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Vasorelaxant activity of *Canavalia grandiflora* seed lectin: A structural analysis

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ABSTRACT

Lectins are comprised of a large family of proteins capable of the specific and reversible recognition of carbohydrates. Legume lectins, the most studied plant lectins, show high structural similarity, but with modifications that imply a variation in the intensity of some biological activities. In this work, the primary and tertiary structures of *Canavalia grandiflora* (ConGF) were determined. ConGF, a lectin isolated from *C. grandiflora* seeds, is able to induce relaxant activity in rat aortic rings. The complete sequence of ConGF comprises 237 amino acids. This particular protein has primary sequence variations commonly found in lectins from *Dioclea* and *Canavalia* genera. The protein structure was solved at 2.3 Å resolution by X-ray crystallography. An X-Man molecule was modeled into the carbohydrate recognition domain. Still, ConGF (30 and 100 $\mu g\,mL^{-1}$) elicited 25% of vasorelaxation (IC_{50} = 34.48 ± 5.07 $\mu g\,mL^{-1}$) in endothelialized aortic rings. A nonselective inhibitor of nitric oxide blocked ConGF relaxant effect, showing mediation by nitric oxide. Key distances between ConGF carbohydrate recognition domain residues were determined in order to explain this effect, in turn revealing some structural aspects that could differentiate lectins from the *Canavalia* genera with respect to different efficacy in vasorelaxant effect.

Introduction

Lectins are proteins able to specifically and reversibly recognize carbohydrates without modifying them through the carbohydrate-recognition domain (CRD)[1] [1]. Closely phylogenetically related lectins from the Legume subtribe *Diocleinae* present small differences in crucial positions of the primary structure, and the impact of these changes has been shown through several biological assays [1–7]. CRDs are highly conserved in *Diocleinae* lectins, and, thus far, only CPL, the lectin of *Camptosema pedicellatum* seeds, has presented a point mutation on the hydrophobic subsite from the carbohydrate-binding domain. Importantly, key distances between residues that comprise the CRD affect its shape and, hence, the volume of each domain, characteristics that directly affect carbohydrate recognition and the ability to elicit biological effects [3,4,7].

Besides the shape and volume of the CRD, the ratio between the dimeric and tetrameric forms of lectins in solution changes by alterations in pH, i.e., pH-dependent oligomerization, reflecting differences in the association of monomers affecting the biological activity of lectins. More specifically, the relative orientation of the CRD amino acids in tetramers affect the spatial arrangement of carbohydrate binding sites and determine the ability of lectins to distinguish saccharides and bind to glycoconjugates of cell surface [1,8].

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[1] *Abbreviations used:* ConGF, *Canavalia grandiflora*; CRD, carbohydrate recognition domain; ConM, *Canavalia maritima*; ConA, *Canavalia ensiformis*; EDRF, endothelium-derived relaxing factors; DVL, *Dioclea violacea*; DRL, *Dioclea rostrata*; DvirL, *Diocleta virgata* lectin; CGL, *Canavalia gladiata* lectin; LLG, log-likelihood-gain; RFZ, rotation function z-score; TFZ, translation function z-score; DDA, data dependent acquisition function; CID, collision-induced dissociation; NOS, nitric oxide synthase; L-NAME, N-nitro-L- arginine methyl ester; RMSD, root mean square deviation.

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Structural analysis of lectins can further clarify their mechanisms of action and the structure–function relationship. An important example is the substitution of Pro_{202} by Ser_{202} in the lectin of *Canavalia maritima* (ConM). This modification leads to conformational alterations in the CRD at position 12 (tyrosine). This is a unique alteration in this *Diocleinae* lectin sequence, and it has high similarity with *Canavalia ensiformis* lectin (ConA). In comparison with ConA, this modification contributes to the extended CRD of ConM, allowing the approximation of Tyr_{12} in the carbohydrate-binding site and the emergence of a recurrent H-bond between the Tyr_{12} hydroxyl oxygen and the hydrogen of the disaccharide outer glucose [9,10]. Thus, the structural features of ConM CRD explain its high affinity for disaccharides [8].

Endothelial cells exhibit surface glycoproteins that can interact with CRD, thereby facilitating lectin cell recognition and the release of endothelium-derived relaxing factors (EDRFs) from blood vessels [11,12]. Homologous lectins from *Dioclea violacea* (DVL) and *Dioclea rostrata* (DRL) seeds exhibit marked differences in their relaxation activities in vascular smooth muscle (43% for DVL and 96% for DRL). Despite their otherwise remarkable similarity, differences in the distance between key residues present in CRD results in a wider and shallower CRD in DRL that favors vasorelaxation [7]. In another example, while *Diocleta virgata* lectin (DvirL) shows better efficacy in NO induction compared to DVL, it is inferior to that of DRL, a phenomenon that may also be explained by the distinct location of the residues in CRD. Specifically, *Diocleinae* lectins with larger distances between Arg_{228} and Leu_{99} and between Arg_{228} and Tyr_{12} present interactions that are numerous enough to stabilize a second unit of a disaccharide. These types of lectins do not display the Tyr_{12} pattern and have affinity for monosaccharides [6].

Different lectins of the *Canavalia* genus, such as ConA, ConBr (from *Canavalia brasiliensis* seeds), CGL (from *Canavalia gladiata* seeds) and ConM all exhibit relaxant effects on precontracted endothelialized rat aorta [9,13]. Bezerra and coworkers suggested CRD as the most important factor in determining NO induction by *Diocleinae* lectins. Based on this, it can be proposed that the low ability of ConBr to induce NO production results from its smaller CRD volume [3]. ConGF has shown modulatory activity in acute inflammation and antinociceptive effects [14,15]. However, the effect of ConGF in the vascular smooth muscle has not been described yet.

In this work, we report the full sequencing of ConGF by mass spectrometry. The 3-dimensional structure is solved by X-ray crystallography, and lectin co-crystallization is solved with X-Man (5-bromo-6-chloro-3-indolyl-α-D-mannopyranoside) in order to analyze lectin-carbohydrate interaction and to stabilize flexible loops in the carbohydrate recognition domain (CRD). The relaxation activity of ConGF in rat aortic rings was additionally performed to analyze the structure and activity relationship.

Materials and methods

Protein purification, crystallization and data collection

ConGF was purified from powdered *Canavalia grandiflora* seeds by affinity chromatography using a Sephadex G-50 [16]. The freeze-dried, purified lectin was resuspended in Milli-Q™ containing 3 mM X-Man (5-bromo-4-chloro-3-indolyl-a-D-mannose) at a final protein concentration of 12 mg mL^{-1} and incubated at 310 K for 1 h prior to the crystallization experiments. As described by Simões and coworkers [15], ConGF crystals were obtained using Crystal Screen II (Hampton Research, USA) reagent N° 34 (0.05 M cadmium sulfate hydrate, 0.1 M HEPES buffer at pH 7.5, and 1.0 M sodium acetate trihydrate) grown in 24-well Linbro plates at room temperature (293 K) by the vapor-diffusion method [17] in hanging drops. Suitable crystals for X-ray diffraction grew in 1 week to maximum dimensions of approximately $0.1 \times 0.3 \times 0.1$ mm. X-ray diffraction data were collected from a single crystal cooled to 100 K with a cold nitrogen-gas stream. Crystals were previously soaked in a cryoprotectant aqueous solution of glycerol (30%) to avoid ice formation. The X-ray diffraction data were collected at a wavelength of 1.47 Å using a synchrotron-radiation source (MX1 station in the National Laboratory of Synchrotron Light (LNLS), Campinas, Brazil), using a MAR CCD-detector of 165 mm (MAR Research) in 180 frames with an oscillation range of 1°.

X-ray diffraction data analysis

The dataset was indexed and integrated, and the intensities were reduced through the XDS package [18]. To solve the phase problem, we performed molecular replacement using maximum-likelihood methods implemented in the PHASER program [19]. The atomic coordinates used as a search model were those of *C. gladiata* lectin (CGL) complexed with dimannoside man1-manOMe (PDB code 2OVU) [20]. The log-likelihood-gain (LLG) solution obtained was 1378.09, and the Rotation Function Z-score (RFZ) and Translation Function Z-score (TFZ) were respectively 18.0 and 32.1. Crystallographic refinement was carried out in cycles of maximum-likelihood refinement using Refmac 5 [21].

ConGF models were submitted to a few cycles of rigid body refinement to verify the relative position of the rigid groups, followed by restrained refinement and correction and/or substitution of amino acid side chains using 2Fo-Fc. The electron density maps were generated and visualized using Coot. Point mutation in the lectin sequence, followed by addition of water molecules to the refined ConGF structure, was performed using Coot, and all the inspection was done with different Fourier maps and stereochemical criteria. Finally, the structures were submitted to restrained isotopic refinement, and the quality of the ConGF model was assessed with the Procheck software [22]. The final ConGF model was visualized with Coot [23] and PyMOL [24]. The atomic coordinates of ConGF complexed with X-man were deposited in the Protein Data Bank labeled as 4L8Q.

Molecular mass and sequence determination by mass spectrometry

The isotopic average molecular mass of ConGF was determined by electrospray ionization using a hybrid mass spectrometer (Synapt HDMS System, Waters Corp., Milford, MA, USA). Protein suspension (10 ρmol/μL) was infused into the system at a flow rate of 10 μL/min. The capillary voltage and the cone voltage were set at 3 kV and 40 V, respectively. The source temperature was maintained at 90 °C, and nitrogen was used as a drying gas (flow rate: 150 L/h). Data were acquired with the Mass Lynx 4.1 software. Multiply charged spectra were deconvoluted using maximum entropy techniques [25].

For primary structure determination, ConGF was subjected 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). For proteolytic cleavage, performed at 37 °C overnight, gels were rehydrated with the following enzyme solutions: 50 mM ammonium bicarbonate containing trypsin (Promega), chymotrypsin (Sigma–Aldrich) and endoproteinase Asp-N (Roche). The obtained peptides were extracted in a solution of 50% acetonitrile with 5% formic acid and concentrated in Speedvac [26]. ConGF was also subjected to chemical digestion with formic acid using conditions described by Hua and coworkers [27]. The peptides were injected into a nanoAcquity system connected to the electrospray source of a mass spectrometer (Synapt HDMS System,