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A rational protocol for the successful crystallization of L-amino-acid oxidase from *Bothrops atrox*

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Despite the valuable contributions of robotics and high-throughput approaches to protein crystallization, the role of an experienced crystallographer in the evaluation and rationalization of a crystallization process is still crucial to obtaining crystals suitable for X-ray diffraction measurements. In this work, the difficult task of crystallizing the flavoenzyme L-amino-acid oxidase purified from *Bothrops atrox* snake venom was overcome by the development of a protocol that first required the identification of a non-amorphous precipitate as a promising crystallization condition followed by the implementation of a methodology that combined crystallization in the presence of oil and seeding techniques. Crystals were obtained and a complete data set was collected to 2.3 Å resolution. The crystals belonged to space group $P2_1$, with unit-cell parameters a = 73.64, b = 123.92, c = 105.08 Å, β = 96.03°. There were four protein subunits in the asymmetric unit, which gave a Matthews coefficient V_M of 2.12 Å^3 Da^{-1}, corresponding to 42% solvent content. The structure has been solved by molecular-replacement techniques.

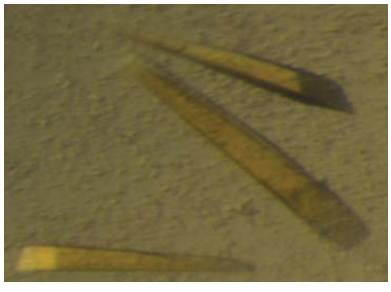
1. Introduction

The benefits of automated high-throughput processes in protein crystallization have already been extensively presented (Mayo *et al.*, 2005; Stevens, 2000; Joachimiak, 2009; Bard *et al.*, 2004; Pusey *et al.*, 2005). In fact, advantages such as speed, reproducibility and the economic amount of protein used in the crystallization trials, among others, have already had a large impact in the development of structural biology in the last few years. Initially, automated crystallization techniques were applied to high-throughput pipelines in industrial and structural genomics projects; nowadays, robotics is more widespread in the community and is found as part of the infrastructure of many academic laboratories.

Despite the unquestionable value of these developments, the usefulness of robotics in protein crystallography still does not replace the expertise of a crystallographer in crystal growth. The adequate visualization and evaluation of crystallization experiments, as well as the development of rational alternatives to traditional trial-and-error screening, still strongly depend on human intervention and play a very important role in the successful crystallization of not so well behaved proteins.

Membrane proteins, protein–protein complexes and multi-domain proteins are examples of challenging problems where meticulous and laborious work is still necessary to achieve crystallization. One such interesting example is reported here, in which a sequence of well known crystallization techniques in combination with a rational approach were necessary to establish a reproducible protocol for the crystallization of the enzyme L-amino-acid oxidase isolated from the venom of the snake *Bothrops atrox* (BatroxLAAO).

L-Amino-acid oxidases (LAAOs) are homodimeric flavoenzymes that catalyze the stereospecific oxidative deamination of a wide range of L-amino acids, generating the corresponding α-keto acids, H_2O_2 and ammonia (Massey & Curti, 1967; Zhang *et al.*, 2003; Sun *et al.*, 2010; Curti *et al.*, 1992). In addition, recent studies have shown that



LAAOs are multifunctional enzymes that exhibit both induction and/or inhibition of platelet aggregation (Li *et al.*, 1994; Sakurai *et al.*, 2001; Alves *et al.*, 2008; Zhong *et al.*, 2009; Sun *et al.*, 2010), anticoagulant activity (Sakurai *et al.*, 2001), stimulation of oedema formation (Stabeli *et al.*, 2004; Izidoro *et al.*, 2006), haemorrhage (Souza *et al.*, 1999; Stabeli *et al.*, 2004), cytotoxicity and apoptotic activities (Alves *et al.*, 2008; Rodrigues *et al.*, 2009) and antibacterial, antiviral and leishmanicidal functions (Zhang *et al.*, 2003; Izidoro *et al.*, 2006; Rodrigues *et al.*, 2009; Zhong *et al.*, 2009; Sun *et al.*, 2010; Costa Torres *et al.*, 2010).

LAAOs have been isolated from sea animals, the mucus of the giant snail *Achatina fulica* Férussac (Ehara *et al.*, 2002), the ink of the sea hare *Aplysia californica* (Butzke *et al.*, 2005), bacteria, fungi and plants (Du & Clemetson, 2002; Arima *et al.*, 2009; Yang *et al.*, 2009; Kasai *et al.*, 2010) and are widely distributed in the venomous snake families Viperidae, Crotalidae and Elapidae.

Few LAAO crystal structures have been reported to date. The crystal structures of LAAO from *Calloselasma rhodostoma* (Moustafa *et al.*, 2006), *Rhodococcus opacus* (Faust *et al.*, 2007) and *Agkistrodon halys pallas* (Zhang *et al.*, 2004) have been determined, allowing the identification of a functional dimer in which each monomer consists of three domains: an FAD-binding domain, a substrate-binding domain and a helical domain that is involved in protein dimerization. An interesting feature of snake-venom LAAOs is the presence of glycosylation sites, as found on the surface of *C. rhodostoma* LAAO (Moustafa *et al.*, 2006). The glycan moiety of LAAO may allow docking of the enzyme to the surface of the host cell, resulting in the production of high concentrations of H_2O_2, which could lead to oxidative damage of cell structural elements (Geyer *et al.*, 2001).

Owing to their involvement in a wide range of cellular functions and the therapeutic potential of L-amino-acid oxidases, we are interested in understanding the structure–function relationship of snake-venom LAAOs in more detail. The crystallization and structure determination of *B. atrox* LAAO presented here constitutes the first step towards this goal.

2. Crystallization

BatroxLAAO was isolated from *B. atrox* snake venom as described previously (Alves *et al.*, 2008). In summary, the purification protocol consisted of three chromatographic steps: samples of crude venom were fractioned by size-exclusion chromatography on Sephadex G-75 resin (GE Healthcare), followed by an anion-exchange chromatographic step performed on ES-502N 7C resin (Shodex) and a final affinity-chromatography step on lentil lectin resin (GE Healthcare).

Prior to crystallization, native BatroxLAAO was dialyzed against 20 m*M* Tris–HCl pH 8.0 and concentrated to 6 mg ml^{-1} as determined by biuret assay (Gornall *et al.*, 1949); the enzyme activity was confirmed by a horseradish peroxidase-coupled assay (Ponnudurai *et al.*, 1994).

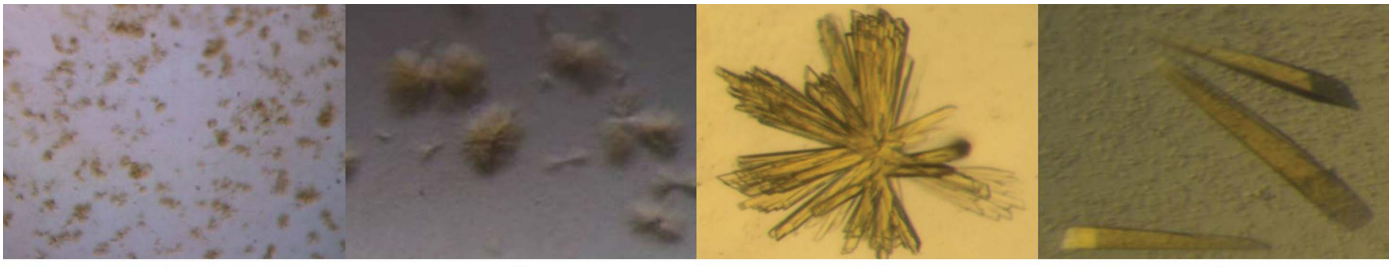
Initial crystallization conditions were screened by the sparse-matrix method (Jancarik & Kim, 1991) using the commercially available screening kits Crystal Screen and Crystal Screen 2 (Hampton Research). PEG/Ion and PEG/Ion 2 screens (Hampton Research) were also tested. The crystallization experiments were performed manually using the sitting-drop vapour-diffusion technique in 24-well Cryschem plates (Hampton Research). Equal volumes (2 µl) of protein and reservoir solution were mixed; the drops were equilibrated against 500 µl reservoir solution at 295 K.

Even though none of the conditions tested showed crystal formation, by careful visualization of the drops it was possible to recognize some small yellow crystalline aggregates that were suspended in the drop after 10 d in the presence of 10 m*M* zinc sulfate heptahydrate, 0.1 *M* MES monohydrate pH 6.5, 25%(*v*/*v*) polyethylene glycol monomethyl ether 550 (Crystal Screen 2 condition No. 27; Hampton Research; Fig. 1*a*).

Efforts were made to optimize the crystallization experiment by altering a wide range of crystallization variables. Systematic variation of various crystallization parameters failed to enhance nucleation and crystal growth. In the best-case scenario, fine manipulation of the pH, the polyethylene glycol molecular weight and concentration, the protein concentration and the temperature and the use of additives resulted in the formation of yellowish whisk/broom-like aggregates (Fig. 1*b*). Under the conditions tested, crystalline growth required the presence of zinc (5–10 m*M*), an acidic pH (pH 5–5.5) and a low concentration of precipitant (10–15%).

To manipulate the rate of vapour diffusion, a 100 µl oil barrier (composed of a mixture of silicone and paraffin oils in different proportions) was placed over 500 µl reservoir solution. After four months of incubation at 295 K, yellow clusters of the enzyme grew in 3 m*M* zinc sulfate heptahydrate, 0.1 *M* sodium cacodylate pH 5, 12%(*v*/*v*) polyethylene glycol monomethyl ether 550 when a 1:1 silicone:paraffin oil mixture was used (Fig. 1*c*). The clusters were then broken apart and small pieces (5–10 µm) were used as seeds for new crystallization experiments. A grid screen based on previous crystallization experiments was designed and the macroseeds were transferred using a cryoloop into a new drop consisting of a mixture of equal volumes (4 µl) of protein solution at 5–6 mg ml^{-1} and reservoir solution equilibrated against 500 µl reservoir solution. The reservoir solution was covered with 100 µl 1:1 paraffin:silicone oil.

Rod-type crystals suitable for X-ray diffraction measurements were obtained at 295 K in the presence of 5 m*M* zinc sulfate hepta-



(*a*) (*b*) (*c*) (*d*)

Figure 1
Steps towards the successful crystallization of BatroxLAAO. (*a*) Crystalline aggregates obtained by the sparse-matrix method. (*b*) Whisk/broom-like aggregates obtained after manipulation of the initial crystallization parameters. (*c*) Clusters of needle-like crystals obtained by using vapour-diffusion techniques in the presence of oil. (*d*) Single crystals obtained by using a macroseeding matrix screen in presence of oil.

Table 1
Data-collection and processing statistics.

Values in parentheses are for the outer resolution shell.

Temperature (K)	100
Wavelength (Å)	1.45860
Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 73.64, b = 123.92, c = 105.08, β = 96.03
Resolution range (Å)	34.84–2.3 (2.42–2.30)
Unique reflections	80024
Multiplicity	2.1 (2.1)
Data completeness (%)	96.5 (93.4)
$\langle I/\sigma(I)\rangle$	6.4 (1.7)
$R_{\rm merge}$† (%)	14.6 (55.7)
Molecules per asymmetric unit	4
$V_{\rm M}$ (Å^3 Da^{-1})	2.12
Solvent content (%)	42

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $\langle I(hkl)\rangle$ is the mean intensity of multiple observations of symmetry-related reflections.

hydrate, 0.1 *M* sodium cacodylate pH 5.0, 15%(*v*/*v*) polyethylene glycol monomethyl ether 550 and 1:1 silicone:paraffin oil barrier (100 µl). The crystals of BatroxLAAO usually reached maximum dimensions of 0.4 × 0.1 × 0.1 mm after 23 d (Fig. 1*d*).

3. Data collection and structure determination

The crystals were transferred to a cryoprotectant solution consisting of 20% glycerol in 5 m*M* zinc sulfate heptahydrate, 0.1 *M* sodium cacodylate pH 5.0, 17%(*v*/*v*) polyethylene glycol monomethyl ether 550 and flash-cooled in a stream of nitrogen gas maintained at 110 K. Diffraction data were collected from a single crystal on the D03B-MX2 beamline of the Laboratório Nacional de Luz Síncrotron (Campinas, Brazil) using a MAR CCD 165 detector with a crystal rotation of 1° per frame. An exposure time of 90 s per image was used, with a crystal-to-detector distance of 120 mm. A total of 108 diffraction images were collected covering 1° of crystal rotation. The data were processed using *MOSFLM* (Leslie, 2006) and scaled with *SCALA* (Evans, 2006). The data-collection and processing statistics are summarized in Table 1.

The crystals of BatroxLAAO belonged to the monoclinic space group $P2_1$, as determined from systematic absences and the symmetry of the diffraction intensities. The unit-cell parameters were a = 73.64, b = 123.92, c = 105.08 Å, β = 96.03° and the crystal diffracted to 2.3 Å resolution. The calculated Matthews coefficient ($V_{\rm M}$) for four molecules of protein in the asymmetric unit is 2.12 Å^3 Da^{-1}, with 42% of the unit cell occupied by solvent (Matthews, 1968).

Initial phases were obtained by molecular replacement as implemented in *Phaser* (McCoy *et al.*, 2007). Initial rotation and translation functions were calculated using the coordinates of a homologous LAAO from *A. halys pallas* (PDB code 1reo; Zhang *et al.*, 2004) as the search model. The model was initially refined by rigid-body refinement followed by simulated annealing; initial rounds of positional and individual *B*-factor refinement using the *PHENIX* suite of crystallographic programs (Adams *et al.*, 2010) resulted in an *R* factor of 24.8% and an $R_{\rm free}$ of 29.9%. Further structural refinement is in progress.

4. Conclusions

The crystal structure of native L-amino-acid oxidase from *B. atrox* has been solved by molecular-replacement techniques at 2.3 Å resolution. The structure determination, together with biological assays and complete biophysical characterization, will be used to develop our understanding of the structure–function relationship of the LAAOs.

In brief, the visualization of a non-amorphous precipitate as a promising crystallization condition in combination with seeding and oil-barrier techniques proved to be an effective means of crystallizing BatroxLAAO and resulted in a dramatic increase in crystal size and quality that was vital for the success of this project.

The results presented here emphasize the importance of understanding and evaluating each step during the crystallization process. In addition, they encourage the exploration of very well established techniques in order to enhance the chances of protein crystallization.

From our point of view, automated processes in crystallization are undoubtedly a great ally in the progress of structural biology, but they are not essential for success. Old-fashioned manual crystallization protocols can be successful as well as satisfying to use.

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