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Research paper

# Structure of *Dioclea virgata* lectin: Relations between carbohydrate binding site and nitric oxide production

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ABSTRACT

The lectin of *Dioclea virgata* (DvirL), both native and complexed with X-man, was submitted to X-ray diffraction analysis and the crystal structure was compared to that of other Diocleinae lectins in order to better understand differences in biological properties, especially with regard to the ability of lectins to induce nitric oxide (NO) production. An association was observed between the volume of the carbohydrate recognition domain (CRD), the ability to induce NO production and the relative positions of Tyr12, Arg228 and Leu99. Thus, differences in biological activity induced by Diocleinae lectins are related to the configuration of amino acid residues in the carbohydrate binding site and to the structural conformation of subsequent regions capable of influencing site–ligand interactions. In conclusion, the ability of Diocleinae lectins to induce NO production depends on CRD configuration.

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## 1. Introduction

Lectins are proteins/glycoproteins with at least one noncatalytic domain binding reversibly to specific monosaccharides or oligosaccharides. By binding to carbohydrate moieties on the cell surface, lectins participate in a range of cellular processes without changing the properties of the carbohydrates involved [1].

Some of the most common and important uses of lectin carbohydrate binding specificity are identification of blood serotypes, preparation of bone marrow for transplantation and recognition of mammalian cell types [2–4].

Lectins of the *Diocleinae* subtribe (Leguminosae; *Papilionoideae*; *Phaseoleae*) are among the most extensively investigated. As observed by several investigators, these lectins are highly homologous in structure but differ considerably with regard to biological activity [5].

As reported for Concanavalin A (ConA, extracted from *Canavalia ensiformis*, the jack bean), Diocleinae lectins are glucose/mannose-specific [6]. ConA is the best known member of the leguminosae lectins due to a wealth of biological applications made possible by its carbohydrate binding properties [7].

Ramos and coworkers [8] investigated the fine sugar specificity of five *Diocleinae* lectins of the genera *Canavalia*, *Dioclea* and *Cratylia* in relation to specific carbohydrates and glycoproteins and found discrepancies which they attributed to the presence of fragmented subunits in the quaternary structure. Legume lectins weighing 25–30 kDa are usually composed of 2 or 4 subunits. The main α-subunit comprises 237 amino acid residues and two other subunits, β and γ (natural fragments of the α-subunit). The quaternary structure of these lectins feature a unique, highly conserved carbohydrate binding site (CBS) as well as conserved metal binding sites for divalent cations ($Ca^{2+}$ and $Mn^{2+}$). The monomers are bound by non-covalent interactions in dimers stabilized as tetramers. This has been described as a rigid β-sandwich containing six β sheets (back) and a curved β-sandwich containing seven β sheets (front) plus one smaller β sheet with five strands [9,10]. The CBS is located on the concave side on the rigid β-sandwich, next to the metal binding site, and consists of diverse loops with different degrees of variability [11].

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Despite minor differences in primary and three-dimensional structure, it remains unclear why Diocleinae lectins differ so much with regard to biological properties [5,12,13].

Knowledge of the recognition mechanism of the CBS, acquired through X-ray crystallographic studies, and of the specific interactions between the CBS and different sugars and glycans, can help establish correlations between binding activity on the cell surface and biological activities in a range of physiological systems.

Lectin was extracted from the seeds of *Dioclea virgata* (DvirL), a Brazilian vine species known locally as "cipó-pixuma" [14]. In a previous study [15], DvirL was submitted to molecular characterization and crystallization, resulting in the determination of 50 residues of the N-terminal sequence and in the formation of tetragonal crystals which diffracted to a maximum resolution of 2.9 Å. However, the data were not sufficient to solve the crystal structure.

In the present study, we performed an X-ray diffraction analysis of DvirL, both native and complexed with X-man (5-bromo-4-chloro-3-indolyl-α-D-mannose), and correlated the structural data obtained for the carbohydrate recognition domain (CRD) with data from other *Diocleinae* lectins, highlighting structural aspects relevant to understanding the ability of *Diocleinae* lectins to induce nitric oxide production in isolated aorta.

## 2. Materials and methods

### 2.1. Protein purification and hemmaglutinating activity (HA)

*D. virgata* seeds were collected at municipality of João Pessoa in Paraíba state in Northeast of 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 4 h, followed by centrifugation at 10,000 × g at 277 K for 20 min. Protein purification was carried by the affinity chromatography protocol previous described by Delatorre and co workers [16] using Sephadex G-50 column (10 × 50 cm). Briefly, the column was previously equilibrated with 0.15 M NaCl containing 5 mM $CaCl_2$ and 5 mM $MnCl_2$ and 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* glycine buffer pH 2.6 and dialyzed exhaustively against Milli-Q™ water. This fraction was freeze-dried and used for further experiments.

HA was determined in plates by double serial dilutions. Each well received 25 μL 0.1 M Tris–HCl buffer pH 7.6 containing 0.15 *M* NaCl. A 25 μL aliquot of supernatant was added to the first well of the column. Subsequently, 50 μL suspension of 2% rabbit or ABO human erythrocytes suspension treated with trypsin, papain or neither, containing 0.15 M NaCl, was added to each well. HA was measured after 30 min of incubation at 37 °C and 30 min of incubation at room temperature. HA was expressed as a titer (the reciprocal value of the highest dilution testing positive) per mg of protein. The X-Man (5-bromo-4-chloro-3-indolyl-α-D-mannose, Sigma–Aldrich, USA) specificity was determined by comparing the ability to inhibit HA. DVirL was incubated with the compound at 37 °C for 30 min prior to determination of the inhibition titer. The initial concentration of the carbohydrate tested was 100 mM. Results were expressed as the minimum concentration of sugar required to inhibit HA.

### 2.2. Protein structure determination

The purified native lectin freeze-dried was resuspended in 1 mM Tris–HCL buffer pH 7.0 containing 5 mM $CaCl_2$ and 5 mM $MnCl_2$ in a final concentration of 10 mg.$mL^{-1}$. X-Man complexed lectin was obtained by resuspension of freeze-dried native purified lectin in Milli-Q™ water containing 3 mM of X-Man (Sigma–Aldrich, USA - 12 mM stock solution in DMSO) in a final concentration of 12 mg.$mL^{-1}$. Both, native and X-Man complexed lectin were incubated at 310 K for 1 h before the crystallization assays.

DvirL crystals were obtained as described by Delatorre [16]. After a week, suitable native crystals for diffraction experiments were obtained on lithium sulfate concentrations from 0.1 to 0.5 M and PEG 8000 from 10 to 12% and complexed crystals grew in 0.5 *M* ammonium sulfate, 0.1 M sodium citrate tribasic dihydrate pH 5.6 and 1.0 M lithium sulfate monohydrate.

X-ray data were collected from a single crystal cooled to a temperature of 100 K. Crystals were soaked in a cryoprotectant solution made of water (70%) and PEG 400 (30%) to avoid ice formation and were submitted to data collection at a wavelength of 1.42 Å using a synchrotron-radiation source [MX1 station, Laboratório Nacional de Luz Síncrotron (LNLS), Campinas, Brazil]. The data were indexed and scaled as reported previously [16].

The structures were solved by molecular replacement with MolRep [17] using the lectin of *Dioclea rostrata* (PDB code: 2ZBJ) [18] as search model. The crystallographic refinement was carried out in cycles of maximum-likelihood refinement using Refmac 5 [19].

The DvirL molecular 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 [20]. Water molecules were added to the refined DvirL structures using Coot and inspection was done with difference Fourier maps and stereochemical criteria. Finally, the structures were submitted to restrained isotropic refinement and the quality of the DvirL models was assessed with the software Procheck [21]. The final DvirL models were visualized with Coot [20] and PyMOL [22]. The atomic coordinates of DvirL, both native and complexed with X-man, were labeled as 3RRD and 3RS6, respectively, and deposited in the Protein Data Bank.

### 2.3. Superposition of carbohydrate recognition domains and site volume calculation

The CRD was characterized by primary sequence analysis using the software Blast [23]. The three-dimensional structures of the *Diocleinae* lectins DvirL, Dros, Dgui, Dviol and Dgran were superimposed using the software Coot [20] and PyMOL [22]. The hydrogen bonds were quantified with Coot [20]. The volume of each CRD was calculated by the program Q-SiteFinder [24]

### 2.4. Biological assays

#### 2.4.1. Animals

Male Wistar rats (250–300 g) were kept in cages (6 in each) in a controlled environment (circadian cycle, 25 °C, food and water *ad libitum*). The experimental protocols were approved by the Institutional Animal Care and Use Committee of the State University of Ceará (UECE, Fortaleza, Ceará, Brazil) under No. 10130208-8/40, following the recommendations of the Guide for the Care and Use of Laboratory Animals of the US Department of Health and Human Services (NIH publication No. 85–23, revised 1985).

#### 2.4.2. Mechanical activity in aortic rings

Animals were euthanized by stunning, followed by prompt excision and cleaning of the thoracic aorta. Ring segments (3–5 mm) were mounted for tension recording (2 *g*) in 10 mL organ

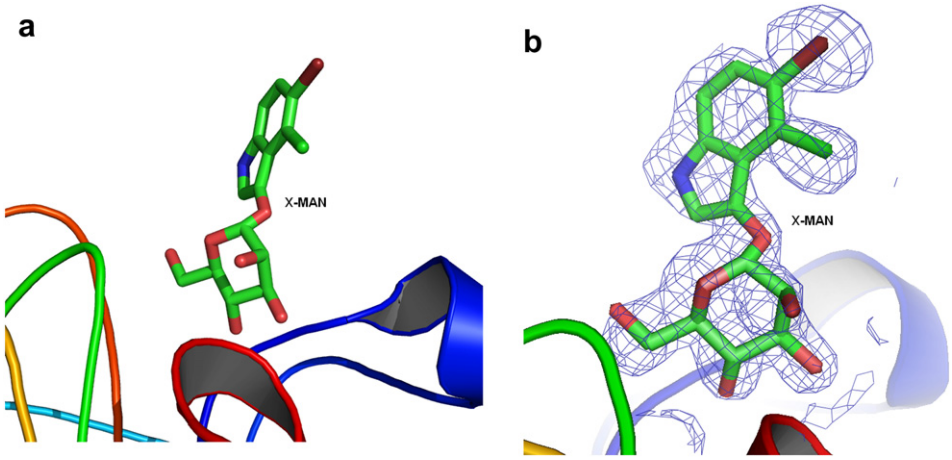


**Fig. 1.** *Dioclea virgata* lectin (DvirL) carbohydrate binding site. (a) Binding of X-man molecule in the carbohydrate binding site of DvirL. (b) X-man 2Fo–Fc electron density map contoured at 2.0 $\sigma$.

baths filled with modified Tyrode solution (in m*M*: 136 NaCl, 5 KCl, 0.98 $MgCl_2$, 2 $CaCl_2$, 0.36 $NaH_2PO_4$, 11.9 $NaHCO_3$, and 5.5 glucose) at 37 °C, 95% $O_2$ and 5% $CO_2$ (pH 7.4). In all experiments, the aortic rings were challenged with 60 m*M* KCl after 45 min of equilibrium to assure tissue viability. The contractile response was measured using a force transducer coupled to a pre-amplifier and computerized data acquisition system (DATAQ).

### 2.4.3. Lectin-induced relaxation

Cumulative concentration curves of DvirL (1–100 μg/mL) were registered at the contraction plateau induced by 0.1 μ*M* phenylephrine (PHE) in either endothelized or de-endothelized aorta. De-endothelization was achieved by mechanical rubbing of the internal aortic surface. The endothelium was considered intact when the relaxation response to 1 μ*M* acetylcholine was above 75% of the PHE induced tone [25]. Control animals received the same amount of Tyrode. To investigate the mechanism of lectin-induced relaxation, 100 μ*M* L-NAME (a nitric oxide synthase inhibitor) was added to tissues with intact endothelium 30 min prior treatment with PHE.

## 3. Results and discussion

### 3.1. Protein purification and ligand specificity

DvirL was purified by affinity chromatography in a single step purification and the hemaglutination assay permitted to determine the hemaglutination titer of 256 HU. This title was inhibited with a minimum concentration of 3.125 m*M* of X-Man. This characteristics of affinity by a dextran column and this inhibition concentration for a modified mannose are wildly described to *Diocleinae* lectins, such as ConA [6] and *Canavalia brasiliensis* lectin – ConBr [5].

### 3.2. General structure

Like ConA, the crystal structure of DvirL consists of three chains (α, β and γ) [16]. It is expressed as a pre-pro-protein (N-terminal signal peptide + γ-chain + linker peptide + β-chain + C-terminal signal peptide) cleaved into a γ-product and β-product. The active protein (α-chain) is formed by the fusion of the two smaller chains (β and γ) in inverse order, without the signal peptides and the linker peptide [26,27]. On mass spectrometry, the α-chain has a molecular mass of 25,412 ± 2 Da. The β-chain fragments and the

**Table 1**
X-ray data collection, final refinement statistics and structure analysis of the crystal structure of the lectin of *Dioclea virgata* (DvirL).

| Parameter | X-Man | Native |
|---|---|---|
| *Data collection* | | |
| Beamline wavelength (Å) | 1.42 | 1.42 |
| Space group | I222 | I222 |
| Unit cell parameters (Å) | | |
| A | 64.5 | 61.89 |
| B | 86.6 | 87.67 |
| C | 90.2 | 88.78 |
| Total reflections | 444,008 | 92,983 |
| Number of unique reflections | 23,748 | 9094 |
| Molecules per Asymmetric Unit | 1 | 1 |
| Resolution Limits (Å) | 21.33 (1.80) | 33.35 (2.45) |
| Rmerge[a] (%) | 5.6 (38.9) | 11.6 (22.6) |
| Completeness (%) | 99.7 (99.3) | 96.7 (77.9) |
| Multiplicity | 9.9 | 4.6 |
| I/sigma (Average) | 8.4 (1.8) | 5.2 (3.1) |
| *Refinement* | | |
| Resolution range (Å) | 20.93/1.80 | 33.36/2.46 |
| Rfactor[b] | 0.184 | 0.184 |
| Rfree[c] | 0.227 | 0.226 |
| Number of residues in asymmetric unit | 237 | 237 |
| Number of water molecules | 163 | 76 |
| *Temperature factors (Å)* | | |
| Average B value for whole protein chain | 21.317 | 15.642 |
| *RMS deviations* | | |
| Bond length (Å) | | |
| COUNT | 1863 | 1799 |
| RMS | 0.027 | 0.018 |
| WEIGHT | 0.022 | 0.022 |
| Bond angle (degree) | | |
| COUNT | 2554 | 2453 |
| RMS | 2.992 | 1.675 |
| WEIGHT | 1.962 | 1.943 |
| *Ramachandran plot* | | |
| Residues in most favored regions (%) | 98.25 | 94.30 |
| Residues in additional allowed regions (%) | 1.75 | 5.70 |
| Residues in generously allowed regions (%) | 0 | 0 |

Values in parenthesis represent the high resolution shell.

[a] $R_{merge} = \frac{\sum_{hkl}\sum_i|\bar{I}(hkl) - \langle I(hkl)i\rangle|}{\sum_{hkl}\sum_i\langle I(hkl)i\rangle}$ where $I(hkl)i$ is the intensity of ith measurement of the reflection h and $I(hkl)$ is the mean value of the $I(hkl)i$ for all I measurements.

[b] $R_{factor} = \frac{|F_{obs}| - |F_{calc}|}{|F_{obs}|}$.

[c] Calculated with 5% of the reflections omitted from refinement.

**Table 2**
H-bond distances in Å between the oxygen molecule of X-man and the atoms of the carbohydrate binding site in DvirL.

| Oxygen | Amino acid residues | | | | | | |
|---|---|---|---|---|---|---|---|
| DvirL | Tyr12–OH | Asn14-ND2 | Leu99-N | Tyr100-N | Asp208-OD1 | Asp208-OD2 | Arg228-N |
| O-3 | – | – | – | – | – | – | 2.7 |
| O-4 | – | 3.1 | – | – | 2.2 | 3.2 | 2.9 |
| O-5 | – | – | 3.2 | – | – | – | – |
| O-6 | – | – | – | 3.2 | – | – | – |
| N-1 | 3.1 | – | – | – | – | – | – |

γ-chain fragments weigh 12,817Da ± 2 and 12,612Da ± 2, respectively. The final protein monomer consists of 237 amino acid residues folded as a β-sandwich [28].

The DvirL diffraction analysis revealed that both the native crystal and the X-Man-complexed form are orthorhombic and belong to space group I222. The corresponding Matthews coefficients were 2.56 and 2.53 $Å^3 \cdot Da^{-1}$, indicating the presence of a monomer in the asymmetric unit of both structures [29]. Native DvirL and X-Man-complexed DvirL contain 76 and 163 water molecules, respectively, one calcium ion and one manganese ion. The X-Man molecule is placed in the CBS (Fig. 1a). The cell parameters were: a = 65.4 Å, *b* = 86.6 Å and *c* = 90.2 Å (native structure), and a = 61.89 Å, *b* = 87.67 Å and *c* = 88.78 Å (X-Man-complexed structure). The stereochemical quality of the structure was assessed with a Ramachandran plot. The results of the final refinement statistics and structure analysis of the native and X-man-complexed DvirL crystal structure are shown in Table 1.

The monomer is prior a jelly-roll domain, consisting of β-sheets connected by turns and loops. The biological assembly is a tetramer composed by dimers of canonic dimers which structurally characterize DvirL as a ConA like lectin in accordance with the oligomeric characterization of legume lectins [10]. Monomers contains manganese and calcium ions in the vicinity of the saccharide-binding site and the amino acids involved in metal binding ($Mn^{2+}$

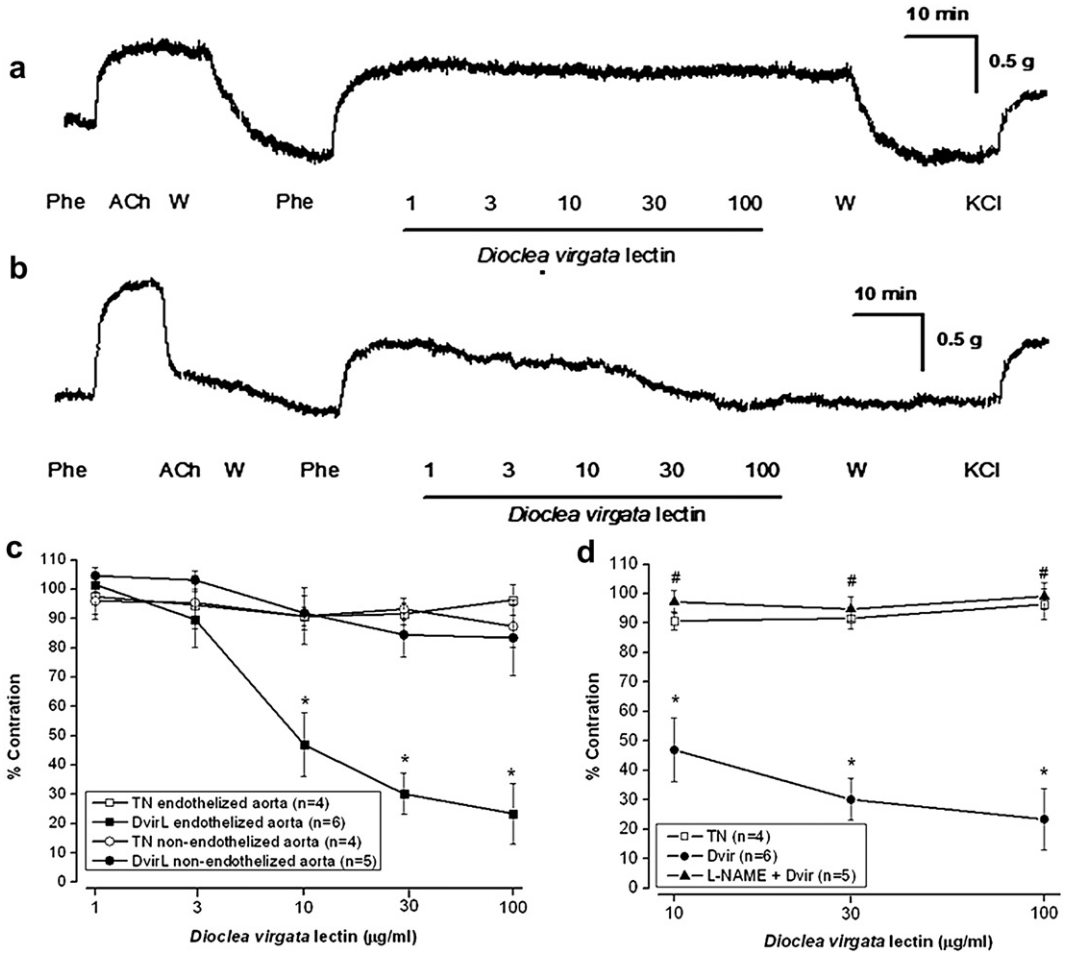


**Fig. 2.** *Dioclea virgata* lectin induces relaxation in endothelialized aortic rings pre-contracted with phenylephrine via nitric oxide. Typical traces showing the effects of DvirL (1–100 µg/mL) in de-endothelized (a) and endothelized (b) aortic rings pre-contracted with PHE. (c) Data comparing the responses of DvirL in de-endothelized and endothelized aortic rings pre-contracted with phenylephrine. (d) Data comparing the effect of DvirL (10–100 µg/mL) on phenylephrine-induced contraction of endothelized aortic rings in absence or presence of L-NAME (100 µM). Values are expressed as mean ± SEM. * = $p < 0.05$ *versus* control; # = $p < 0.05$ *versus* lectin alone; PHE = Phenylephrine (0.1 µM); ACh = Acetylcholine (1 µM); KCl = Potassium chloride (60 mM); 'W' = washing of the preparation with Tyrode's solution; "TN" = control group receiving Tyrode's solution (same volume as lectin preparation); DvirL = lectin of *D. virgata*.

**Table 3**
Relaxant activity of lectins from subtribe Diocleinae in rat aorta contracted with phenylephrine.

| Lectin | Maximal effect | $IC_{50}$ (μg/mL) | Nitric oxide participation |
|---|---|---|---|
| ConBr** | 74% (100 μg/mL) | 32.98 ± 3.66 | Yes |
| CGL** | 108% (100 g/mL) | 32.96 ± 4.92 | Yes |
| ConM* | 110% (30 μg/mL | 9.80 ± 0.60 | Yes |
| ConA* | 85% (100 μg/mL) | 26.18 ± 5.70 | n.d. |
| DvirL | 70% (30 μg/mL) | 10.47 ± 4.66 | Yes |

Lectins of *C. brasiliensis* (ConBr), *C. gladiata* (CGL), *C. maritima* (ConM), *C. ensiformis* (ConA) and *D. virgata* (DvirL). Gadelha *et al.* 2005 (*) and Assreuy *et al.*, 2009 (**). N.d.:not determined.

and $Ca^{2+}$) are conserved, and the structures of the binding sites showed similarities with those of other legume lectins. The calcium ion coordination (Asp10, Tyr12, Asn14 and Asp19) induces a trans-cis peptide bond isomerization between Ala207-Asp208. The manganese binding also establishes some coordination interactions (Glu8, Asp10, Asp19 and His24) to stabilize the carbohydrate binding site. The main amino acid residues of the carbohydrate binding site (Asn14, Leu99, Tyr100, Asp208 and Arg228) participate in establishes hydrogen bonds and Van der Waals interactions with X-Man in DvirL complexed structure.

X-man was properly placed in the CBS of the complexed structure, as shown by the 2Fo–Fc electron density map (Fig. 1b). The mannose residue is directed to the site and stabilized by a network of seven H bonds connecting oxygen atoms O-3, O-4, O-5 and O-6 of the mannoside to residues Asn14, Leu99, Tyr100, Asp208, Arg228 (Table 2). The indolyl group is stabilized by interaction with the hydroxyl group of Tyr12 and several Van der Walls interactions with the hydrophobic subsite, involving the residues Tyr12, Leu99 and Tyr100.

As a common trait, *Dioclea* lectins display no electron density in unstable regions of the structure, usually loops 117–123 and 149–151. However, in DvirL only loop 117–123 is unstable. All other regions are stable and consistent with the amino acid sequences.

### 3.3. Biological assays

PHE induced tonic contractions in aortic rings in the absence and presence of endothelium, with an amplitude of 0.649 ± 0.35 g ($n = 9$; Fig. 2a) and 0.613 ± 0.249 g ($n = 10$; Fig. 2b), respectively. DvirL ($IC_{50}$ = 10.47 ± 4.66 μg/mL) relaxed pre-contracted endothelized aortic rings by 53.04 ± 10.8% and 69.85 ± 7.1%, respectively, at 10 μg/mL (initial concentration) and 30 μg/mL (maximal effect) (Fig. 2b and c). Increasing concentration of DvirL from 30 μg/mL to 100 μg/mL did not enhance relaxation (76.57 ± 10.35%). No relaxation was observed for de-endothelized samples (Fig. 2a and c).

The influence of endothelium-derived nitric oxide, the main endothelium-derived relaxant factor, on the effect of DvirL was also evaluated. In aortic rings pre-incubated with L-NAME, an inhibitor of nitric oxide synthase, PHE induced contractions with an amplitude of 0.851 ± 0.356 g ($n = 5$). L-NAME blocked the relaxation effect of DvirL at 10, 30 and 100 μg/mL (Fig. 2d), showing that nitric oxide participates in the lectin relaxation. Another *Diocleinae* lectins also induced aorta relaxation with nitric oxide participation [13,30].

DvirL did not affect tissue responsiveness in any protocol: by the end of each experiment, the contractile response to KCl was similar to the initial tone (Fig. 2a and b).

### 3.4. CRD and biological activity

Co-crystallization of DvirL was tested using an inhibitory carbohydrate (mannose linked with the cluster X). Crystal was obtained of the lectin in complex with a derivative of mannose (5-bromo-4-chloro-3-indolyl-α-D-mannose) suitable for making an electron density map. The sugar-binding specificity of this lectin towards mannose, an abundant building block of surface-exposed glycoconjugates of viruses, bacteria and fungi, suggests a relation with defense against pathogens.

Mannose-binding affinity and biological properties are directly related to scaffolding and protein structure of binding sites for carbohydrates (*eg*, β-sheet sandwich of leguminous lectins, β-barrel of monocots) [31] and to the different quaternary structures formed from identical subunits (or monomers) [32]. The number and spatial arrangement of carbohydrate binding sites confers distinct biological properties to different oligomers with the same nominal carbohydrate binding specificity.

The ability of ConA-like lectins to induce nitric oxide production is variable (Table 3) [30,33]. Based in the present data and in other unpublished from our laboratory, in which lectins from genus *Dioclea* showed lower $IC_{50}$ compared to lectins isolated from the genus *Canavalia*, we suggest that *Dioclea* lectins are more potent nitric oxide inducers than *Canavalia* lectins. Interestingly, the structural behavior of the lectin of *Canavalia maritima* (ConM) is

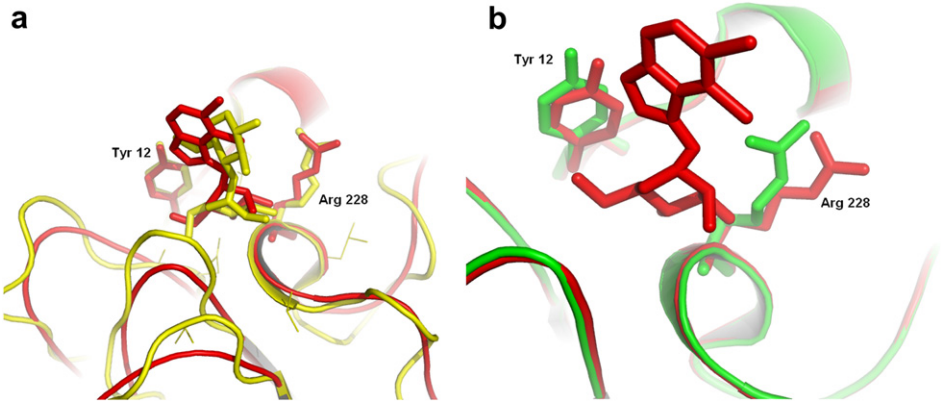


**Fig. 3.** Structural alignement of DvirL crystal structures (DvirL X-Man and DvirL Native) and ConM complexed with trehalose. (a) Superposition of DvirL X-Man (red) and ConM Trehalose (yellow). Notice that in both lectins Arg228 adopts a similar orientation. (b) Superposition of DvirL Native (green) and DvirL X-Man (red). The Arg228 adopts a proximal orientation in native molecule and a distal orientation in complexed molecule. The Arg228 in distal orientation translocates the Tyr12 to the center of carbohydrate binding site. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

anomalous for the genus: a simple mutation substituting Pro202 for Ser202 reconfigures the topology of the CRD, making it structurally close to the CRD of lectins of the genus *Dioclea* [32].

In the CRD of native DvirL, Leu99 and Arg228 are closer to each other and Leu99 and Asn14 are further apart than in ConM. The topology of ConM complexed with trehalose and of DvirL complexed with X-man is different from the topology of the respective native structures based on the r.m.s.d for main chains and side chains. The comparison between these two complexed structures reveals many differences in the arrangement of the loops involved in carbohydrate recognition (Fig. 3a). The relative distance between key residues (Table 4) is fundamental for the connection with disaccharides. In weak nitric oxide inducers of the *Diocleinae* family, the relative distance between the residues diverges from the ideal measure.

In trehalose-ConM, the second glucose ring is stabilized with an H-bond by the OH molecule of Tyr12 [32]. The same is observed for DvirL, in which Tyr12 stabilizes the X-man ring. A comparison of the two structures reveals the important role Tyr12 plays in stabilizing sugar conjugates, allowing DvirL to act as a potential ligand for mannose disaccharide derivatives, despite having a greater affinity for monosaccharides.

In a study on lectins of the genus *Canavalia*, Bezerra and coworkers [33] found an association between the volume of the CRD and its ability to induce NO production. The authors concluded that there is an ideal volume range for protein–glycan interaction, making some lectins better NO inducers than others. Several co-crystallization tests of ConBr complexed with disaccharides were performed but—in support of the authors' hypothesis—high-accuracy X-ray diffraction analysis revealed no protein–ligand complex. In other *Dioclea* lectins, biological activity also depends on CRD volume. The volume is known for the following types: Dgui = 138 $Å^3$ (PDB code 2JDZ), DvirL = 134 $Å^3$ (PDB code 3RS6), DRL = 157 $Å^3$ (PDB code 2ZBJ) and DVL = 179 $Å^3$ (PDB code 2GDF).

Differences in the CRD before and after binding to X-man are evident (Fig. 3b). In complexed DvirL, residue Arg228 is located away from the center of the CRD, the site is enlarged along the longitudinal axis, and the structure is narrowed along the transverse axis due to the approximation of Tyr12, allowing better interaction with endothelial cell surface glycans. Because the structure is expanded in one direction and contracted in another, native and complexed DvirL do not differ substantially in volume.

The fact that DvirL is a better NO inducer than other *Dioclea* lectins may be explained by the strategic location of the residues, that allows a greater number of interactions with the first saccharide unit and the approaching Tyr12, facilitating contact with the glycan. In Diocleinae CRDs with greater distances between Arg228 and Leu99, and between Arg228 and Tyr12, interactions are numerous enough to stabilize a second carbohydrate unit. In lectins with large CRD volume, the residue Arg228 is positioned distally on the CRD, allowing interaction with only one glycoside unit. Such lectins do not display the Tyr12 pattern and have affinity for monosaccharides.

Tetrameric biological assembly of legume lectins is as important as the volume of the CDR in determining the potential to induce nitric oxide production. Lectins present dimer–tetramer equilibrium depends on pH and those which have a composition of dimers and tetramers at pH between 7.0 and 8.0 are less efficient in inducing nitric oxide production. Dgui lectin has pH-dependent dimer–tetramer equilibrium [18], and although the volume site is very close to the DVirL, Dgui does not present the same efficiency in stimulates NO production.

**Table 4**
Relative distances between key amino acid residues for interaction with disaccharide for the structures of DvirL and ConM in the native forms and complexed forms.

| Amino acid residues | Native DvirL | DvirL X-man | Native ConM | ConM maltose |
|---|---|---|---|---|
| Gly 227–Tyr 12 | 10.0 | 9.3 | 9.0 | 8.9 |
| Leu 99–Tyr 12 | 9.2 | 7.7 | 9.0 | 7.1 |
| Leu 99–Asn 14 | 9.6 | 10.9 | 9.1 | 10.4 |
| Tyr 100–Tyr 12 | 6.6 | 5.2 | 5.3 | 5.4 |
| Leu 99–Arg 228 | 9.4 | 12.2 | 11.2 | 12.3 |

## 4. Conclusion

Our results indicate an association between CRD volume, oligomerization and the ability to induce NO production. In addition, the relative position of Tyr12, Arg228 and Leu99 were found to interfere with the ability of *Diocleinae* lectins to interact with glycans, explaining differences in biological properties despite their considerable homology. In conclusion, biological activities induced by lectins depend on the configuration of the amino acid residues in the carbohydrate binding site and on the structural conformation of surround regions capable of influencing site–ligand interactions. Thus, the ability of *Diocleinae* lectins to induce NO production depends on CRD reconfiguration, increasing the affinity for disaccharides. Summarizing, lectins with Arg228 in the proximal position have greater affinity for glycans and are therefore better NO inducers.

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