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Structural analysis of *Centrolobium tomentosum* seed lectin with inflammatory activity

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ABSTRACT

A glycosylated lectin (CTL) with specificity for mannose and glucose has been detected and purified from seeds of *Centrolobium tomentosum*, a legume plant from *Dalbergieae* tribe. It was isolated by mannose-sepharose affinity chromatography. The primary structure was determined by tandem mass spectrometry and consists of 245 amino acids, similar to other *Dalbergieae* lectins. CTL structures were solved from two crystal forms, a monoclinic and a tetragonal, diffracted at 2.25 and 1.9 Å, respectively. The carbohydrate recognition domain (CRD), metal-binding site and glycosylation site were characterized, and the structural basis for mannose/glucose-binding was elucidated. The lectin adopts the canonical dimeric organization of legume lectins. CTL showed acute inflammatory effect in paw edema model. The protein was subjected to ligand screening (dimannosides and trimannoside) by molecular docking, and interactions were compared with similar lectins possessing the same ligand specificity. This is the first crystal structure of mannose/glucose native seed lectin with proinflammatory activity isolated from the *Centrolobium* genus.

1. Introduction

Structural variability and complexity allow cell-surface glycans to act as signaling, recognition and adhesion molecules. The interaction of cell-surface glycans with proteins is a crucial step in many biological processes, such as cell-cell recognition, intracellular trafficking and localization, adhesion and host-pathogen interaction [1].

Lectins are proteins or glycoproteins that specifically bind to carbohydrates and form complex glycosylated molecules, but without altering the covalent structure of the glycosyl ligands [2]. Lectins differentiate among glycosylated structures and participate in cell recognition. The biological information contained in carbohydrates needs to be deciphered, and the lectins are tasked with reading the glycocode [3,4].

Lectins possess diverse biotechnological applications as, for example, antitumor and antiviral drugs [5], drug delivery systems [6], and lectin-related diagnostics to detect a broad spectrum of diseases [7]. Consequently, lectins are attracting increasing interest in biotechnology, and the continuous investigation of lectins may yield new biotechnological tools.

Studies on legume lectins typically focus on the subfamily *Papilonoideae*, whereas few studies have reported on lectins from primitive tribes, such as *Dalbergieae* [8–10]. A few lectins from the *Dalbergieae* tribe possessing either galactose- [11,12] or mannose-

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binding [13,14] affinities have been purified and biochemically characterized. Some structural analyses have also been performed [8,11,15,16], including the tridimensional structure of three *Dalbergieae* lectins [10,15,17].

Although they differ in carbohydrate-binding specificity, leguminous lectins have similar fold at the monomer level [16]. These lectins have a dome-like jelly-roll fold motif characterized by a β-sandwich of 25–30 kDa containing a carbohydrate recognition domain (CRD) and metal-binding sites for divalent cations (Ca^{2+} and Mn^{2+}) [18]. Based on quaternary structure, some variations in dimeric and tetrameric assembly occur, specifically in the carbohydrate-binding domain. The site consists of a few conserved amino acids and depends on five loops with variable size and sequence that control carbohydrate specificity [19–21]. Lectin structures complexed with glycans provide information about carbohydrate recognition sites and protein-carbohydrate interactions around the CRD that are fundamental to such biological events as immune defense [10,22].

Previous *in vitro* results have demonstrated that plant lectins are able to trigger events related to inflammatory processes, such as lymphocyte proliferation [23], histamine release from mast cells [24], production of nitric oxide and apoptosis [25,26]. *In vivo*, local application of some plant lectins elicits paw edema, increased vascular permeability and neutrophil migration. These effects are usually dose-dependent and have the participation of CRD [27–29].

Centrolobium tomentosum Guill. ex Benth (*Fabaceae-Papilionoideae-Dalbergieae*) is typically distributed in Brazil where it is known as “araribá”. The bark and leaves are used as a plaster covering wounds and bruises. The wood is used in carpentry, construction and ship building. Up to now, the research of this species has been limited to studies of resprouting from roots and inoculation with Rhizobium and mycorrhizal fungi [30,31].

This study aimed to determine the primary and tridimensional structure of *Centrolobium tomentosum* lectin and investigate its acute inflammatory effect on a paw edema model.

2. Materials and methods

2.1. Protein purification

CTL was purified from powdered *C. tomentosum* seeds as described previously [13]. *Centrolobium tomentosum* seeds were ground into a fine powder using a coffee mill and defatted in the presence of n-hexane. The treated flour was suspended 1:10 (w/v) in 100 mM Tris-HCl buffer (pH 7.6) containing 150 mM NaCl and incubated at room temperature under continuous stirring for 4 h before centrifugation at 12,000 × *g* for 20 min at 4 °C (Eppendorf Centrifuge 5810R). The supernatant (crude extract) was applied to a column (10.0 × 60.0 mm; 2.5 mL bed volume) of Sepharose 4B-mannose matrix (GALAB Technologies GmbH, Geesthacht, Germany) equilibrated with 100 mM Tris-HCl buffer, pH 7.6, containing 150 mM NaCl. The unbound proteins were washed from the column with the equilibrium solution and the lectin desorbed by eluting with 100 mM D-mannose in equilibrium solution. The purified lectin was dialyzed against ultrapure water and freeze-dried for further analysis.

2.2. Primary structure determination by tandem mass spectrometry (MS/MS)

Protein digestion was performed as previously described [32]. To accomplish this, CTL was submitted to SDS-PAGE, and the bands were excised and bleached with 50 mM ammonium bicarbonate in 50% acetonitrile. Bands were dehydrated in 100% acetonitrile and dried in a Speedvac (LabConco, Kansas City, MO, USA). For proteolytic cleavage, performed at 310 K overnight, gels were rehydrated in 50 mM ammonium bicarbonate containing trypsin (Sigma-Aldrich) and chymotrypsin (Sigma-Aldrich), at 1:50 w/w (enzyme/substrate). Endoproteinase Asp-N (Sigma-Aldrich) digestion was performed in ammonium bicarbonate 25 mM at 1:50 w/w (enzyme/substrate). Pepsin (Sigma-Aldrich) digestion was performed in 100 mM HCl at 1:50 w/w (enzyme/substrate). The obtained peptides were extracted in a solution of 50% acetonitrile with 5% formic acid and concentrated in Speedvac.

Peptides obtained in different proteolytic cleavages were separated on a BEH300C_{18} column (100 × 100 mm) using a nanoAcquity™ system (Waters Corp.). The column was equilibrated with 0.1% formic acid and eluted with acetonitrile gradient (10–85%) containing 0.1% formic acid at 600 μL/min. The liquid chromatography system was connected to a nanoelectrospray mass spectrometer source (SYNAPT HDMS System, Waters Corp., Milford, USA). The mass spectrometer was operated in positive mode, using a source temperature of 363 K and capillary voltage of 3.5 kV. The lock mass used in acquisition was m/z 785.84 ions of the [Glu1]-fibrinopeptide B. The LC-MS/MS experiment was performed according to the data dependent acquisition (DDA) method, selecting MS/MS doubly or triply charged precursor ions, which were fragmented by collision-induced dissociation (CID) using argon as the collision gas and ramp collision energy that varied according to the charge state of the selected precursor ion. CID spectra were interpreted manually using the Peptide Sequencing tool from MassLynx 4.0 software (Waters). Using BLAST, CTL peptide sequences were compared to all non-redundant proteins deposited in the National Center of Biotechnology Information [33], and the proteins with the lowest e-values were selected for sequence alignments using ESPript 3.0 [34].

2.3. Crystallization and data collection

The freeze-dried purified lectin was suspended in Milli-Q™ ultrapure water containing 5 mM Methyl-O3-(α-D-mannose)-α-D-mannose, named Methyl-Dimannoside, at a final concentration of 15 mg/mL. The carbohydrate in solution was used for characterization and as a stabilizing agent of CRD. The sample was incubated at 310 K for 1 h prior to the crystallization experiments. Crystallization trials were performed using “Crystallization Extension Kit for Proteins” (Sigma-Aldrich). The crystals grown in 24-well Linbro plates at room temperature (293 K) by the vapor-diffusion method [35] in hanging drops. Suitable crystals for X-ray diffraction grew in 2 weeks. X-ray diffraction data were collected from crystals cooled to 100 K.

The X-ray diffraction data were collected at 100 K on MX2 Station of the National Laboratory of Synchrotron Light (LNLS–Campinas, Brazil), using a PILATUS2M™ detector (Dectris, Swiss). Prior to the diffraction crystals were soaked in a cryoprotectant aqueous solution of glycerol (30%) to avoid ice formation. A number of 180 images were collected with an oscillation angle of 1°.

2.4. X-ray diffraction analysis

The dataset of images was indexed and integrated, and the intensities were reduced by the iMosflm program in the CCP4 suite [36]. Molecular replacement was conducted to solve the phase problem, using maximum-likelihood methods implemented in the MOLREP program [37]. For the monoclinic form, the atomic coordinates used as the search model were from *Platypodium elegans* lectin [15] complexed with trimannoside (PDB code 3ZVX), which shows 89% of identity with CTL. The number of copies to find were set to 2. For the tetragonal form, monoclinic structure was used as search model with number of copies set to 1. Crystallographic