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

Crystallization and preliminary X-ray diffraction analysis of three myotoxic phospholipases A_2 from *Bothrops brazili* venom

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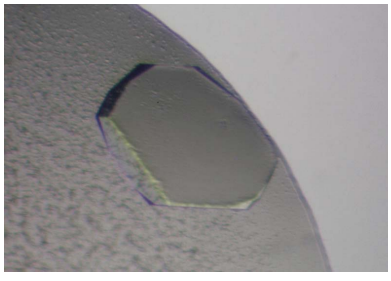
Received 28 November 2011
Accepted 8 June 2012



Two myotoxic and noncatalytic Lys49-phospholipases A_2 (braziliantoxin-II and MT-II) and a myotoxic and catalytic phospholipase A_2 (braziliantoxin-III) from the venom of the Amazonian snake *Bothrops brazili* were crystallized. The crystals diffracted to resolutions in the range 2.56–2.05 Å and belonged to space groups $P3_121$ (braziliantoxin-II), $P6_522$ (braziliantoxin-III) and $P2_1$ (MT-II). The structures were solved by molecular-replacement techniques. Both of the Lys49-phospholipases A_2 (braziliantoxin-II and MT-II) contained a dimer in the asymmetric unit, while the Asp49-phospholipase A_2 braziliantoxin-III contained a monomer in its asymmetric unit. Analysis of the quaternary assemblies of the braziliantoxin-II and MT-II structures using the *PISA* program indicated that both models have a dimeric conformation in solution. The same analysis of the braziliantoxin-III structure indicated that this protein does not dimerize in solution and probably acts as a monomer *in vivo*, similar to other snake-venom Asp49-phospholipases A_2.

1. Introduction

Envenomation resulting from snakebites is an important public health problem in rural areas of Asia, Africa and Latin America. A recent study estimated that at least 421 000 envenomations and 20 000 deaths from ophidian accidents occur each year (Kasturiratne *et al.*, 2008). However, owing to scarce statistical data on this topic, these numbers may be as high as 1 841 000 envenomations and 94 000 deaths (Kasturiratne *et al.*, 2008). The mortality caused by snake bites is much greater than those caused by several neglected tropical diseases, including dengue haemorrhagic fever, cholera, leishmaniasis, schistosomiasis and Chagas disease (Williams *et al.*, 2010). Only in recent years has this subject attracted massive attention from the scientific community, with the publication of important articles and reviews on the real impact of snakebites on health services (Gutiérrez *et al.*, 2006; Kasturiratne *et al.*, 2008; Williams *et al.*, 2010). The World Health Organization (WHO) has recognized snakebites as a neglected tropical disease and the International Society on Toxinology has created The Global Snakebite Initiative (Williams *et al.*, 2010). Both initiatives aim to reduce snakebite morbidity and mortality through a programme of sustainable approaches and outcome-oriented strategies. Although the majority of deaths owing to snakebite envenomation occur in south and south-east Asia and in sub-Saharan Africa (Kasturiratne *et al.*, 2008), these accidents are also an important health problem in Latin America (Gutiérrez & Lomonte, 1995). They may result in drastic tissue damage and permanent disability (Gutiérrez & Lomonte, 1995), leading to economic and social problems. Snakes of the *Bothrops* genus (Viperidae family) are responsible for more than 85% of all reported ophidian accidents in Latin America (Fundação Nacional de Saúde, 2001; de Oliveira, 2009), and phospholipases A_2 (PLA_2s) are one of the main components of their venoms (Fox & Serrano, 2008). These enzymes promote Ca^{2+}-dependent hydrolysis of the *sn*-2 acyl groups of membrane phospholipids, releasing fatty acids and lysophospholipids (Schaloske & Dennis, 2006). In addition to their catalytic activity, these proteins are involved in a wide spectrum of pharmacological activities, including neurotoxicity, myotoxicity and cardiotoxicity (Bon *et al.*, 1979; Fletcher *et al.*, 1981; Gutiérrez *et al.*, 1991).

The catalytic site of these proteins is formed by a histidine at position 48 (according to the numbering system proposed by Renetseder *et al.*, 1985) and the conserved residues Asp49, Tyr52 and Asp99. Two catalytic mechanisms of phospholipid hydrolysis by PLA_2s have been proposed: the single-water mechanism (Scott, Otwinowski *et al.*, 1990; Scott, White *et al.*, 1990) and the assisting-water mechanism (Rogers *et al.*, 1996; Yu *et al.*, 1998). Briefly, the single-water mechanism proposes that after phospholipid binding His48 $N^{\delta1}$ abstracts a proton from a structurally conserved water, initiating nucleophilic attack on the *sn*-2 position of the substrate and forming an intermediate tetrahedral oxyanion which is stabilized by the Ca^{2+} cofactor (Scott, Otwinowski *et al.*, 1990; Scott, White *et al.*, 1990). The assisting-water mechanism suggests that two different water molecules are involved in the formation and breakdown of the tetrahedral intermediate. In this model, a water molecule coordinated by the Ca^{2+} ion performs the nucleophilic attack. This catalytic water is also stabilized by a second water molecule that is hydrogen bonded to His48 and Asp99 (Rogers *et al.*, 1996; Yu *et al.*, 1998).

In snakes of the Viperidae family, an important subgroup of PLA_2s, the Lys49-PLA_2s, are found that exhibit natural replacements of the Tyr28 and Asp49 residues by Asn28 and Lys49, respectively (Holland *et al.*, 1990; Fernandes *et al.*, 2010). These substitutions hinder binding of the Ca^{2+} ion, the essential cofactor for PLA_2 catalysis, resulting in an inability to promote phospholipid hydrolysis (Arni & Ward, 1996; Fernandes *et al.*, 2010). Despite their catalytic inactivity, Lys49-PLA_2s play an important role in ophidic accidents, inducing drastic local myonecrosis by a Ca^{2+}-independent mechanism which is not efficiently neutralized by conventional serum therapy, the action of which is related to systemic mechanisms (Gutiérrez & Lomonte, 1995). Synthetic peptides and site-directed mutagenesis experiments have shown that the 115–129 segment of the C-terminal region is responsible for this myotoxic activity (Ward *et al.*, 2002; Lomonte *et al.*, 2003; Chioato *et al.*, 2007). Recently, a myotoxic site in Lys49-PLA_2s that is specific to venoms from snakes of the *Bothrops* genus has been proposed that contains three residues: Lys115, Arg118 (C-terminus) and Lys20 (N-terminus) (dos Santos, Soares *et al.*, 2009).

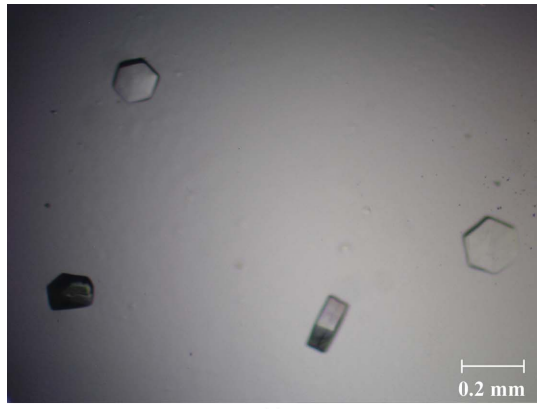
B. brazili is a snake that lives in the Amazonian region, being found in Brazil, Colombia, Ecuador, Guyana, Peru, Suriname and French Guiana (Campbell & Lamar, 2004). Access to antivenoms in the remote areas of this region is very limited owing to natural geographic barriers and the vast territory. Moreover, the commercial therapeutic anti-bothropic serum produced by the Butantan Institute (Brazil) has a low efficacy against Amazonian snakes (Muniz *et al.*, 2000). Despite the large amount of protein structural data available for snakes of the *Bothrops* genus (Magro *et al.*, 2004; Murakami *et al.*, 2007; dos Santos, Fernandes *et al.*, 2009; Fernandes *et al.*, 2010; dos Santos *et al.*, 2011), no structural studies related to Amazonian snakes have been reported to date.

In this work, we report the crystallization, X-ray diffraction data collection and molecular-replacement solution of three myotoxic phospholipases A_2 (the Asp49-PLA_2 braziliantoxin-III and the Lys49-PLA_2s braziliatoxin-II and MT-II) from the venom of the Amazonian snake *B. brazili*.

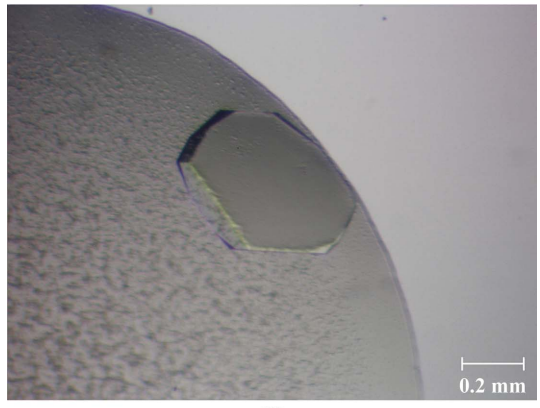
2. Materials and methods

2.1. Protein purification and crystallization

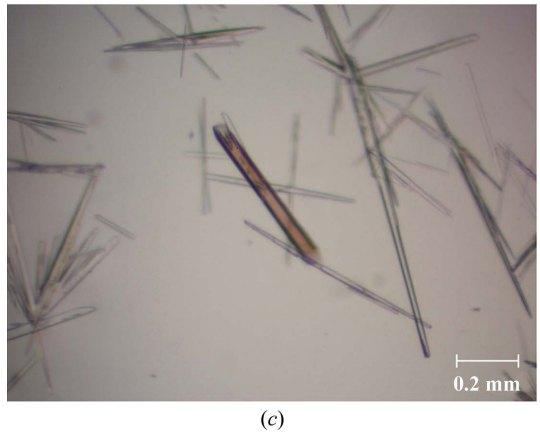
Braziliantoxin II (BbTX-II) and braziliantoxin-III (BbTX-III) were isolated from *B. brazili* venom by single-step reverse-phase HPLC as described previously (Huancahuire-Vega *et al.*, 2009). MT-II was obtained by the fractionation of *B. brazili* venom on a CM-Sepharose column (2 × 20 cm) as described previously (Costa *et al.*, 2008). Lyophilized samples of BbTX-III and MT-II were dissolved in ultrapure water to a concentration of 12 mg ml^{-1}. A lyophilized sample of BbTX-II was dissolved in 300 m*M* phosphate buffer to the same concentration. The sparse-matrix method (Jancarik & Kim, 1991) was used to perform initial screening of the crystallization conditions (Crystal Screen, Hampton Research). All crystals were obtained using the conventional hanging-drop vapour-diffusion method (McPherson, 2003), in which 1 µl protein solution and 1 µl reservoir solution were mixed and equilibrated against the following reservoir solutions (500 µl): 30%(*w*/*v*) polyethylene glycol 4000, 0.25 *M* lithium sulfate, 0.1 *M* Tris–HCl pH 8.5 for BbTX-II, 2%(*w*/*v*) polyethylene glycol 400, 2.0 *M* ammonium sulfate, 0.1 *M* Na HEPES



(*a*)



(*b*)



(*c*)

Figure 1
Crystals of the three myotoxic phospholipases A_2 from *B. brazili* venom. (*a*) Braziliantoxin-II, (*b*) braziliantoxin-III, (*c*) MT-II.

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

Values in parentheses are for the highest resolution shell.

	BbTX-II	BbTX-III	MT-II
Unit-cell parameters (Å, °)	$a = b = 56.43$, $c = 129.08$	$a = b = 70.818$, $c = 105.83$	$a = 39.01$, $b = 71.41$, $c = 44.42$, $\beta = 102.5$
Space group	$P3_121$	$P6_522$	$P2_1$
Resolution (Å)	40–2.11 (2.19–2.11)	50–2.70 (2.80–2.70)	20–2.08 (2.15–2.08)
Unique reflections	13825 (1381)	4562 (443)	13821 (1396)
Completeness (%)	95.9 (98.7)	97.2 (99.1)	96.4 (97.5)
R_{merge}† (%)	6.3 (49.0)	13.4 (48.4)	12.9 (35.3)
Radiation source	MX1 station, LNLS		
Data-collection temperature (K)	100		
Average $I/\sigma(I)$	21.09 (2.95)	11.05 (3.48)	6.63 (2.05)
Multiplicity	4.9 (4.8)	7.5 (8.9)	2.8 (2.8)
Matthews coefficient V_M (Å^3 Da^{-1})	2.12	2.74	2.16
Molecules in the asymmetric unit	2	1	2
Solvent content (%)	41.99	55.07	43.02

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ 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 were processed using the *HKL* suite (Otwinowski & Minor, 1997).

pH 7.5 for BbTX-III and 30%(*w*/*v*) polyethylene glycol 8000, 0.25 *M* ammonium sulfate, 0.1 *M* sodium cacodylate pH 6.5 for MT-II. Crystals were grown at 291 K for approximately three weeks for MT-II and BbTX-II and for three months for BbTX-III (Fig. 1).

2.2. X-ray data collection and processing

X-ray diffraction data from all crystals were collected at a wavelength of 1.435 Å using a synchrotron-radiation source (MX1 station, Laboratório Nacional de Luz Síncrotron, LNLS, Campinas, Brazil) and a MAR CCD imaging-plate detector (MAR Research). The crystals were mounted in a nylon loop and flash-cooled in a stream of nitrogen gas at 100 K without using a cryoprotectant. The data were processed using the *HKL* program package (Otwinowski & Minor, 1997).

3. Results and discussion

Data-collection statistics are shown in Table 1. The crystals diffracted to resolutions in the range 2.56–2.05 Å and belonged to space groups $P3_121$ (BbTX-II), $P6_522$ (BbTX-III) and $P2_1$ (MT-II). The crystal structures were determined by molecular-replacement techniques as implemented in the program *MOLREP* (Vagin & Teplyakov, 2010) using the coordinates of piratoxin-I (PrTX-I; PDB entry 2q2j; dos Santos, Soares *et al.*, 2009) from *B. pirajai* venom for BbTX-II, of acid phospholipase A_2 (BthA-I; PDB entry 1zlb; Murakami *et al.*, 2006) from *B. jararacussu* venom for BbTX-III and of bothropstoxin-I (BthTX-I) from *B. jararacussu* venom complexed with polyethylene glycol 4000 (PDB entry 3iq3; Fernandes *et al.*, 2010) for MT-II.

Calculations based on the protein molecular weight indicated the presence of one molecule of BbTX-III and two molecules of BbTX-II and MT-II in the asymmetric unit, corresponding to Matthews coefficients (V_M; Matthews, 1968) of 2.12 Å^3 Da^{-1} for BbTX-II, 2.74 Å^3 Da^{-1} for BbTX-III and 2.16 Å^3 Da^{-1} for MT-II. These values are within the range for typical protein crystals, assuming a value of 0.74 cm^3 g^{-1} for the protein partial specific volume. Analysis of the quaternary assemblies of the BbTX-II and MT-II crystallographic models using the *PISA* program (Krissinel & Henrick, 2007) showed a complexation significance score of 1.0, indicating that both models present a dimeric conformation in solution. The same analysis of the BbTX-III structure indicated that this protein does not dimerize in solution and probably acts as a monomer *in vivo*, similar to other snake-venom Asp49-PLA_2s (Carredano *et al.*, 1998; Xu *et al.*, 2003; Murakami *et al.*, 2006).

In conclusion, BbTX-II, BbTX-III and MT-II from *B. brazili* were crystallized and X-ray diffraction data were collected. The structures of the Lys49-PLA_2s BbTX-II and MT-II showed a dimeric conformation, while the Asp49-PLA_2 BbTX-III presented a monomeric conformation. Elucidation of the native structures and of structures of possible complexes with different ligands may be useful for the development of effective inhibitors that can be used as supplemental treatments to serum therapy and as important models for synthesis of new drugs.

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) and CAPES (Coordenadação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil). We acknowledge the use of the Laboratório Nacional de Luz Síncrotron (LNLS, Brazil).

References

Arni, R. K. & Ward, R. J. (1996). *Toxicon*, **34**, 827–841.
Bon, C., Changeux, J.-P., Jeng, T.-W. & Fraenkel-Conrat, H. (1979). *Eur. J. Biochem.* **99**, 471–481.
Campbell, J. & Lamar, W. (2004). *The Venomous Reptiles of the Western Hemisphere*. Ithaca: Cornell University Press.
Carredano, E., Westerlund, B., Persson, B., Saarinen, M., Ramaswamy, S., Eaker, D. & Eklund, H. (1998). *Toxicon*, **36**, 75–92.
Chioato, L., Aragão, E. A., Lopes Ferreira, T., Medeiros, A. I., Faccioli, L. H. & Ward, R. J. (2007). *Biochim. Biophys. Acta*, **1768**, 1247–1257.
Costa, T. R., Menaldo, D. L., Oliveira, C. Z., Santos-Filho, N. A., Teixeira, S. S., Nomizo, A., Fuly, A. L., Monteiro, M. C., de Souza, B. M., Palma, M. S., Stábeli, R. G., Sampaio, S. V. & Soares, A. M. (2008). *Peptides*, **29**, 1645–1656.
Fernandes, C. A., Marchi-Salvador, D. P., Salvador, G. M., Silva, M. C., Costa, T. R., Soares, A. M. & Fontes, M. R. (2010). *J. Struct. Biol.* **171**, 31–43.
Fletcher, J. E., Rapuano, B. E., Condrea, E., Yang, C. C. & Rosenberg, P. (1981). *Toxicol. Appl. Pharmacol.* **59**, 375–388.
Fox, J. W. & Serrano, S. M. (2008). *Proteomics*, **8**, 909–920.
Fundação Nacional de Saúde (2001). *Manual de Diagnóstico e Tratamento de Acidentes por Animais Peçonhentos*. Brasília: Ministério da Saúde.
Gutiérrez, J. M. & Lomonte, B. (1995). *Toxicon*, **33**, 1405–1424.
Gutiérrez, J. M., Núñez, J., Díaz, C., Cintra, A. C., Homsi-Brandeburgo, M. I. & Giglio, J. R. (1991). *Exp. Mol. Pathol.* **55**, 217–229.
Gutiérrez, J. M., Theakston, R. D. & Warrell, D. A. (2006). *PLoS Med.* **3**, e150.
Holland, D. R., Clancy, L. L., Muchmore, S. W., Ryde, T. J., Einspahr, H. M., Finzel, B. C., Heinrikson, R. L. & Watenpaugh, K. D. (1990). *J. Biol. Chem.* **265**, 17649–17656.
Huancahuire-Vega, S., Ponce-Soto, L. A., Martins-de-Souza, D. & Marangoni, S. (2009). *Toxicon*, **54**, 818–827.
Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.
Kasturiratne, A., Wickremasinghe, A. R., de Silva, N., Gunawardena, N. K., Pathmeswaran, A., Premaratna, R., Savioli, L., Lalloo, D. G. & de Silva, H. J. (2008). *PLoS Med.* **5**, e218.
Krissinel, E. & Henrick, K. (2007). *J. Mol. Biol.* **372**, 774–797.
Lomonte, B., Angulo, Y. & Santamaría, C. (2003). *Toxicon*, **42**, 307–312.
Magro, A. J., Murakami, M. T., Marcussi, S., Soares, A. M., Arni, R. K. & Fontes, M. R. (2004). *Biochem. Biophys. Res. Commun.* **323**, 24–31.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
McPherson, A. (2003). *Introduction to Macromolecular Crystallography*. Hoboken: Wiley.
Muniz, E. G., Maria, W. S., Estevão-Costa, M. I., Buhrnheim, P. & Chávez-Olórtegui, C. (2000). *Toxicon*, **38**, 1859–1863.
Murakami, M. T., Gabdoulkhakov, A., Genov, N., Cintra, A. C., Betzel, C. & Arni, R. K. (2006). *Biochimie*, **88**, 543–549.
Murakami, M. T., Viçoti, M. M., Abrego, J. R., Lourenzoni, M. R., Cintra, A. C., Arruda, E. Z., Tomaz, M. A., Melo, P. A. & Arni, R. K. (2007). *Toxicon*, **49**, 378–387.

Oliveira, R. C. W. de (2009). *Animais Peçonhentos do Brasil: Biologia, Clínica e Terapêutica dos Envenenamentos*. São Paulo: Sarvier.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Renetseder, R., Brunie, S., Dijkstra, B. W., Drenth, J. & Sigler, P. B. (1985). *J. Biol. Chem.* **260**, 11627–11634.
Rogers, J., Yu, B.-Z., Serves, S. V., Tsivgoulis, G. M., Sotiropoulos, D. N., Ioannou, P. V. & Jain, M. K. (1996). *Biochemistry*, **35**, 9375–9384.
dos Santos, J. I., Cintra-Francischinelli, M., Borges, R. J., Fernandes, C. A., Pizzo, P., Cintra, A. C., Braz, A. S., Soares, A. M. & Fontes, M. R. (2011). *Proteins*, **79**, 61–78.
dos Santos, J. I., Fernandes, C. A., Magro, A. J. & Fontes, M. R. (2009). *Protein Pept. Lett.* **16**, 887–893.
dos Santos, J. I., Soares, A. M. & Fontes, M. R. (2009). *J. Struct. Biol.* **167**, 106–116.
Schaloske, R. H. & Dennis, E. A. (2006). *Biochim. Biophys. Acta*, **1761**, 1246–1259.
Scott, D. L., Otwinowski, Z., Gelb, M. H. & Sigler, P. B. (1990). *Science*, **250**, 1563–1566.
Scott, D. L., White, S. P., Otwinowski, Z., Yuan, W., Gelb, M. H. & Sigler, P. B. (1990). *Science*, **250**, 1541–1546.
Vagin, A. & Teplyakov, A. (2010). *Acta Cryst.* D**66**, 22–25.
Ward, R. J., Chioato, L., de Oliveira, A. H., Ruller, R. & Sá, J. M. (2002). *Biochem. J.* **362**, 89–96.
Williams, D., Gutiérrez, J. M., Harrison, R., Warrell, D. A., White, J., Winkel, K. D. & Gopalakrishnakone, P. (2010). *Lancet*, **375**, 89–91.
Xu, S., Gu, L., Jiang, T., Zhou, Y. & Lin, Z. (2003). *Biochem. Biophys. Res. Commun.* **300**, 271–277.
Yu, B.-Z., Rogers, J., Nicol, G. R., Theopold, K. H., Seshadri, K., Vishweshwara, S. & Jain, M. K. (1998). *Biochemistry*, **37**, 12576–12587.