ions, do not show significant changes in the Ca^{2+}-binding loops, in contrast, PLA_2s with distorted Ca^{2+}-binding loops cannot bind Ca^{2+} ions, consequently we hypothesize BthTX-II cannot bind Ca^{2+} and its low catalytic function is based in a alternative way compared with other Asp49-PLA_2s.

In order to test this hypothesis, we crystallized BthTX-II in the same conditions, however we add $CaCl_2$. An X-ray data collection has been performed and processed at 2,35 Å resolution. The initial refinement of the structure did not show the presence of this ion in the calcium binding loop, The position of residues of this region are in the same position as in the native protein.

In conclusion, we showed BthTX-II is not able to bind Ca^{2+}, consequently, we suggest that its low catalytic function is based in an alternative way compared with other phospholipases. Further studies are in progress in order to establish the details of catalytic and myotoxic activities of this class of protein. These studies include co-crystallization with inhibitors, functional studies and site-directed mutagenesis.

5. References

Andrião-Escarso, S. H., Soares, A. M., Fontes, M. R. M., Fuly, A. L., Corrêa, F. M. A., Rosa, J. C., Greene, L. J. & Giglio, J. R. (2002). Biochem. Pharmacol. **64**, 723–732.

Angulo, Y., Guitiérrez, J. M., Soares, A. M., Cho, W., Lomonte, B. (2005). Toxicon, **46**, 291-296.

Arni, R. K., Fontes, M. R. M., Barberato, C., Gutiérrez, J. M., Díaz-Oreiro, C. & Ward, R. J. (1999). Arch. Biochem. Biophys. **366**, 177–182.

Arni, R. K, Ward, R. J. (1996). Toxicon, **34**, 827-841.

Arni, R. K., Ward, R. J. & Gutiérrez, J. M. (1995). Acta Cryst. D**51**, 311–317.

Brunger, A. T., Adams, P. D., Clore, G. M., DeLano, W. Gros, L. P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T. & Warren, G. L. (1998) Acta Crystallogr. D**54**, 905–921.

Brunger, A. T. (1992). *X-PLOR Version 3.1: A System for Crystallography and NMR*, Yale University Press, New Haven.

Chen,Y.C., Maraganore,J.M., Reardon,I. and Heinrikson,R.L. (1987). Toxicon, **25**, 401-409.

Cintra, A. C., Marangoni, S., Oliveira, B. and Giglio, J. R. (1993). J. Protein Chem. **12**, 57-64.

Corrêa, L.C., Marchi-Salvador, D.P., Cintra, A.C.O., Soares, A.M. & Fontes, M.R.M. (2006). Acta Cryst. F**62**, 765-767.

Deenen, L. L. M. van & de Haas, G. H. (1963). Biochem. Biophys. Acta, **70**, 538–553.

Daniele, J. J., Bianco, I. D. & Fidelio, G. D. (1995). Arch. Biochem. Biophys. **318**, 65–70.

da Silva-Giotto, M. T., Garratt, R. C.,Oliva,G., Mascarenhas, Y. P., Giglio, J. R., Cintra, A. C. O., de Azevedo, W. F. Jr, Arni, R. K. & Ward, R. J. (1998). Proteins: Structure, Function, and Genetics, **30**, 442–454.

Dekker, N. (2000). Mol. Microbiol. **35**, 711– 717.

DeLano, W. L. (2002). Proteins, **30**, 442–454.

de Oliveira, A. H., Giglio, J. R., Andrião-Escarso, S. H., Ito, A. S., Ward, R. J. (2001). Biochemistry. **40**, 6912–6920.

Fuly, A. L., Soares, A. M., Marcussi, S., Giglio, J. R. & Guimarães, J. A. (2004). Biochimie, **86**, 731–739.

Gui,L., Niu,X., Bi,R., Lin,Z. & Chen,Y. (1992) Chin.Sci.Bull. **37**, 1394.

Gutiérrez, J. M. & Lomonte, B. (1997). *Venom Phospholipase A_2 Enzymes: Structure, Function and Mechanism*, edited by R. M. Kini, pp. 321–352. Chichester: Wiley & Sons.

Gutiérrez, J. M., Nunez, J.,Diaz, C., Cintra, A. C.O.,Homsi-Brandeburgo,M. I. & Giglio, J. R. (1991). Exp. Mol. Pathol. **55**, 217–229.

Higgins D., Thompson J., Gibson T.Thompson J.D., Higgins D.G., Gibson T.J. (1994). Nucleic Acids Res. **22**, 4673-4680.

Homsi-Brandeburgo, M. I., Queiroz, L. S., Santo-Neto, H., Rodrigues-Simioni, L. & Giglio, J. R. (1988). Toxicon, **26**, 615–627.

Jancarik, J. & Kim, S.-H. (1991). J. Appl. Cryst. **24**, 409–411.

Jones, T. A., Bergdoll, M. & Kjeldgaard, M. *O: a macromolecule modeling environment*, in: C.E. Bugg, S.E. Ealick (Eds.), *Crystallographic and Modeling Methods in Molecular Design*, Springer-Verlag, New York, 1990, pp. 189–195.

Kashima, S., Roberto, P. G., Soares, A. M, Astolfi-Filho, S., Pereira, J.O., Giuliati, S., Faria, M. Jr., Xavier, M. A., Fontes, M. R., Giglio, J. R. & Franca, S. C. (2004). Biochimie. **86**, 211-219.

Kini, R. M. & Evans, H. J. (1989). Toxicon, **27**, 613–635.

Laskowski, R. A., MacArthur, M. W., Moss, D. S. & Thornton, J. M. (1993) J. Appl. Crystallogr. **26**, 283–291.

Lee, W. H., da Silva-Giotto,M. T., Marangoni, S., Toyama, M. H., Polikarpov, I. & Garratt, R. C. (2001). Biochemistry, **40**, 28–36.

MacPherson, A. (1982). *Preparation and Analysis of Protein Crystals*. New York: Wiley.

Magro, A. J., Murakami, M. T., Marcussi, S., Soares, A. M., Arni, R. K. & Fontes, M. R. M. (2004). Biochem. Biophys. Res. Commun. **323**, 24–31.

Magro, A. J., Soares, A. M., Giglio, J. R. & Fontes, M. R. M. (2003). Biochem. Biophys. Res. Commun. **311**, 713–720.

Magro, A. J., Takeda, A. A. S., Soares, A. M. & Fontes, M. R. M. (2005a). Acta Cryst. D **61**, 1670–1677.

Magro, A. J., Takeda, A. A. S., Santos, J. I., Marcussi, S., Soares, A. M., Fontes, M.R.M. (2005b). The FEBS Journal, **272**, 13.

Marchi-Salvador, D. P., Fernandes, C. A. H., Amui, S. F., Soares, A. M. & Fontes, M. R. M. (2006). Acta Cryst. F**62**, 600–603.

Marchi-Salvador, D. P., Silveira, L. B., Soares, A.M. & Fontes,M. R.M. (2005). Acta Cryst. F**61**, 882–884.

Murakami, M. T., Arruda, E. Z., Melo, P A., Martinez, A. B., Eliás, S. C. ,Tomaz, M. A., Lomonte, B, Gutiérrez, J. M., & Arni, R. K. (2005). J. Mol. Biol. **350**, 416–426.

Murakami, M.T., Gabdoulkhakov, A., Genov, N., Cintra, A.C., Betzel, C., Arni, R.K. (2006). Biochimie, **88**, 543-549.

Navaza., J. (1994). Acta Crystallogr. A **50**, 157–163.

Needleman, P., Turk, J., Jakschik, B. A., Morrison, A. R. & Lefkowith, J. B. (1986). Annu. Rev. Biochem. **55**, 69–102.

Otwinowski, Z. & Minor, W. (1997). Methods Enzymol. **276**, 307–326.

Ownby, C. L. (1998). J. Toxicol. Toxin Rev. **17**, 1003–1009.

Pan, Y. H., Epstein, T. M., Jain, M. K., Bahnson, B. J. (2001). Biochemistry. **40**, 609–617.

Pereira, M. F., Novello, J. C., Cintra, A. C. O., Giglio, J. R., Landucci, E. T., Oliveira, B. & Marangoni, S. (1998). J. Protein Chem. **17**, 381–386.

Renetseder, R., Brunie, S., Dijkstra, B. W., Drenth, J., Sigler, P. B. (1985). J. Biol. Chem. **260**, 11627–11636.

Rigden, D. J., Hwa, L. W., Marangoni, S., Toyama, M. H. & Polikarpov, I. (2003). Acta Cryst. D**59**, 255–262.

Roberto, P. G., Kashima, S., Marcussi, S., Pereira, J. O., Astolfi-Filho, S., Nomizo, A., Giglio, J. R., Fontes, M. R. M., Soares, A. M. & França, S. C. (2004). Protein J. **23**, 273–285.

Ruller, R., Aragão, E. A, Chioato, L., Ferreira, T. L, Oliveira, A. H.C. , Sá, J. M, Ward, R. J, (2005). *Biochimie*. **87**, 993-1003.

Sanchez, S. A., Chen, Y., Muller, J. D., Gratton, E., Hazlett, T. L. (2001). Biochemistry, **40**, 6903–6911.

Scott, D. L., Achari, A., Vidal, J. C. & Sigler, P. B. (1992). J. Biol. Chem. **267**, 22645–22657.

Scott, D. L. & Sigler, P. B. (1994). Adv. Protein Chem. **45**, 53, 88.

Serrano, S. M. T., Reichl, A. P., Mentele, R., Auerswald, E. A., Santoro, M. L., Sampaio, C. A. M., Camargo, A. C. M. & Assakura, M. T. (1999). Arch. Biochem. Biophys. **367**, 26–32.

Singh, G., Gourinath, S., Sharma, S., Paramasivam, M., Srinivasan, A., Singh, T.P. (2001). . J.Mol.Biol. **307**, 1049-1059.

Snijder, H. J., Ubarretxena-Belandia, I., Blaauw, M., Kalk, K. H., Verheij, H. M., Egmond, M. R., Dekker, N., Dijkstra, B. W. (1999). Nature, **401**, 717–721.

Soares, A. M., Andrião-Escarso, S.H.,Bortoleto, R.K.,Rodrigues-Simioni, L., Arni, R.K., Ward, R.J., Gutiérrez, J.M., Giglio, J.R. (2001). Arch. of Biochem. Biophys. **387**, 188-196.

Soares, A. M., Fontes, M. R. M. & Giglio, J. R. (2004). Curr. Org. Chem. **8**, 1677–1690.

Soares, A. M. & Giglio, J. R. (2003). Toxicon, **42**, 855–868.

Soares, A. M., Guerra-Sá, R., Borja-Oliveira, C. R., Rodrigues, V. M., Rodrigues-Simioni, L., Rodrigues, V., Fontes, M. R. M., Lomonte, B., Gutiérrez , J. M., Giglio J.R. (2000). Archives of Biochemistry and Biophysics, **378**, 201-209

Snijder, H. J., Ubarretxena-Belandia, I., Blaauw, M., Kalk, K. H., Verheij, H. M., Egmond, M. R., Dekker, N., Dijkstra, B. W. (1999). Nature, **401**, 717–721.

Toyama, M.H., Soares, A.M., Wen-Hwa, L., Polikarpov, I., Giglio, J.R., Marangoni, S. (2000). Biochimie, **82**, 245-250.

Volwerk, J. J., Pieterson, W. A. & de Haas, G. H. (1974). B iochemistry, **13**, 1446, 1454.

Wallace A C, Laskowski R A & Thornton J M (1995). Prot. Eng. **8**, 127-134.

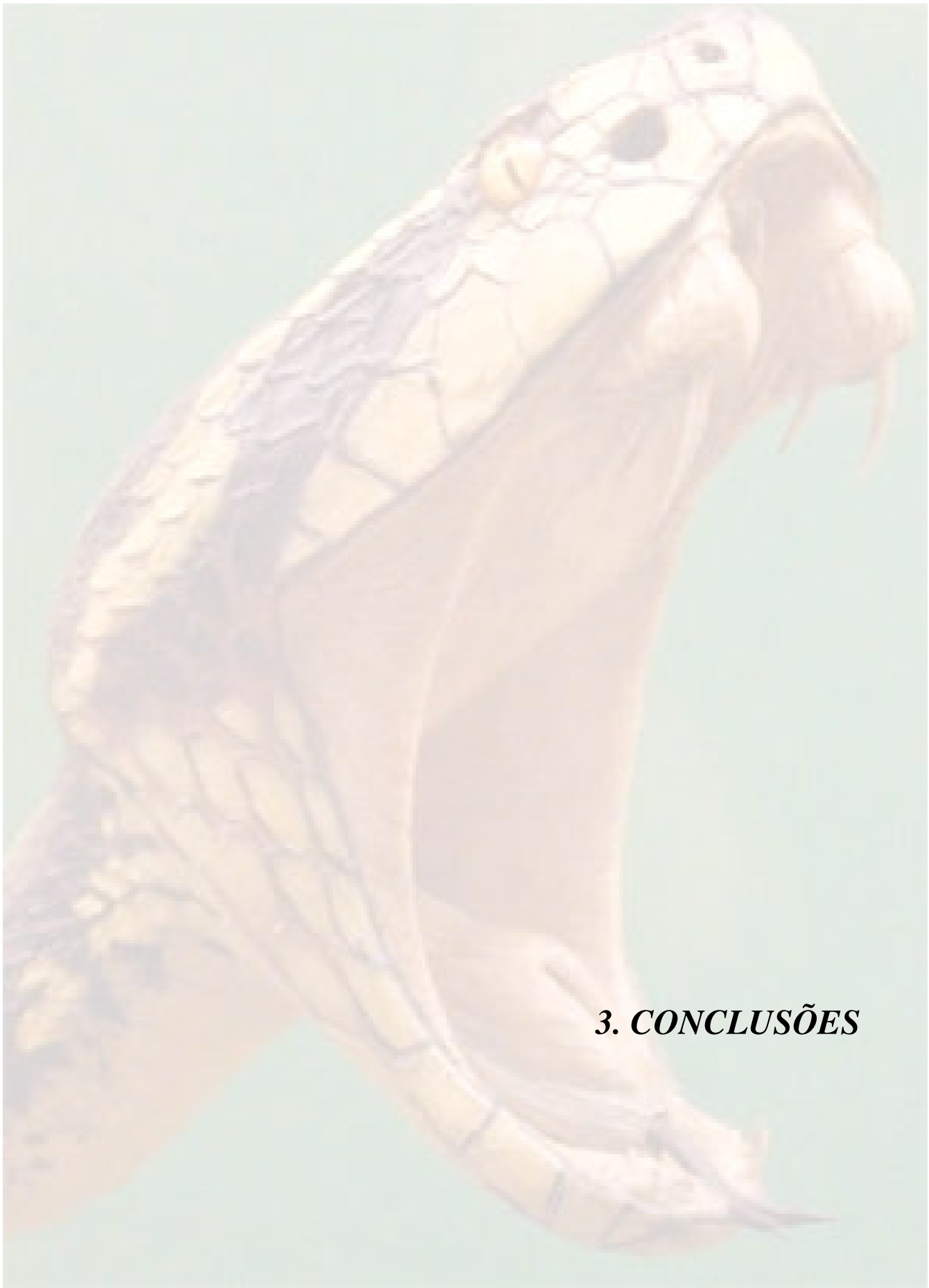
Wang, X.Q., Yang, J., Gui, L.L., Lin, Z.J., Chen, Y.C., Zhou, Y.C. (1996). J.Mol.Biol. **255**, 669-676.

Watanabe, L., Soares, A. M., Ward, R. J., Fontes, M. R. M. & Arni, R. K. (2005). Biochimie, **87**, 161–167.

Zhang, D. L., Zheng, L., Lin, N. Q., Ruan, K. C., Zhou, Y. C. (1994). Chinese Biochemical Journal, **10**, 330-334.

Zhao, K., Song, S., Lin,Z. & Zhou, Y. (1997). Shengwu Huaxue Zazhi. **13**, 765.

Zhao, K., Song, S., Lin, Z., Zhou, Y. (1998). Acta Crystallogr. D**54**, 510-521.



3. CONCLUSÕES

O entendimento dos sistemas biológicos ao nível molecular tem apresentado avanços importantes, em grande parte, devido aos conhecimentos gerais e detalhados de estruturas tridimensionais obtidos com o avanço da área cristalográfica, a ponto de. Companhias químicas e farmacêuticas se tornarem mais ativas nesta área estrutural devido a interesses no desenvolvimento de novos produtos industriais e no desenho racional de novas drogas (Drenth, 1994, Kubinyi, 1998 e Souza *et al*., 2000).

Nos estudos de cristalização de BthTX-II, foi necessário um mês para serem encontrados os primeiros cristais, os quais foram levados para a coleta de dados de difração de raios-X, no entanto, só após mais três meses, foram obtidos monocristais com qualidade suficiente para a coleta dos dados. Nesse período, foram realizados inúmeros testes com variações das concentrações dos componentes do sistema, bem como na temperatura de incubação e nos métodos utilizados *(sitting-drop e hanging-drop).* Após a coleta do conjunto de dados de difração de raios-X, outros problemas foram encontrados. Com a resolução da estrutura cristalina, passou-se ao refinamento da estrutura tridimensional e para isso, foi utilizada a seqüência de aminoácidos de BthTX-II com 120 resíduos de aminoácidos, proposta por Pereira *et al.* (1998). Após vários ciclos de refinamento sem muito êxito, passou-se a utilizar o sequenciamento realizado por Kashima *et al.* (2004), que compreende 122 resíduos, uma vez que não se tinha conhecimento deste, até então. Uma varredura foi realizada e constatou-se que espaços no mapa de densidade eletrônica, até então não preenchidos pela primeira seqüência, foram ocupados satisfatoriamente pela segunda, uma vez que esta favorecia a existência de mais dois resíduos, além do fato de que cadeias laterais mal preenchidas pelos resíduos até então utilizados, passaram a ser bem ocupadas após a modificação pelos propostos na segunda seqüência. Após essa modificação, foi possível dar seguimento ao refinamento até a conclusão da estrutura tridimensional de BthTX-II.

A determinação da estrutura tridimensional de uma proteína é apenas um passo na elucidação dos mecanismos envolvidos nas relações estrutura/função de uma proteína. BthTX-II é uma Asp49-PLA_2 que no entanto, apresenta baixa atividade catalítica, diferente do observado em BthA-I, extraída do veneno da mesma espécie. Assim, um estudo comparativo com outras fosfolipases A_2, tanto Asp49 como Lys49, foi fundamental para aumentar a compreensão das bases estruturais dessa variação na atividade catalítica, levando-nos a propor que a posição significativamente deslocada da região do cálcio binding loop em BthTX-II impede a coordenação do íon cálcio, sendo responsável pela baixa atividade catalítica dessa enzima. Uma nova fase de estudos, já em andamento, que

tem por objetivo, confirmar essa proposta, envolve a cristalização de BthTX-II na presença de íons cálcio, bem como a resolução de sua estrutura cristalina. Estudos preliminares com um conjunto de dados de difração de raios-X de cristais obtidos nessa condição dão fortes indícios da inexistência do íon Ca^{2+} na região do cálcio *binding loop*, no entanto, é necessária a obtenção de um conjunto de dados com melhor estatística, a fim de se concluir essa análise. Esse estudo deverá ser concluído nos próximos meses, como parte do projeto de doutorado. A partir destes dados, possivelmente poderemos propor um mecanismo catalítico independente de cálcio para fosfolipases A_2, porposta que seria inédita e impensável até o momento. Para este estudo, serão necessários possíveis estudos de complexos desta proteína com inibidores, bem como outros estudos bioquímicos, biofísicos e de mutação sítio dirigida.

4. REFERÊNCIAS BIBLIOGRÁFICAS

ANDRIÃO-ESCARSO, S.H. *et al*. Myotoxic phospholipases A_2 in *Bothrops* snake venoms: effects of chemical modifications on the enzymatic and pharmacological properties of Bothropstoxins from *Bothrops jararacussu*. **Biochimie**, v.82, p.755-763, 2000.

ANDRIÃO-ESCARSO, S.H. *et al*. Structural and functional characterization of an acidic platelet aggregation inhibitor and hypotensive phospholipase A_2 from *Bothrops jararacussu* snake venom. **Biochem. Pharmacol**, v.64, p.723-732, 2002.

ARNI, R.K. *et al*. Structure of a calcium-independent phospholipase-like myotoxic protein from *Bothrops asper*. **Acta Crystallogr. D**, v.51, p.311-317, 1995.

ARNI, R.K.; WARD, R.J. Phospholipase A_2 - a structural review. **Toxicon,** v.34, n.8, p.827-841, 1996.

ARNI, R.K. *et al*. Crystal structure of myotoxin II, a monomeric Lys49-phospholipase homologue isolated from the venom of *Cerrophidion* (*Bothrops*) *godmani*. **Arch. Biochim. Biophys.**, v.366, p.177-182, 1999.

de AZEVEDO JR. *et al*. Crystal structure of myotoxin-II: a myotoxic phospholipase A_2 homologue from *Bothrops moojeni* venom. **Protein. Pept. Lett.**, v.4. p.329-334, 1997.

de AZEVEDO JR. *et al*. Structure of a Lys49-phospholipase A_2 homologue isolated from the venom of *Bothrops nummifer* (Jumping Viper). **Toxicon**, v.37, p.371-384, 1999.

BONFIM, V. L. *et al*. Isolation and enzymatic characterization of a basic phospholipase A_2 from *Bothrops jararacussu* venom**. Journal of Protein Chemistry**, v. 20, p. 239-245, 2001.

BRAUD, S.; BON, C.; WISNER, A. Snake venoms proteins acting on homeostasis. **Biochimie**, v.82, p.851-859, 2000.

CAMPBELL, J.A.; LAMAR, W.W. **The venomous reptiles of Latin America.** New York: Cornell University, 1989. 425p.

CHANDRA, V. *et al*. First structural evidence of a specific inhibition of phospholipase A_2 by α–tocopherol (vitamin E) and its implications in inflammation: crystal structure of the complex formed between phospholipase A_2 and α–tocopherol at 1.8Å resolution. **J. Mol. Biol.,** v.320, p.215-222, 2002.

CHIOATO, L. *et al.* Distinct sites for myotoxic and membrane-damaging activities in the C-terminal region of a Lys49-phospholipase A_2. **Biochem. J.**, v.366, p. 971-976, 2002.

CINTRA, A. C. O. *et al*. Bothropstoxin-I: Amino Acid Sequence and Function**. Journal of Protein Chemistry**, v. 12, p. 57-64, 1993.

CONDREA, E.; YANG, C. C.; ROSENBERG, P. Lack of correlation between the anticoagulant activity and phospholipase hydrolysis by snake venom phospholipases A_2. **Thromb. Hemostasis**, v. 45, p. 82-89, 1981.

DELANO, W.S. **The PyMOL molecular graphics system**. San Carlos: Delano Scientific, 2002. Disponível em <http://www.pymol.org>. Acesso em: 26/01/2006.

DENNIS, E.A. Phospholipases A_2. **The enzymes**, v.16, p.307-353, 1983.

DENNIS, E.A. Diversity of group types, regulation and function of phospholipase A_2. **J. Biol. Chem.**, v.269, p.13057-13060, 1994.

DÍAZ, C. *et al*. The effect of myotoxins isolated from *Bothrops* snake venoms on multilamellar liposomes: relationship to phospholipase A_2, anticoagulant and myotoxic Activities. **Biochim. Biophys. Acta**, v.1070, p.455-460, 1991.

DÍAZ, C.; GUTIÉRREZ, J.M.; LOMONTE, B. Isolation and characterization of basic myotoxic phospholipase A_2 from *Bothrops godmani* (Godman's Pit Viper) snake venom. **Arch. Biochem. Biophys.**, v.298, p.135-142, 1992.

DRENTH, J. **Principles of protein X-ray crystallography.** New York: Springer-Verlag, 1994. 311p.

DUFTON, M.J.; HIDER, R.C. Classification of phospholipases A_2 according to sequence evolutionary and pharmacological implications **Eur. J. Biochem**.,v. 137, p. 545-551, 1983.

EBADI, M.; SRINIVANASAN, S. K.; BAXI, M. D. Oxidative stress and antioxidant therapy in Parkinson's disease. **Prog. Neurobiol**., v. 48, P. 1–19, 1996.

EDMAN, P.; BEGG, G. A protein sequenator. **Eur. J. Biochem**, v.1, p. 80-91, 1967.

EVANS, H.J.; KINI, R.M. Effects of phospholipase A_2 enzymes on platelet aggregation. In: KINI, R.M. (Ed.). **Venom phospholipase A_2 enzymes:** structure, function and mechanism. Chichester: Wiley, 1997. p.1-28.

FERREIRA, M.L. *et al*. Toxic activities of venoms from nine *Bothrops* species and their correlation with lethality and necrosis. **Toxicon,** v.30, p.1603-1608,1992.

FRANCIS, B.; GUTIERREZ, J.M.; LOMONTE, B.; KAISER, I.I. Myotoxin II from *Bothrops asper* (Terciopelo) venom in a lysine-49 phospholipase A_2. **Arch. Biochem. Biophys**., v.284, p.352-359, 1991.

GERRARD, J. M.; *et al.* Increased phosphatidic acid and decreased lysophosphatidic acid in response to thrombin is associated with inhibition of platelet aggregation**. Biochem. cell. Biol**., v. 71, p. 432-439, 1993.

GUTITRREZ, J. M.; ARROYO, O.; BOLANOS, R. Mionecrosis, hemorragia y edema inducidos por el veneno de ***Bothrops asper*** en raton blanco. **Toxicon**, v. 18, p. 603-610, 1980.

GUTIÉRREZ, J.M.; LOMONTE, B. Phospholipase A_2 myotoxins from *Bothrops* snake venoms. **Toxicon**, v.33, p.1405-1424, 1995.

GUTIÉRREZ, J.M.; LOMONTE, B. Phospholipase A_2 myotoxins from *Bothrops* snake venoms. In: KINI, R.M. (Ed). **Venom phospholipase A_2 enzymes:** structure, function and mechanism. Chichester: Wiley, 1997. p.321-352.

HEINRIKSON, R.L.; KRUEGER, E.T.; KEIM, P.S. Amino acid sequence of phospholipase A_2-alpha from the venom of Crotalus adamanteus. A new classification of phospholipases A_2 based upon structural determinants. **J. Biol. Chem**., v. 252, p. 4913-4921, 1977.

HOLLAND, D.R. *et al*. The crystal structure of a lysine 49 phospholipase A_2 from the venom of the cottonmouth snake at 2.0Å. **J. Biol. Chem.**, v.266, p.17649-17656, 1990.

HOMSI-BRANDEBURGO, M.I. *et al*. Fractionation of *Bothrops jararacussu* snake venom: partial chemical characterization and biological activity of bothropstoxin. **Toxicon**, v.26, p.615-627, 1988.

KASHIMA, S. *et al*. Analysis of *Bothrops jararacussu* venomous gland transcriptome focusing on structural and functional aspects: I-gene expression profile of highly expressed phospholipases A_2**. Biochimie**, v. 86, p. 211-219, 2004.

KINI, R.M.; EVANS, H.J. A model to explain the pharmacological effects of snake venom phospholipase A_2. **Toxicon**, v.27, p.613-635, 1989.

KINI, R.M. Phospholipase A_2: a complex multifunctional protein puzzle. In: KINI, R.M. (Ed.). **Venom phospholipase A_2 enzymes**: structure, function and mechanism. Chichester: Wiley, 1997. p.1-28.

KOBE, B. *et al*. Crystallization of protein kinases and phosphatases. In: HARDIE D.G. (Ed). **Protein phosphorylation:** a practical approach. 2nd Ed. Oxford: Oxford University Press. 1999. p.127- 151.

KUBINYI, H. Structure-based design of enzyme inhibitors and receptor ligands. **Curr. Opin. Drug Discov. Devel.,** v.1, p.4-15, 1998.

LEE, W.H. *et al*. Structural basis for low catalytic activity in Lys49-phospholipase A_2 – a hypothesis: the crystal structure of Piratoxin II complexed to fatty acid. **Biochemistry**, v.40, p.28-36, 2001.

LEHNINGER, A.L.; NELSON, D.L.; COX, M.M. **Principles of biochemistry.** 2nd Ed. New York: Worth Publishers, 1993. 725p.

LEONARDO, S. D. Serpentário. 2006. Disponível em: <http://univap.br/cen/serpentario/fotos/crotalidae_bothrops/04.htm> Acesso em 20 fev. 2007.

LLORET, S.; MORENO, J. J. Oedema formation and degranulation of mast cells by phospholipase A_2 purified from porcine pancreas and snake venoms. **Toxicon**, v. 31, 8, p. 949-956, 1993.

LOMONTE, B.; CARMONA, E. Individual Expression Patterns of Myotoxin Isoforms in the Venom of the Snake *Bothrops asper*. **Comp. Biochem. Physiol. B**, v.102, p.325-329, 1992.

LOMONTE, B.; TARKOWSKI, A.; HANSON, L. A. Broad cytolytic specificity of myotoxin II, a lysine 49 phospholipase A_2 of *Bothrops asper* snake venom. **Toxicon**, v. 32, p. 1359-1369, 1994a.

LOMONTE, B. *et al*. Neutralizing interaction between heparin and myotoxin II, a Lys49 phospholipase A_2 from *Bothrops asper* snake venom. Identification of a heparin-binding and cytolitic toxin region by the use of synthetic peptides and molecular modeling. **J. Biol. Chem.**, v.269, p.29867-29873, 1994b.

MAGRO, A.J. **Determinação estrutural das fosfolipases homólogas BnSP-7 e BnSP-6:** considerações a respeito de suas estruturas quaternárias e mecanismos de inibição. 2003.

49p. Dissertação (Mestrado) – Instituto de Biociências, Universidade Estadual de São Paulo, Botucatu, 2003.

MAGRO, A.J. *et al.* Crystal structure of an acidic platelet aggregation inhibitor and hypotensive phospholipase A_2 in the monomeric and dimeric states: insights into its oligomeric state. **Biochem. Biophys. Res. Commun.,** v.323, p.24-31, 2004.

McREE, D.E. **Practical protein crystallography.** San Diego: Academic Press, 1993. 477p.

MELGAREJO, A. R. SERPENTES PEÇONHENTAS: PRINCIPAIS GRUPOS, IDENTIFICAÇÃO, VENENO, ACIDENTES E PRIMEIROS SOCORROS. Disponível em<http://www.ivb.rj.gov.br/palestras/roteiro.doc-> Acesso em 20 fev. 2007

MONTEIRO, R.Q *et al*. *Bothrops jararaca* snakes produce several bothrojaracin isoforms following an individual pattern. **Comp. Biochem Physiol.**, v.120, n.4, p.791-798, 1998.

MOOLENAAR, W.H.; et al. **Cell Biol**., v. 9, p. 168-173 1997.

MOREIRA, L.A. *et al.* Bee venom phospholipase inhibits malaria parasite development in transgenic mosquitoes. **J. Biol. Chem.,** v.277, n.43, p.40839-40843, 2002.

MUKHERJEE, A.B.; MIELE, L.; PATTABIRAMAN, N. Phospholipase A_2 enzymes: regulation and physiological role. **Biochem. Pharmacol.**, v.48, p.1-10, 1994.

MURAKAMI *et al*. Insights into metal ion binding in phospholipase A_2: ultra high-resolution crystal structures of an acidic phospholipase A_2 in the Ca^{2+} free and bound states. **Biochimie**. v. 88, p. 543-549, 2006.

OWNBY, C.L.H.; SELISTRE-DE-ARAUJO, S.; WHITE, S.P.; FLECTHER, J.E. Lysine 49 phospholipase A_2 proteins. **Toxicon,** v.37, p.411-445, 1999.

PENTLAND, A.P. *et al*. Tocopherol analogs supress arachidonic acid metabolism via phospholipase inhibition. **J. Biol. Chem.**, v.267, p.15578-15584, 1992.

PEREIRA, M. F. *et al*. The amino acid sequence of bothropstoxin-II, an Asp49 myotoxin from *Bothros jararacussu* (jararacussu) venom with low phospholipase A_2 activity. **J. Protein Chem**. v. 17, p. 381-386, 1998.

RENETSEDER, R.; BRUNIE, S.; DIJKSTRA, B.W.; DRENTH, J.; SIGLER, P.B. A comparison of the crystal structures of phospholipases A_2 from bovine pancreas and *Crotalus atrox* venom. **J. Biol. Chem.**, v.260, p.11627-11636, 1985.

RIBEIRO, L.A. *et al*. Acidente ofídico no estado de São Paulo. **Rev. Assoc. Med. Bras.**, v.39, p.4-7, 1993.

RODRIGUES, V.M. *et al*. Geographic variations in the composition of myotoxins from *Bothrops neuwiedi* snake venoms. **Comp. Biochem. Physiol. A**, v.121, p.215-222, 1998.

ROSENBERG, P. Phospholipases. In: SHIER, W.T.; MEBS, D. (Eds.). **Handbook of toxinology**. New York: Marcel Dekker, 1990. p.67-277.

ROSENFELD, G. Symptomathology, pathology and treatment of snake bites in South America. In: BÜCHERL, W.; BUCKLEY, E.; DEULOFEU, V. (Eds). **Venomous animals and their venoms.** New York: Academic Press, 1971. v.2, p.345-384.

RUFINI, S. *et al*. Calcium ion independent membrane leakage induced by phospholipase-like myotoxins. **Biochemistry**, v.31, p.12424-12430, 1992.

SANO, M., *et al*. A controlled trial of selegiline, alpha-tocopherol, or both as treatment for Alzheimer's disease. The Alzheimer's Disease Cooperative Study. **N. Engl. J. Med**. 336, 1216–1222,1997.

SCHALOSKE, R. H.; DENNIS, E. A. The phospholipase A_2 superfamily and its group numbering system. **Biochimica et Biophysica Acta**, v. 1761, p. 1246- 1259, 2006.

SCOTT, D.L. *et al*. Interfacial catalysis the mechanism of phospholipase A_2. **Science**, v.250, p.1541-1546, 1990.

da SILVA-GIOTTO, M.T. *et al*. Crystallographic and spectroscopic characterization of a molecular hinge: conformational changes in Bothropstoxin I, a dimeric Lys49-phospholipase A_2 homologue. **Proteins**, v.30, p.442-454, 1998.

SIX, D.A.; DENNIS, E.A. The expanding superfamily of phospholipase A_2 enzymes: classification and characterization. **Biochem. Biophys. Acta**, v.1488, p.1-19, 2000.

SOARES, A.M. *et al*. Polyacrylamide gel electrophoresis as a tool for the taxonomic identification of snakes from the Elapidae and Viperidae families. **J. Venom. Anim. Toxins,** v.4, p.137-142, 1998.

SOARES, A.M. *et al*. Structural and functional characterization of Miotoxin I, a Lys49 phospholipase A_2 homologue from *Bothrops moojeni* (Caissaca) snake venom. **Arch. Biochem. Biophys.**, v.373, n.1, p. 7-15, 2000a.

SOARES, A.M. *et al*. Structural and functional characterization of BnSP-7, a Lys49 myotoxic phospholipase A_2 homologue from *Bothrops neuwiedi pauloensis* venom. **Arch. Biochem. Biophys.**, v.378, n.2, p. 201-209, 2000b.

SOARES, A.M.; *et al.* Effects of chemical modifications of crotoxin B, the phospholipase A_2 subunit of crotoxin from *Crotalus durissus terrificus* snake venom, on its enzymatic and pharmacological activities**. Int. J. Biochem. Cell Biol**., v. 33, p. 877–888, 2001a.

SOARES, A.M.; *et al*. Dissociation of enzymatic and pharmacological properties of piratoxins-I and -III, two myotoxic phospholipases A_2 from Bothrops pirajai snake venom. **Arch. Biochem. Biophys**., v. 387, p. 188–196, 2001b.

SOARES, A. M.; GIGLIO, J. R. Chemical modifications of phospholipases A_2 from snake venoms: effects on catalytic and pharmacological properties. **Toxicon**, v. 42, p. 855–868, 2003.

SOARES, A.M.; FONTES, M.R.M.; GIGLIO, J. R. Phospholipase A_2 Myotoxins from *Bothrops* Snake Venoms: Structure-Function Relationship. **Curr. Org. Chem.**, v.8, n.17, p.1677-1690, 2004.

SOUZA, D.H.F.; SELISTRE-DE-ARAUJO, H.S.; GARRATT, R.C. Determination of the tree-dimensional structure of toxins by protein crystallography. **Toxicon**, v.38, p.1307-1353, 2000.

STEPHENS, W.W.; WALKER, J.L.; MYERS, W. **J. Pathol. Bacteriol**., v. 5, p. 279-301, 1898.

TAKEDA, A. A. S. *et al*. Crystallization and preliminary X-ray diffraction analysis of an acidic phospholipase A2 complexed with p-bromophenacyl bromide and α-tocopherol inhibitors at 1.9- and 1.45-Å resolution. **Biochimica et Biophysica Acta**, v. 1699, p. 281–284, 2004

TRABER, M.G.; PACKER, L. Vitamin E: beyond antioxidant function. **Am. J. Clin. Nutr.**, v.62, p.1501-9, 1995.

UHL,W.; NEVALAINEN,T.J.; BUCHLER, M.W. **Phospholipase A_2 basic and clinical aspects in inflammatory diseases.** Karger: Switzerland, 1997. v.24, 250p.

VALENTIN, E.; LAMBEAU, G. What can venom phospholipase A_2 tell us about the functional diversity of mammalian secreted phospholipase A_2. **Biochimie**, v.82, p.815-831, 2000.

VALIENTE, C. *et al*. An electrophoretic study on phospholipase A_2 isoenzymes in the venoms of Central American Crotalinae snakes. **Toxicon**, v.30, p.815-823, 1992.

WAITE, M. The phospholipases. In: WAITE, M (Ed) **Handbook of lipid research**. New York: Plenum Press, 1987. p.155-241.

WARD, R. J. *et al*. Sequence of a cDNA encoding bothropstoxin I, a myotoxin from the venom of ***Bothrops jararacussu*. Gene**, v. 156, p. 305-306, 1995**.**

WARD, R.J.; de AZEVEDO JR., W.F.; ARNI R.K. At the interface: crystal structures of phospholipases A_2. **Toxicon**, v.36, p.1623-1633, 1998.

WARD, R.J. *et al*. Active-site mutagenesis of a Lys49-phospholipase A_2: biological and membrane-disrupting activities in the ausence of catalysis. **Biochem. J.,** v.362, p. 89-96, 2002.

YANG, C.C.; HUANG, C.S.; LEE, H.J. Studies on the status of tyrosyl residues in phospholipases A_2 from Naja naja atra and Naja nigricollis snake venoms. **J. Protein Chem.** v. 4, p. 87–94. 1985.

ZHAO, H.; TANG, L.; WANG, X.; ZHOU, Y.; LIN, Z. Structure of a snake venom phospholipase A_2 modified by *p*-bromo-phenacyl-bromide. **Toxicon**, v.36, n.6 p.875-886, 1998.

APÊNDICE

Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

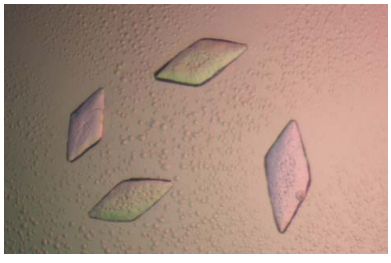
Preliminary X-ray crystallographic studies of BthTX-II, a myotoxic Asp49-phospholipase A_2 with low catalytic activity from *Bothrops jararacussu* venom

L. C. Corrêa,[a] D. P. Marchi-Salvador,[a] A. C. O. Cintra,[b] A. M. Soares[b] and M. R. M. Fontes[a]*

[a]Departamento de Física e Biofísica, Instituto de Biociências, UNESP, CP 510, CEP 18618-000, Botucatu-SP, Brazil, and [b]Departamento de Análises Clínicas, Toxicológicas e Bromatológicas, FCFRP, USP, Ribeirão Preto/SP, Brazil

Correspondence e-mail: fontes@ibb.unesp.br

Received 17 May 2006
Accepted 29 June 2006



© 2006 International Union of Crystallography
All rights reserved

For the first time, a complete X-ray diffraction data set has been collected from a myotoxic Asp49-phospholipase A_2 (Asp49-PLA_2) with low catalytic activity (BthTX-II from *Bothrops jararacussu* venom) and a molecular-replacement solution has been obtained with a dimer in the asymmetric unit. The quaternary structure of BthTX-II resembles the myotoxin Asp49-PLA_2 PrTX-III (piratoxin III from *B. pirajai* venom) and all non-catalytic and myotoxic dimeric Lys49-PLA_2s. In contrast, the oligomeric structure of BthTX-II is different from the highly catalytic and non-myotoxic BthA-I (acidic PLA_2 from *B. jararacussu*). Thus, comparison between these structures should add insight into the catalytic and myotoxic activities of bothropic PLA_2s.

1. Introduction

Phospholipases A_2 (PLA_2s; EC 3.1.1.4) belong to a superfamily of proteins which hydrolyze the *sn*-2 acyl groups of membrane phospholipids to release fatty acids, arachidonic acid and lysophospholipids (van Deenen & de Haas, 1963). The coordination of the Ca^{2+} ion in the PLA_2 calcium-binding loop includes an Asp at position 49 which plays a crucial role in the stabilization of the tetrahedral transition-state intermediate in catalytically active PLA_2s (Scott *et al.*, 1992). In the genus *Bothrops*, PLA_2s are the main components of the venoms produced by species classified into this animal group. In addition to their primary catalytic role, snake-venom PLA_2s show other important toxic/pharmacological effects, including myonecrosis, neurotoxicity, cardiotoxicity and haemolytic, haemorrhagic, hypotensive, anticoagulant, platelet-aggregation inhibition and oedema-inducing activities (Gutiérrez & Lomonte, 1997; Ownby, 1998; Andrião-Escarso *et al.*, 2002). Some of these activities are correlated with the enzymatic activity, but others are completely independent (Kini & Evans, 1989; Soares & Giglio, 2004). It has been suggested that some specific sites of these molecules have biochemical properties that are responsible for the pharmacological and toxic actions, including the anticoagulant and platelet-inhibition activities (Kini & Evans, 1989). PLA_2s are also one of the enzymes involved in the production of eicosanoids. These molecules have physiological effects at very low concentrations; however, increases in their concentration can lead to inflammation (Needleman *et al.*, 1986). Thus, the study of specific PLA_2 inhibitors is important in the production of structure-based anti-inflammatory agents.

Many non-catalytic homologous PLA_2s (Lys49-PLA_2s) have been purified from *Bothrops* snake venoms and have been structurally and functionally characterized (Marchi-Salvador *et al.*, 2005, 2006; Watanabe *et al.*, 2005; Soares *et al.*, 2004; Magro *et al.*, 2003; Lee *et al.*, 2001; Arni *et al.*, 1995, 1999; da Silva-Giotto *et al.*, 1998). However, little is known about the bothropic catalytic PLA_2s (Asp49-PLA_2s; Magro *et al.*, 2004, 2005; Rigden *et al.*, 2003; Serrano *et al.*, 1999; Pereira *et al.*, 1998; Daniele *et al.*, 1995; Homsi-Brandeburgo *et al.*, 1988).

Despite the structures of a large number of PLA_2s having been solved by crystallography to date, many questions still need to be clarified. For example, there are PLA_2s with high, moderate and no catalytic activity (Magro *et al.*, 2004; Rigden *et al.*, 2003; da Silva-Giotto *et al.*, 1998). However, for all these 'classes' of PLA_2s the

majority of residues of the catalytic machinery are conserved. Similarly, toxic (*e.g.* myotoxity, cytotoxity) and pharmacological effects (*e.g.* anticoagulant, hypotensive and platelet-aggregation activities) are far from being completely understood.

An acidic catalytic PLA_2 (BthA-I) has been isolated from *B. jararacussu* venom and characterized (Andrião-Escarso *et al.*, 2002; Roberto *et al.*, 2004). BthA-I is three to four times more catalytically active than BthTX-II (bothropstoxin-II from *B. jararacussu*) and other basic Asp49-PLA_2s from *Bothrops* venoms, but is not myotoxic, cytotoxic or lethal (Andrião-Escarso *et al.*, 2002). Other activities demonstrated by this enzyme are time-independent oedema induction, hypotensive response in rats and platelet-aggregation inhibition (Andrião-Escarso *et al.*, 2002). The crystal structure of BthA-I has been recently described in two conformational states: monomeric and dimeric (Magro *et al.*, 2004). Additionally, Magro *et al.* (2005) solved the structure of BthA-I chemically modified with BPB (*p*-bromophenacyl bromide) and showed important tertiary and quaternary structural changes in this enzyme. This novel oligomeric structure is more energetically and conformationally stable than the native structure and the abolition of pharmacological activities (including anticoagulant, hypotensive effect and platelet-aggregation inhibition) by the ligand may be related to the oligomeric structural changes.

The isolation, biochemical/pharmacological characterization and amino-acid sequence of bothropstoxin II from *B. jararacussu* (BthTX-II) have been reported (Homsi-Brandeburgo *et al.*, 1988; Gutiérrez *et al.*, 1991; Pereira *et al.*, 1998). Protein sequencing indicated that BthTX-II is an Asp49-PLA_2 and consists of 120 amino acids (MW = 13 976 Da). The protein shows myotoxic, oedematogenic and haemolytic effects and low phospholipase activity (Homsi-Brandeburgo *et al.*, 1988; Gutiérrez *et al.*, 1991). Recently, it has been shown that BthTX-II induces platelet aggregation and secretion through multiple signal transduction pathways (Fuly *et al.*, 2004). Despite BthTX-II having been crystallized more than ten years ago (Bortoleto *et al.*, 1996), the structure has not been solved to date, probably owing to the low completeness of the data set (50–60% completeness). The crystals belonged to the tetragonal crystal system and preliminary analysis indicated the presence of three molecules in the asymmetric unit (Bortoleto *et al.*, 1996). However, a careful analysis of the Matthews coefficient indicated that a tetrameric conformation is also possible (V_M = 2.4 Å^3 Da^{-1}), which also occurs in the Lys49-PLA_2 M_jTX-I (myotoxin I from *B. moojeni* venom) structure formed of two Lys49-PLA_2 dimers (Marchi-Salvador *et al.*, 2005; personal communication).

In the present paper, we describe the crystallization of BthTX-II (bothropstoxin-II) from *B. jararacussu* venom in the monoclinic system, the collection of a complete X-ray diffraction data set and molecular-replacement solution. This study should improve the understanding of the relation of the myotoxic and low catalytic activity mechanisms to the structural features of this protein when compared with BthTX-I (Lys49-PLA_2 from *B. jararacussu* venom) and BthA-I, which possess no and high catalytic activity, respectively.

2. Experimental procedures

2.1. Purification

BthTX-II was isolated from *B. jararacussu* snake venom by gel-filtration and ion-exchange chromatography as previously described (Homsi-Brandeburgo *et al.*, 1988).

2.2. Crystallization

A lyophilized sample of BthTX-II was dissolved in ultrapure water at a concentration of 12 mg ml^{-1}. The sparse-matrix method (Jancarik & Kim, 1991) was used to perform initial screening of the crystallization conditions (Crystal Screens I and II; Hampton Research). Large crystals of BthTX-II were obtained by the conventional hanging-drop vapour-diffusion method (MacPherson, 1982), in which 1 µl protein solution and 1 µl reservoir solution were mixed and equilibrated against 500 µl of the same precipitant solution. The BthTX-II was crystallized using a solution containing 20%(*v*/*v*) 2-propanol, 13%(*w*/*v*) polyethylene glycol 4000 and 0.1 *M* sodium citrate pH 5.6. The best crystals measured approximately 0.4 × 0.2 × 0.1 mm after two months at 291 K (Fig. 1).

2.3. X-ray data collection and processing

X-ray diffraction data from BthTX-II crystals were collected using a wavelength of 1.427 Å at a synchrotron-radiation source (Laboratório Nacional de Luz Síncrotron, LNLS, Campinas, Brazil) with a MAR CCD imaging-plate detector (MAR Research). A crystal was mounted in a nylon loop and flash-cooled in a stream of nitrogen at 100 K using no cryoprotectant. The crystal-to-detector distance was 100 mm and an oscillation range of 1° was used; 149 images were collected. The data were processed to 2.13 Å resolution using the *HKL* program package (Otwinowski & Minor, 1997).

3. Results and discussion

The data-collection statistics are shown in Table 1. The data set is 96.1% complete at 2.13 Å resolution, with R_{merge} = 9.1%. The crystals belong to space group *C*2, with unit-cell parameters *a* = 58.9, *b* = 98.5, *c* = 46.7 Å, β = 125.9°.

Packing parameter calculations based on the protein molecular weight indicate the presence of a dimer in the asymmetric unit. This corresponds to a Matthews coefficient (Matthews, 1968) of 2.0 Å^3 Da^{-1} with a calculated solvent content of 37.4%, which are within the expected range for typical protein crystals (assuming a value of 0.74 cm^3 g^{-1} for the protein partial specific volume).

The crystal structure was determined by molecular-replacement techniques implemented in the program *AMoRe* (Navaza, 1994) using the coordinates of a monomer of PrTX-III (PDB code 1gmz). The quaternary structure of BthTX-II is very similar to those of PrTX-III and all dimeric bothropic Lys49-PLA_2s (Rigden *et al.*, 2003; Soares *et al.*, 2004) and totally different from those of native dimeric

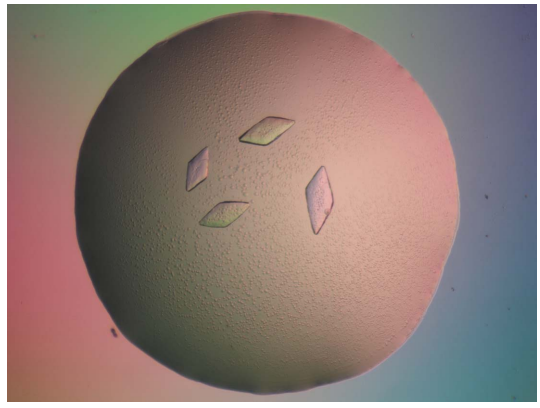


Figure 1
Crystals of BthTX-II from *B. jararacussu* venom

Table 1
X-ray diffraction data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

Unit-cell parameters (Å, °)	a = 58.9, b = 98.5, c = 46.7, β = 125.9
Space group	$C2$
Resolution (Å)	40–2.13 (2.21–2.13)
Unique reflections	11560 (1127)
$R_{\rm merge}$† (%)	9.1 (26.4)
Completeness (%)	96.1 (94.2)
Radiation source	Synchrotron (LNLS-MX1)
Data-collection temperature (K)	100
$\sigma(I)$ cutoff for data processing‡	−3
Average $I/\sigma(I)$	10.6 (3.7)
Redundancy	3.0 (2.9)
Matthews coefficient $V_{\rm M}$ (Å^3 Da^{-1})	2.0
Molecules in the ASU	2
Solvent content (%)	37.4

† $R_{\rm merge} = \sum_{hkl}[\sum_i(|I_{hkl,i} - \langle I_{hkl}\rangle|)]/\sum_{hkl,i}\langle I_{hkl}\rangle$, where $I_{hkl,i}$ is the intensity of an individual measurement of the reflection with Miller indices hkl and $\langle I_{hkl}\rangle$ is the mean intensity of that reflection. Calculated for $I > -3\sigma(I)$. ‡ Data processing used the *HKL* suite (Otwinowski & Minor, 1997).

BthA-I and BthA-I–bromophenacyl bromide and BthA-I–α-tocopherol complexes (Magro *et al.*, 2004, 2005; Takeda *et al.*, 2004).

In conclusion, a complete X-ray diffraction data set has been collected from a low catalytic activity Asp49-PLA$_2$ for the first time (to 2.13 Å) and a molecular-replacement structure solution has been obtained. The quaternary structure of BthTX-II resembles those of the myotoxin PrTX-III (which does not bind Ca^{2+} ions) and all non-catalytic and myotoxic dimeric Lys49-PLA$_2$s (Rigden *et al.*, 2003; Soares *et al.*, 2004). In contrast, the oligomeric structure of BthTX-II is different from that of the high catalytic activity and non-myotoxic BthA-I (Magro *et al.*, 2004). Thus, comparison between these structures should add insight into the catalytic and myotoxic activities of bothropic PLA$_2$s.

The authors gratefully acknowledge financial support from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação para o Desenvolvimento da UNESP (FUNDUNESP) and Laboratório Nacional de Luz Síncrontron (LNLS, Campinas-SP).

References

Andrião-Escarso, S. H., Soares, A. M., Fontes, M. R. M., Fuly, A. L., Corrêa, F. M. A., Rosa, J. C., Greene, L. J. & Giglio, J. R. (2002). *Biochem. Pharmacol.* **64**, 723–732.
Arni, R. K., Fontes, M. R. M., Barberato, C., Gutiérrez, J. M., Díaz-Oreiro, C. & Ward, R. J. (1999). *Arch. Biochem. Biophys.* **366**, 177–182.
Arni, R. K., Ward, R. J. & Gutiérrez, J. M. (1995). *Acta Cryst.* D**51**, 311–317.
Bortoleto, R. K., Ward, R. J., Giglio, J. R., Cintra, A. C. O. & Arni, R. K. (1996). *Toxicon*, **34**, 614–617.
Deenen, L. L. M. van & de Haas, G. H. (1963). *Biochem. Biophys. Acta*, **70**, 538–553.
Daniele, J. J., Bianco, I. D. & Fidelio, G. D. (1995). *Arch. Biochem. Biophys.* **318**, 65–70.
da Silva-Giotto, M. T., Garratt, R. C., Oliva, G., Mascarenhas, Y. P., Giglio, J. R., Cintra, A. C. O., de Azevedo, W. F. Jr, Arni, R. K. & Ward, R. J. (1998). *Proteins*, **30**, 442–454.
Fuly, A. L., Soares, A. M., Marcussi, S., Giglio, J. R. & Guimarães, J. A. (2004). *Biochimie*, **86**, 731–739.
Gutiérrez, J. M. & Lomonte, B. (1997). *Venom Phospholipase A_2 Enzymes: Structure, Function and Mechanism*, edited by R. M. Kini, pp. 321–352. Chichester: Wiley & Sons.
Gutiérrez, J. M., Nunez, J., Diaz, C., Cintra, A. C. O., Homsi-Brandeburgo, M. I. & Giglio, J. R. (1991). *Exp. Mol. Pathol.* **55**, 217–229.
Homsi-Brandeburgo, M. I., Queiroz, L. S., Santo-Neto, H., Rodrigues-Simioni, L. & Giglio, J. R. (1988). *Toxicon*, **26**, 615–627.
Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.
Kini, R. M. & Evans, H. J. (1989). *Toxicon*, **27**, 613–635.
Lee, W. H., da Silva-Giotto, M. T., Marangoni, S., Toyama, M. H., Polikarpov, I. & Garratt, R. C. (2001). *Biochemistry*, **40**, 28–36.
MacPherson, A. (1982). *Preparation and Analysis of Protein Crystals.* New York: Wiley.
Magro, A. J., Soares, A. M., Giglio, J. R. & Fontes, M. R. M. (2003). *Biochem. Biophys. Res. Commun.* **311**, 713–720.
Magro, A. J., Murakami, M. T., Marcussi, S., Soares, A. M., Arni, R. K. & Fontes, M. R. M. (2004). *Biochem. Biophys. Res. Commun.* **323**, 24–31.
Magro, A. J., Takeda, A. A. S., Soares, A. M. & Fontes, M. R. M. (2005). *Acta Cryst.* D**61**, 1670–1677.
Marchi-Salvador, D. P., Fernandes, C. A. H., Amui, S. F., Soares, A. M. & Fontes, M. R. M. (2006). *Acta Cryst.* F**62**, 600–603.
Marchi-Salvador, D. P., Silveira, L. B., Soares, A. M. & Fontes, M. R. M. (2005). *Acta Cryst.* F**61**, 882–884.
Matthews, B. W. (1968) *J. Mol. Biol.* **33**, 491–497.
Navaza, J. (1994). *Acta Cryst.* A**50**, 157–163.
Needleman, P., Turk, J., Jakschik, B. A., Morrison, A. R. & Lefkowith, J. B. (1986). *Annu. Rev. Biochem.* **55**, 69–102.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Ownby, C. L. (1998). *J. Toxicol. Toxin Rev.* **17**, 1003–1009.
Pereira, M. F., Novello, J. C., Cintra, A. C. O., Giglio, J. R., Landucci, E. T., Oliveira, B. & Marangoni, S. (1998). *J. Protein Chem.* **17**, 381–386.
Rigden, D. J., Hwa, L. W., Marangoni, S., Toyama, M. H. & Polikarpov, I. (2003). *Acta Cryst.* D**59**, 255–262.
Roberto, P. G., Kashima, S., Marcussi, S., Pereira, J. O., Astolfi-Filho, S., Nomizo, A., Giglio, J. R., Fontes, M. R. M., Soares, A. M. & França, S. C. (2004). *Protein J.* **23**, 273–285.
Scott, D. L., Achari, A., Vidal, J. C. & Sigler, P. B. (1992). *J. Biol. Chem.* **267**, 22645–22657.
Serrano, S. M. T., Reichl, A. P., Mentele, R., Auerswald, E. A., Santoro, M. L., Sampaio, C. A. M., Camargo, A. C. M. & Assakura, M. T. (1999). *Arch. Biochem. Biophys.* **367**, 26–32.
Soares, A. M., Fontes, M. R. M. & Giglio, J. R. (2004). *Curr. Org. Chem.* **8**, 1677–1690.
Soares, A. M. & Giglio, J. R. (2004). *Toxicon*, **42**, 855–868.
Takeda, A. A. S., dos Santos, J. I., Marcussi, S., Silveira, L. B., Soares, A. M. & Fontes, M. R. M. (2004). *Biochim. Biophys. Acta*, **1699**, 281–284.
Watanabe, L., Soares, A. M., Ward, R. J., Fontes, M. R. M. & Arni, R. K. (2005). *Biochimie*, **87**, 161–167.