Crystallization and preliminary X-ray diffraction
analysis of the lectin from *Canavalia boliviana* Piper
seeds

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Plant lectins are the most studied group of carbohydrate-binding proteins. Despite the high similarity between the members of the Diocleinae subtribe (Leguminosae) group, they present differing biological activities. *Canavalia boliviana* lectin (Cbol) was purified using a Sephadex G-50 column and crystallized in the presence of X-Man by hanging-drop vapour diffusion at 293 K. After optimization, crystals suitable for diffraction were obtained under the condition 0.1 M HEPES pH 7.5 and 3.0 M sodium formate. The crystal belonged to the monoclinic space group C2, with unit-cell parameters $a = 126.70$, $b = 66.64$, $c = 64.99$ Å, $\alpha = 90.0$, $\beta = 120.8$, $\gamma = 90.0^\circ$. Assuming the presence of a dimer in the asymmetric unit, the solvent content was estimated to be about 46%. A complete data set was collected at 1.5 Å resolution.

1. Introduction

Lectins are one of the most important groups of proteins responsible for deciphering the sugar code (Gabijs, 2000). They play a central role in a variety of biological events, functioning as recognition molecules in cell–molecule and cell–cell interactions (Sharon & Lis, 2004). These cellular-mediated events are diverse, spanning from mitogenic processes to plant defence mechanisms (Weis & Drickamer, 1996).

The best studied group of carbohydrate-binding proteins are the plant lectins; within this group, those purified from species of the Leguminosae family are the most studied members (Van Damme *et al.*, 1998). These proteins are used as models for the study of not only protein–sugar interactions but also protein–protein interactions (Lowry *et al.*, 1997; Del Sol *et al.*, 2007).

Despite the high degree of identity observed between the lectins isolated from seeds of the Diocleinae subtribe, they exhibit different biological activities (Alencar *et al.*, 1999; Andrade *et al.*, 1999; Assreuy *et al.*, 1999; Cavada *et al.*, 2001; Havt *et al.*, 2003; Lopes *et al.*, 2005). For instance, although concanavalin A (ConA) and *Canavalia maritima* lectin (ConM) possess 98% identity in primary sequence, they present different histamine-releasing activities, which is related to their affinity for biantennary complex oligosaccharides (Dam *et al.*, 1998). Through studying the crystallographic complexes between both proteins and specific disaccharides, Bezerra *et al.* (2007) provided structural insights regarding these affinity differences: a difference in the conformation of His205 ('turned up' or 'turned down') provokes conformational changes in the active site.

The distinct biological activities reported for Diocleinae lectins have also been attributed to differences in their pH-dependent oligomerization (Calvete *et al.*, 1999; Nagano *et al.*, 2008). Recently, based on comparison of the three-dimensional structures of *Dioclea rostrata* lectin (DRL), *D. guianensis* (Dguia) and *D. grandiflora* (Dgran), Oliveira *et al.* (2008) were able to explain the different behaviours presented in their quaternary association, which must be related to the differences in promotion of the synthesis of INF- γ and histamine secretion that have been observed for these lectins.

Some proteins with lectin domains, called selectins, are known to be involved in the inflammatory process (Kansas, 1996). This supports the use of leguminous lectins as anti-inflammatory molecules and also as tools for better understanding the role of sugar residues in leukocyte recruitment (Assreuy *et al.*, 1997; Alencar *et al.*, 1999). Recent analysis showed that Cbol, a lectin purified from the seeds of *C. boliviana* Piper, a plant found in the Amazon rainforest, possesses anti-inflammatory activity with significant differences compared with other Diocleinae lectins (unpublished data). Solution of the three-dimensional structure of this lectin may contribute to understanding the different behaviours observed in this highly similar class of proteins. The present study reports the purification, crystallization and X-ray diffraction of Cbol.

2. Material and methods

2.1. Purification

C. boliviana Piper seeds were collected in the Amazon rainforest in the Amazon state in Brazil. The seeds were ground to a fine powder in a coffee mill and the soluble proteins were extracted at 298 K by continuous stirring with 0.15 M NaCl [1:10(w:v)] for 1 h, followed by centrifugation at 10 000g at 277 K for 20 min. The supernatant was applied onto a Sephadex G-50 column (10 × 50 cm) previously equilibrated with 0.15 M NaCl containing 5 mM CaCl₂ and 5 mM MnCl₂. The unbound material was eluted with 0.15 M NaCl at a flow rate of 45 ml h⁻¹ until the absorbance at 280 nm of the effluent stabilized at 0.05. The retained fraction was eluted with 0.1 M D-glucose and dialyzed exhaustively against Milli-Q water. This fraction was freeze-dried and used for further experiments. A haemagglutination-activity test was carried out according to a standard procedure (Farias *et al.*, 2004) using native and enzyme-treated rabbit erythrocytes. The purity of all Cbol preparations was monitored by SDS-PAGE (Laemmli, 1970). SDS-PAGE was carried out in a 12.5% gel and molecular-mass markers purchased from Fermentas (#SM0441) were used. The purified lectin was also compared with other previously described Diocleinae lectins (ConBr, Moreira &

Cavada, 1984; CGL, Ceccatto *et al.*, 2002). Protein bands were stained with 0.05% Coomassie Brilliant Blue R-250.

2.2. Crystallization

The freeze-dried purified lectin was resuspended in Milli-Q water containing 3 mM X-Man (5-bromo-4-chloro-3-indolyl- α -D-mannose, 12 mM stock solution in DMSO) at a final concentration of 12 mg ml⁻¹ and incubated at 310 K for 1 h prior to crystallization experiments. The crystals were grown in 24-well Linbro plates at room temperature (293 K) by the vapour-diffusion/sparse-matrix method (Jancarik & Kim, 1991) in hanging drops using Crystal Screen I from Hampton Research (Riverside, California, USA). Each well contained 300 μ l reservoir solution and the drops were composed of equal volumes (1 μ l) of protein solution and reservoir solution.

2.3. Data collection and processing

X-ray data were collected from a single crystal cooled to a temperature of 100 K. Crystals were soaked in a cryoprotectant solution made up of reservoir solution (85%) and glycerol (15%) to avoid ice formation and were submitted to data collection at a wavelength of 1.4 Å using a synchrotron-radiation source [MX2 station, Laboratório Nacional de Luz Síncrotron (LNLS), Campinas, Brazil]. A complete data set was obtained using a CCD (MAR Research) in 360 frames with an oscillation range of 0.5°. The data set was indexed and integrated using *MOSFLM* (Leslie, 1992). The intensities were reduced using *SCALA* (Evans, 1997). The phase problem was solved by the molecular-replacement method using the program *MOLREP* (Vagin & Teplyakov, 1997). The initial model was submitted to several cycles of rigid-body refinement using the program *REFMAC5* and monitored using the R_{free} and R -factor values (Brünger, 1992). All the programs mentioned above belong to the *CCP4* package (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

The lectin Cbol was purified to apparent homogeneity by affinity chromatography on Sephadex G-50, in which Cbol was quantitatively retained in the cross-linked dextran gel column and was desorbed with D-glucose, providing strong evidence of its carbohydrate-binding properties (Fig. 1*a*). This procedure has widely been used for the purification of Diocleinae lectins (Cavada *et al.*, 2001). About 80 mg purified lectin was obtained from 1 g powdered seeds. The purified protein showed haemagglutination activity towards native and enzyme-treated rabbit erythrocytes. SDS-PAGE confirmed the purity of the lectin and its similarity to other Diocleinae lectins, showing a

Figure 1
Affinity chromatography and SDS-PAGE of the purified *C. boliviana* lectin (Cbol). (a) Sephadex G-50 chromatogram; the bonded protein was eluted with 0.1 M D-glucose. (b) Lane 1, SDS-PAGE showing protein markers of ~120 kDa (β -galactosidase), ~86 kDa (bovine serum albumin), ~47 kDa (ovalbumin), ~34 kDa (carbonic anhydrase), ~26 kDa (β -lactoglobulin), ~20 kDa (lysozyme); lane 2, 20 μ g Cbol; lane 3, 20 μ g ConBr; lane 4, 20 μ g CGL. The main-chain α and β and γ fragments are clearly seen in lanes 2–4. The high-molecular-weight bands present in lanes 2–4 are the preproprotein with a molecular mass of approximately 65 kDa.

Figure 2
Crystals of *C. boliviana* lectin.

Table 1

Statistics of data collection.

Values in parentheses are for the highest resolution shell.

R_{merge}	5.7 (22.6)
Resolution limit (Å)	46.56 (1.60)
$I/\sigma(I)$	9.6 (2.07)
Completeness (%)	88.9 (88.9)
Redundancy	2.6
Unit-cell parameters (Å, °)	$a = 126.7$, $b = 66.6$, $c = 64.9$, $\alpha = 90.0$, $\beta = 120.8$, $\gamma = 90.0$
Matthews coefficient (Å ³ Da ⁻¹)	2.3
Solvent content (%)	46.7
Space group	C2
Wavelength (Å)	1.458 Å
Total No. of reflections	493651
Total No. of unique observations	54517

main α chain and β and γ fragments (Fig. 1b). These results suggest that Cbol is a ConA-like lectin and undergoes the same post-translational process of circular permutation as first described by Carrington *et al.* (1985). This process involves proteolytic cleavage of the precursor at an internal site, resulting in the γ and β fragments. These fragments are religated in an inverse DNA-coded order, producing the active α chain ($\alpha = \beta + \gamma$; Carrington *et al.*, 1985; Chrispeels *et al.*, 1986; Min & Jones, 1994). The β and γ fragments observed in the SDS-PAGE (Fig. 1b) are non-religated products of this process and the α chain is the mature protein. The Diocleinae lectins used for comparison, ConBr and CGL, have previously been characterized and their three-dimensional structures have been determined (PDB code 1azd, Sanz-Aparicio *et al.*, 1997; PDB code 2d7f, Delatorre *et al.*, 2007).

Crystals were obtained after a week using condition Nos. 30, 31, 34 and 38 of Hampton Crystal Screen I. Condition No. 34 was chosen to be optimized; it contained 0.1 M sodium acetate trihydrate pH 4.6 and 2.0 M sodium formate. To optimize the crystallization conditions, the concentration of sodium formate was modified from 1 to 6 M and buffers of differing pH were tested. Crystals suitable for diffraction experiments (Fig. 2) were obtained under the condition 0.1 M HEPES pH 7.5 and 3.0 M sodium formate. The crystals obtained provided a data set extending to 1.5 Å resolution, which was scaled in the resolution range 46.56–1.60 Å. The *C. boliviana* lectin crystal belongs to the centred monoclinic space group C2, with unit-cell parameters $a = 126.7$, $b = 66.6$, $c = 64.9$ Å, $\alpha = 90.0$, $\beta = 120.8$, $\gamma = 90.0^\circ$. The calculated value of the Matthews coefficient (Matthews, 1968), based on the molecular weight of 25.5 kDa, indicated a solvent content of 46.7%, which corresponds to the presence of a dimer in the asymmetric unit. Data-collection statistics are shown in Table 1. The preliminary crystal structure of Cbol was determined by standard molecular-replacement methods using the program *MOLREP* (Vagin & Teplyakov, 1997). Various monomers were tested for molecular replacement and the best result was obtained using CGL (PDB code 2d7f; Delatorre *et al.*, 2007) as a structural model. The best solution had a final correlation coefficient of 0.705 and an R factor of 0.424. After placing the molecule in the unit cell, rigid-body refinement was performed using the *REFMAC5* program. Refinement resulted in a model with an R_{free} of 0.363 and an R factor of 0.359. Primary sequence determination by mass spectrometry and Edman degradation as well as solution of the crystal structure are in progress.

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