

Anwar Ullah,<sup>a</sup> Monika Coronado,<sup>a</sup> Mário T. Murakami,<sup>b</sup> Christian Betzel<sup>c</sup> and Raghuvir K. Arni<sup>a\*</sup>

<sup>a</sup>Centro Multiusuário de Inovação Biomolecular, Departamento de Física, Universidade Estadual Paulista (UNESP), 15054-000 São José do Rio Preto-SP, Brazil, <sup>b</sup>Laboratório Nacional de Biociências (LNBio), Centro Nacional de Pesquisa em Energia e Materiais, 13083-970 Campinas-SP, Brazil, and

<sup>c</sup>Laboratory of Structural Biology of Infection, Institute of Biochemistry and Molecular Biology, University of Hamburg, c/o DESY, Notkestrasse 85, Building 22a, D-22603 Hamburg, Germany

Correspondence e-mail: arni@sjrp.unesp.br

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## Crystallization and preliminary X-ray diffraction analysis of an L-amino-acid oxidase from *Bothrops jararacussu* venom

Snake-venom L-amino-acid oxidases (SV-LAAOs) trigger a wide range of local and systematic effects, including inhibition of platelet aggregation, cytotoxicity, haemolysis, apoptosis and haemorrhage. These effects mainly arise from the uncontrolled release of the hydrogen peroxide that is produced by the redox reaction involving L-amino acids catalyzed by these flavoenzymes. Taking their clinical relevance into account, few SV-LAAOs have been structurally characterized and the structural determinants responsible for their broad direct and indirect pharmacological activities remain unclear. In this work, an LAAO from *Bothrops jararacussu* venom (BJu-LAAO) was purified and crystallized. The BJu-LAAO crystals belonged to space group  $P2_1$ , with unit-cell parameters  $a = 66.38$ ,  $b = 72.19$ ,  $c = 101.53$  Å,  $\beta = 90.9^\circ$ . The asymmetric unit contained two molecules and the structure was determined and partially refined at 3.0 Å resolution.

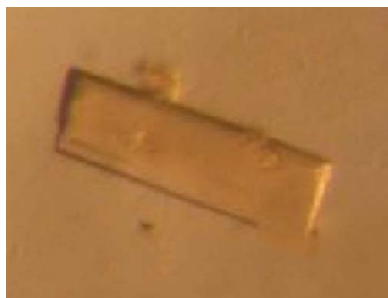
### 1. Introduction

The flavoenzyme L-amino-acid oxidase (LAAO; EC 1.4.3.2) catalyses the oxidative deamination of L-amino acids to the corresponding  $\alpha$ -keto acids with the concomitant liberation of ammonia ( $\text{NH}_3$ ) and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) (Zhang *et al.*, 2003; Sun *et al.*, 2010). These enzymes have been isolated from organisms such as bacteria (Arima *et al.*, 2009), fungi (Yang *et al.*, 2009) and plants (Du & Clemetson, 2002; Kasai *et al.*, 2010), fish-skin mucus (Kitani *et al.*, 2007; Nagashima *et al.*, 2009) and snake venoms (Stábeli *et al.*, 2007). A number of snake venoms (SV-LAAOs) contain LAAO as the major component, which results in the characteristic yellow colour often exhibited by these venoms (Stábeli *et al.*, 2007; Tempone *et al.*, 2001).

LAAOs display many pathological activities such as cytotoxicity (Rodrigues *et al.*, 2009; Alves *et al.*, 2008), inhibition of platelet aggregation (Alves *et al.*, 2008; Li *et al.*, 1994), haemolysis, apoptosis and haemorrhage (Stábeli *et al.*, 2007; Zhang *et al.*, 2003). The pathway or mechanism by which SV-LAAOs participate in venom toxicity remains unclear. However, the liberation of the  $\text{H}_2\text{O}_2$  formed by the re-oxidation of the transiently reduced cofactor FAD by molecular oxygen (MacHeroux *et al.*, 2001) is likely to play an important role in the toxicity of the venoms since the bactericidal activity of LAAO is inhibited by catalase, thereby suggesting that hydrogen peroxide might play a central role in these processes (Stábeli *et al.*, 2007).

LAAOs are homodimeric and glycosylated, with a relative molecular weight of between 110 and 150 kDa; they consist of two identical subunits of 65 kDa and have an isoelectric point in the range 4.7–8.8 (Stábeli *et al.*, 2007; Du & Clemetson, 2002). Typically, snakes possess more than one gene encoding LAAO (Stiles *et al.*, 1991).

Currently, structural information is only available for four LAAOs: a bacterial LAAO from *Rhodococcus opacus* (Faust *et al.*, 2007) and three SV-LAAOs from *Agkistrodon halys pallas* (Zhang *et al.*, 2004), *Calloselasma rhodostoma* (Moustafa *et al.*, 2006) and *Vipera ammodytes ammodytes* (Georgieva *et al.*, 2011). Previous studies have shown that SV-LAAOs exist as homodimers and are formed of three domains: an FAD-binding domain, a substrate-binding domain and a helical domain (Georgieva *et al.*, 2011; Zhang *et al.*, 2004; Faust *et al.*, 2007; Kang *et al.*, 2011).



This structural study is aimed at elucidating the first structure of an SV-LAAO from a snake belonging to the *Bothrops* genus and will help in understanding the differences in the specificities of these enzymes.

## 2. Materials and methods

### 2.1. Venom collection and purification

Desiccated crude *B. jararacussu* venom was purchased from a local serpentarium (Sanmaru Ltda, Taquaral, São Paulo, Brazil). A 125 mg sample of desiccated crude venom was suspended in 1.5 ml Tris–HCl buffer consisting of 0.02 M Tris, 0.15 M NaCl pH 8.0 and centrifuged at 10 000g for 10 min. The clear supernatant (1 ml) was applied onto a 16 × 60 cm Sephacryl S-100 column previously equilibrated with 0.02 M Tris–HCl pH 8.0 buffer containing 0.15 M NaCl. The protein was eluted at a flow rate of 0.2 ml min<sup>-1</sup> and 1 ml fractions were collected. The absorption at 280 nm was monitored and all fractions were analyzed by SDS–PAGE. The above procedure was repeated twice (data not presented).

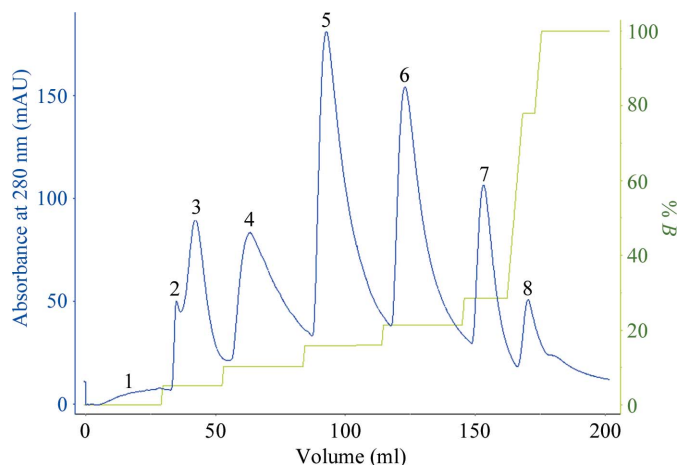
The fractions corresponding to the second peak in the size-exclusion chromatography step were pooled and concentrated to 0.5 ml using a microconcentrator (Amicon) with a membrane cutoff of 30 kDa. This fraction was then applied onto a Mono Q 5/50 GL column. The column was washed with 0.02 M Tris–HCl buffer pH 8.0 (eluent A) and the bound fractions were eluted with an NaCl step gradient using 0.02 M Tris–HCl pH 8.0 plus 1 M NaCl as eluent B. All purification steps were performed at room temperature.

Electrophoresis in polyacrylamide gels in the presence of 0.01% SDS and β-mercaptoethanol was performed using the methodology described by Laemmli (1970) and the gels were stained with silver.

Protein concentrations were determined according to the micro-biuret method (Itzhaki & Gill, 1964) using bovine serum albumin as the standard.

### 2.2. Crystallization

The LAAO sample was concentrated to 9 mg ml<sup>-1</sup> using micro-concentrators and stored in 0.02 M Tris–HCl pH 8.0 buffer at 253 K. Crystallization was performed by the hanging-drop vapour-diffusion method in 24-well tissue-culture plates (Jancarik & Kim, 1991) using commercially available crystallization screens such as Crystal Screen,



**Figure 1** Ion-exchange chromatographic profile (Mono Q 5/50 GL) of the second peak from size-exclusion chromatography. Blue and green lines indicate the absorbance at 280 nm and the elution buffer concentration, respectively.

**Table 1**

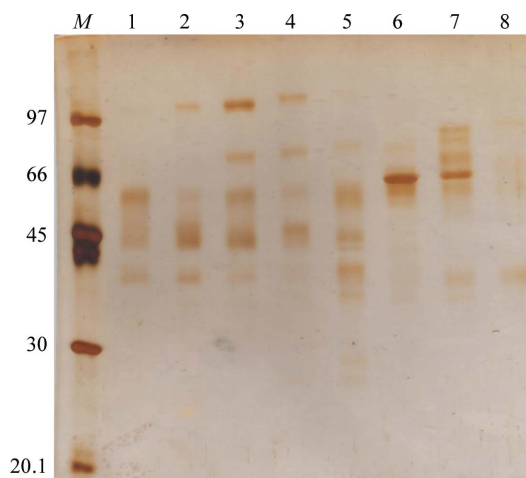
Data-collection and rigid-body refinement statistics.

Data collection	
Temperature (K)	100
Radiation source	Brazilian Synchrotron Light Laboratory
Data-processing software	DENZO/SCALEPACK
Beamline	W01B-MX2
Wavelength (Å)	1.458
Detector	MAR Mosaic 225 mm
Space group	<i>P</i> 2 <sub>1</sub>
Unit-cell parameters (Å, °)	<i>a</i> = 66.38, <i>b</i> = 72.19, <i>c</i> = 101.53, β = 90.9
Resolution range (Å)	30.0–3.0 (3.11–3.00)
No. of unique reflections	16155 (1403)
Multiplicity	2.4 (2.3)
<i>R</i> <sub>merge</sub> (%)	13.2 (35.1)
<i>I</i> /σ( <i>I</i> )	5.1 (1.8)
Data analysis	
Matthews coefficient (Å <sup>3</sup> Da <sup>-1</sup> )	1.8
Solvent content (%)	33.3
Molecules per asymmetric unit	2

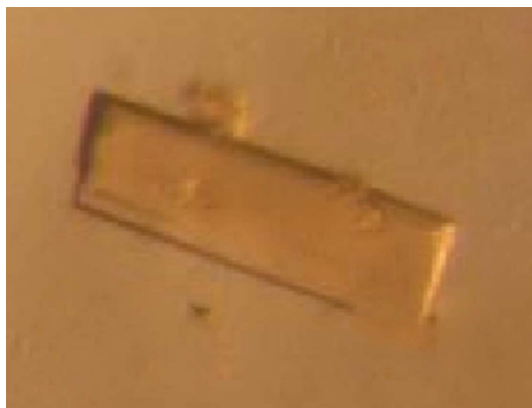
Crystal Screen 2, Grid Screen PEG 6000 and Grid Screen Ammonium Sulfate kits (Hampton Research) and The PEGs Suite (Qiagen). Typically, 1 μl drops of protein solution were mixed with an equal volume of the screening solution and equilibrated over a reservoir containing 0.5 ml of the latter solution. Thin crystals that were suitable for X-ray diffraction experiments were obtained when the reservoir consisted of 0.1 M sodium acetate trihydrate pH 4.6 and 25% (w/v) PEG 1000.

### 2.3. Data collection and processing

An LAAO crystal was directly flash-cooled in a 100 K nitrogen-gas stream. X-ray diffraction data were collected on the W01B-MX2 beamline at the Brazilian Synchrotron Light Laboratory (LNLS, Campinas, Brazil). The wavelength of the radiation source was set to the maximum flux at 1.458 Å (Guimarães *et al.*, 2009) and a MAR Mosaic 225 mm CCD detector was used to record the X-ray diffraction intensities. The LAAO crystal was exposed for 90 s per 2° rotation in φ; the crystal-to-detector distance was set to 100 mm and a total of 180 images were collected. The data were indexed, integrated and scaled using the DENZO and SCALEPACK programs from the HKL-2000 package (Otwinowski & Minor, 1997). Data-collection and processing statistics are summarized in Table 1. Molecular replacement was carried out using the program MOLREP (Vagin &



**Figure 2** SDS-PAGE of protein fractions obtained from ion-exchange chromatography. Lane M, molecular-weight markers (labelled in kDa); lanes 1–8, corresponding protein peaks (5 μg). Peak 6 represents the purified *B. jararacussu* LAAO.



**Figure 3**  
Photomicrograph of an LAAO crystal.

Tepljakov, 2010) and a model based on the atomic coordinates of native LAAO from *V. a. ammodytes* (PDB entry 3kve; Georgieva *et al.*, 2011).

### 3. Results and discussion

An LAAO was isolated from the crude venom of *B. jararacussu* by a two-step procedure consisting of size-exclusion and ion-exchange chromatography. Size-exclusion chromatography resulted in six peaks. The second peak displayed the typical yellow colour of LAAOs and SDS-PAGE indicated a molecular weight of around 65 kDa, which probably corresponds to the protein of interest. Thus, fractions from peak 2 were pooled, concentrated to 0.5 ml and applied onto an ion-exchange column, which resulted in the further separation of eight peaks (Fig. 1); the presence of LAAO with a purity of >95% was confirmed in peak 6 (Fig. 2). This procedure yielded 6.2 mg LAAO from 125 mg crude *B. jararacussu* venom. The purified protein was concentrated to 9 mg ml<sup>-1</sup> in 20 mM Tris-HCl buffer pH 8.0 and submitted to crystallization trials. Large single crystals were obtained when the enzyme solution was mixed with and equilibrated against a reservoir solution consisting of 0.1 M sodium acetate trihydrate pH 4.6 and 25% (w/v) PEG 1000 (Fig. 3).

Thin plate-shaped LAAO crystals with a maximum dimension of 300 µm diffracted to a maximum resolution of 3.0 Å and the reflections were indexed in space group *P*<sub>2</sub><sub>1</sub>. Taking into consideration the molecular weight (65 kDa) and the presence of two molecules in the asymmetric unit, this results in a Matthews coefficient (Matthews, 1968) of 1.84 Å<sup>3</sup> Da<sup>-1</sup>, which corresponds to a solvent content of 33.3%. Data-collection and processing statistics are presented in Table 1.

The atomic coordinates of the LAAO from *V. a. ammodytes* (PDB code 3kve; Georgieva *et al.*, 2011), which shares a sequence identity of 86.5% with the LAAO from *B. jararacussu*, were used to generate a search model for molecular-replacement calculations carried out using the program *MOLREP* (Vagin & Tepljakov, 2010). A clear solution was obtained for two molecules in the asymmetric unit in space group *P*<sub>2</sub><sub>1</sub>. *REFMAC5* (Murshudov *et al.*, 2011) was used for

rigid-body refinement and resulted in an *R* factor of 25.4% and an *R*<sub>free</sub> of 31.5%. Structure determination of the native LAAO and the preparation of complexes with substrate analogues are currently in progress.

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