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A novel vasorelaxant lectin purified from seeds of *Clathrotropis nitida*: partial characterization and immobilization in chitosan beads

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ABSTRACT

A novel lectin from seeds of *Clathrotropis nitida* (CNA) was purified and characterized. CNA is a glycoprotein containing approximately 3.3% carbohydrates in its structure. CNA promoted intense agglutination of rabbit erythrocytes, which was inhibited by galactosides and porcine stomach mucin (PSM). The lectin maintained its hemagglutinating activity after incubation in a wide range of temperatures (30–60 °C) and pH (6.0–7.0), and its binding activity was dependent on divalent cations (Ca^{+2} and Mg^{+2}). SDS-PAGE showed an electrophoretic profile consisting of a single band of 28 kDa, as confirmed by electrospray ionization mass spectrometry, which indicated an average molecular mass of 27,406 ± 2 Da and the possible presence of isoforms and glycoforms. In addition, CNA exhibited no toxicity to *Artemia* sp. nauplii and elicited reversible and dose-dependent vasorelaxation in precontracted aortic rings. CNA was successfully immobilized on chitosan beads and was able to capture PSM in solution. This study demonstrated that CNA is a lectin that has potential as a biotechnological tool in glycomics and glycoproteomics applications.

1. Introduction

Lectins are defined as a structurally heterogeneous group of proteins or glycoproteins of non-immune origin containing at least one non-catalytic domain that selectively recognizes and reversibly binds to specific monosaccharides or oligosaccharides without altering the carbohydrate structure [57]. These proteins can be found in all life kingdoms from the simplest living being, such as bacteria and fungi, to more complex organisms, such as plants and animals [1,13,56].

Plant lectins play several biological roles in, for example, cell signaling and plant defense [51]. In addition, these proteins elicit antitumor [61], antiviral [29], vasorelaxant [7,10,53], and pro- and anti-inflammatory [3,7,45,49] activities. Lectins have also been used for several biotechnological applications, including drug delivery [23] and disease diagnosis [24], as well as facilitating glycomic studies. Furthermore, these lectins immobilized on support material can be used in affinity chromatography for analytical testing [46] or for enrichment of glycoconjugate preparates, such as *Dioclea lasiophylla* and *Canavalia virosa* lectins, which were

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immobilized on CNBr-Sepharose 4B, enabling the capture of glycoproteins in solution [37,41]. ConA, WGA and ricin (RCA_{120}) were efficiently immobilized on silica microparticles and could bind to glycoproteins and glycopeptides [38]. PHA was conjugated with fluorescent chitosan nanoparticles, and the bioconjugates were shown to be relatively stable [31].

Most studies describing lectins of the subfamily *Papilionoideae* (*Leguminosae*) are related to members of the *Phaseoleae* and *Vicieae* tribes, whereas only few have focused the *Sophoreae* tribe [62]. Among lectins of the *Sophoreae* tribe, *Bowringia mildbraedii and Luetzelburgia auriculata* [36], as well as species of the *Sophora* genus (*Sophora japonica*, *Sophora alopecuroides*, *Sophora flavencens*), are representative of lectins isolated and biochemically characterized [30,32].

The present study reports the purification and physicochemical characterization of a novel galactose-binding lectin isolated from seeds of *Clathrotropis nitida* of the Sophoreae tribe. Lectin immobilization on chitosan beads was demonstrated, as well as cytotoxicity and vasorelaxant effects.

2. Materials and methods

2.1. Plant material

The mature seeds of *C. nitida* were collected in the Amazon rainforest, Brazil (Amazonas) and identified by the National Institute for Research of the Amazon (MCTI-INPA). Voucher: HUEFS 3334 (AM).

2.2. Soluble protein extract preparation and hemagglutination assay

Mature seeds from *C. nitida* were peeled and ground into a fine powder using a coffee mill. The obtained flour was diluted 1:10 (w/v) in 100 mM Tris–HCl buffer (pH 7.6) containing 150 mM NaCl, stirred for 4 h at room temperature, and centrifuged at 9000 × *g* at 4 °C for 20 min. The supernatant was then collected and filtrated.

The hemagglutination assays were performed in microtitration plates by serial dilution of rabbit and human (ABO system) erythrocytes, either native or treated with proteolytic enzymes (trypsin or papain) [44]. Hemagglutination unit (H.U.) was expressed as a titer (the value of the highest dilution giving a positive hemagglutination) per mL of sample. Specific activity was defined as the number of hemagglutinating units per mg of protein (H.U./mg).

The specificity of CNA for carbohydrates was determined by the minimal sugar concentration able to inhibit hemagglutination. Monosaccharides, disaccharides and glycoproteins were used. Serial dilutions of carbohydrates, starting with an initial concentration of 100 mM for sugars and 5 mg/mL for glycoproteins, were performed, and the lectin (64 H.U.) was added to each dilution. After incubation, rabbit erythrocytes were added and incubated for 1 h at 37 °C.

2.3. Lectin purification

Proteins of the soluble extract were precipitated by addition of solid ammonium sulfate until 80% saturation in the solution (fraction 0–80%). The sample was centrifuged at 9000 × *g* at 4 °C for 20 min, followed by resuspension of the obtained pellet in ultrapure water (Milli-Q system, Millipore Corporation) and dialyzation against distilled water. This dialyzed fraction was lyophilized, solubilized in 100 mM Tris–HCl buffer (pH 7.6) with 150 mM NaCl and centrifuged at 4000 × *g* for 10 min at room temperature. The obtained supernatant was applied into a guar gum affinity matrix (Sigma–Aldrich) equilibrated with the same buffer. The unbound proteins (unretained peak (PI)) were desorbed with the previous buffer. The lectin was eluted using 200 mM galactose in the equilibrium buffer, and the retained peak (PII) was collected in 1 mL fractions and monitored by spectrophotometry at 280 nm. The PII was dialyzed against ultra-pure water and freeze-dried. Protein content was determined as described by [14] using bovine serum albumin as standard protein.

2.4. Effect of the temperature, pH and metal ions on lectin hemagglutinating activity

The influence of different temperatures on CNA hemagglutinating activity was measured by incubation of the lectin at different temperatures ranging from 30 °C up to 100 °C with 10 °C increments for 1 h. The influence of pH on lectin stability was determined by hemagglutinating activity after dialysis of lectin against buffers in different pH (4.0–10.0) containing 150 mM NaCl for 24 h. The buffer solutions used in this experiment were 100 mM sodium citrate (pH 4.0 and 6.0), 100 mM sodium acetate (pH 5.0), 100 mM sodium phosphate (pH 7.0), 100 mM Tris–HCl (pH 8.0), and 100 mM glycine–NaOH (pH 9.0 and 10.0). To evaluate the dependence of divalent cations on CNA activity, a CNA sample (200 μg/mL) was demetalized by dialysis against 100 mM ethylenediaminetetraacetic acid (EDTA) containing 150 mM NaCl for 24 h. For the removal of excess EDTA, the sample was dialyzed against 150 mM NaCl. The change in activity was determined after addition of 5 mM $CaCl_2$ and 5 mM $MnCl_2$. In both cases, hemagglutinating activity of the samples was determined.

2.5. Polyacrylamide gel electrophoresis (SDS-PAGE) and carbohydrate content

Lectin apparent mass and purification grade were determined by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (150 V, 25 mA and 10 W) in the presence or absence of β-mercaptoethanol [28]. Afterwards, electrophoresis gels were stained with Coomassie brilliant blue (R-350), and lectin apparent molecular masses were estimated. The presence of carbohydrates in the lectin structure was determined by periodic acid-Schiff staining [59]. Moreover, the neutral sugar content of the lectin was estimated by the phenol–sulfuric acid method [18].

2.6. Molecular mass determination by MS and N-terminal analysis

The isotopic average molecular mass of the lectin was determined by electrospray ionization-mass spectrometry (ESI-MS) using a hybrid quadrupole/ion mobility separator/orthogonal acceleration–time of flight mass spectrometer (Synapt HDMS system; Waters Corp., Milford, USA). The protein was solubilized in 50% acetonitrile and 0.1% formic acid at approximately 10 pmol/μL. The sample was applied at a flow rate of 10 mL/min, and the voltages on the capillary and on the cone were adjusted to 3.0 kV and 40 V, respectively. Data acquisition was performed using MassLynx 4.1 software, and the multiply charged spectra were deconvoluted using maximum entropy techniques [20]. The N-terminal sequence was obtained by Edman degradation using a Model PPSQ-31A automated sequencer (Shimadzu Corp, Japan). The cleaved phenylthiohydantoin-amino acids were separated using a C18 column (Wakosil; 2.0 × 250 mm) connected to a Model LC20AT pump and detected by absorbance at 269 nm. The comparison between the obtained sequence and database sequences was performed by BLASTP (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.7. Gel filtration chromatography

Lectin native mass was estimated by gel filtration in BioSuite™

Table 1
Inhibitory effect of saccharides and glycoproteins on the hemagglutinating activity of *Clathrotropis nitida* lectin.

Sugar and glycoproteins	MIC[a]
D-glucose	N.I[b]
D-mannose	N.I
α-Methyl-D-mannoside	N.I
N-acetyl-D-glucosamine	N.I
D-galactose	12.5 mM
α-Methyl-D-galactopyranoside	12.5 mM
β-Methyl-D-galactopyranoside	12.5 mM
N-acetyl-D-galactosamine	6.25 mM
4-Nitrophenyl-α-D-galactopyranoside	1.56 mM
4-Nitrophenyl-β-D-galactopyranoside	1.56 mM
α-Lactose	12.5 mM
Sucrose	N.I
Porcine stomach mucin	0.125 mg/mL
Fetuin	N.I
Ovalbumin	N.I

[a] MIC, minimum inhibitory concentration.
[b] NI, sugar and glycoproteins not inhibitory until a concentration of 100 mM and 1 mg/mL, respectively.

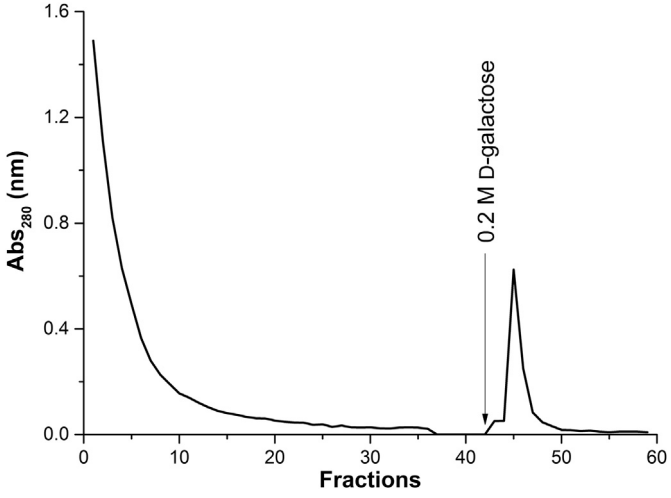

Fig. 1. Elution profile of CNA in guar gum affinity cromatography. The fraction (0–80%) was applied in column and equilibrated with 100 mM Tris–HCl buffer (pH 7.6) containing 150 mM NaCl. Lectin was eluted with 200 mM D-galactose in the equilibrium buffer at a flow rate of 1 mL/min. Fractions (1.5 mL) were collected and monitored at $A_{280\ nm}$.

250 (7.8 × 300 mm, Waters), equilibrated with 50 mM Tris–HCl buffer (pH 7.6) containing 500 mM NaCl. A CNA sample (2 mg/mL; 0.5 mL) was loaded in the column, and chromatography was carried out at a flow rate of 500 μL/min in a nanoACQUITY UPLC System (Waters Corp.), calibrated with standard proteins: conalbumin (75 kDa), ovalbumin (44 kDa) carbonic anhydrase (29 kDa), ribonuclease (13.7 kDa) and aprotinin (6.5 kDa).

2.8. Artemia lethality test

CNA toxicity was determined by *Artemia* lethality test, as described by [42].

2.9. Evaluation of relaxant effects on aorta rings

Male Wistar rats (250–300 g) were manipulated using protocols approved by the Ethics Committee of UECE (CEUA N° 10130208-8/40). Ring segments of thoracic aorta (3–5 mm) were mounted for tension recording (2 g) in 10-ml organ baths filled with modified Tyrode's solution (in mM: 136 NaCl, 5 KCl, 0.98 $MgCl_2$, 2 $CaCl_2$, 0.36 NaH_2PO_4, 11.9 $NaHCO_3$, 5.5 glucose) at pH = 7.4, 37 °C, 95% O_2, and 5% CO_2. After an equilibrium period of 60 min, the rings were challenged with 60 mM KCl to ensure tissue viability. The contractile response was measured using a force transducer, connected to a preamplifier and computerized data acquisition system (Chart 4.1; PowerLab ADInstruments, Inc., Australia). CNA was added in cumulative concentrations (1, 3, 10, 30 and 100 μg/ml) at 10 min intervals to tissues precontracted with phenylephrine (0.1 μM), in either endothelium intact or denuded aorta. De-endothelialization was assessed by mechanical rubbing of the internal aortic surface and intact endothelium considered for relaxant responses to acetylcholine (1 μM) greater than 75% [21]. Participation of the endothelium-derived relaxant factor (EDRF) nitric oxide on CNA relaxant effect was assessed by incubation of endothelialized aorta with N-(G)-nitro-L-arginine methyl ester (L-NAME; 100 μM) and 1H-[1,2,4]oxadiazole-[4,3-a]quinoxaline-1-one (ODQ; 10 μM) 30 min before addition of lectin on the plateau of phenylephrine-induced contraction. Further, CNA (100 μg/mL) was incubated with D-galactose (0.1 M) for 1 h at 37 °C to allow lectin-sugar interaction prior to experiments.

2.10. Preparation of chitosan beads and lectin immobilization

Chitosan beads were prepared with protocol adapted from [54].

For immobilization, the activated beads were incubated with CNA (1.0 mg/mL) in 50 mM sodium phosphate buffer, pH 7.0, under stirring for 24 h at 25 °C. The beads were washed with 50 mM Tris–HCl buffer, pH 7.6, containing 0.5 M NaCl to remove unbound protein. To analyze immobilization performance and the capacity of CNA-chitosan beads to purify glycoproteins, trials were carried out with porcine stomach mucin (PSM). PSM (1.2 mg) was loaded on a CNA-Chitosan column (0.8 × 2.7 cm) previously equilibrated with 50 mM Tris–HCl buffer, pH 7.6, containing 0.5 M NaCl (flow rate of 0.33 mL/min). The lectin affinity support was washed with the same buffer, and bound proteins were eluted with 0.1 M sodium acetate, pH 4.0, containing 0.5 M NaCl. Eluted fractions were monitored by absorbance at 280 nm.

3. Results

3.1. Hemagglutination and inhibition assay

The soluble protein extract of *C. nitida* seeds showed relatively high levels of hemagglutinating activity against native rabbit erythrocytes natives (128 H.U.) and those treated with papain (128 H.U.) or trypsin (256 H.U.). Hemagglutinating activity in human erythrocytes of ABO system was also observed: type A (4 H.U.), type B (8 H.U.) and type O (16 H.U.) with or without enzymatic

Table 2
Purification of *Clathrotropis nitida* lectin.

Fraction	Total protein (mg/ml)[a]	Total H.U.[b]	Specific activity (H.U./mg)[c]	Purification (fold)
Crude extract	1.85	256	186.6	1
Fraction 0–80%	3.06	1024	334.6	1.79
Peak II (Guar gum)	0.078	1024	13128.20	70.35

[a] Protein content.
[b] Hemagglutinating activity expressed in hemagglutinating units (H.U.).
[c] Specific activity calculated as the ratio between hemagglutinating activity and protein content.

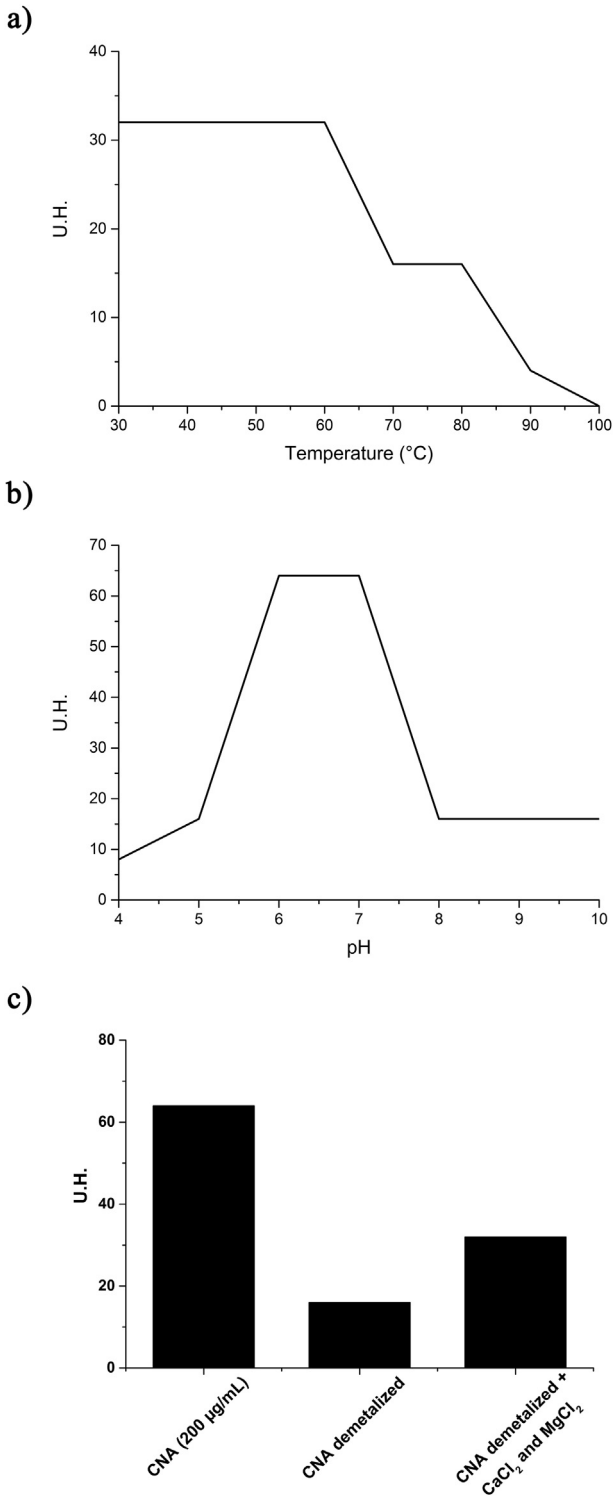

Fig. 2. Effect of various physicochemical parameters on CNA hemagglutinating activity. **A)** Different temperatures, **B)** different pH, **C)** demetallization and addition of divalent cations.

treatment. Hemagglutinating activity was inhibited by galactosides and PSM (Table 1).

3.2. Lectin purification

Ammonium sulfate precipitation of the protein extract produced a protein fraction (fraction 0–80%) that showed strong hemagglutinating activity (data not shown). This fraction was applied to a guar gum affinity column. The unbound material (peak I) was eluted with equilibrium buffer and showed no hemagglutinating activity. The retained peak (peak II), showing hemagglutinating activity, was eluted with 200 mM D-galactose in the equilibrium buffer resulting in a pure lectin which was named CNA (*C. nitida* agglutinin) (Fig. 1). Protein content, hemagglutinating titer and specific activity are shown in Table 2.

3.3. Effect of temperature, pH and divalent cations on lectin hemagglutinating activity

CNA was shown to be thermostable, and its hemagglutinating activity was maintained after incubation for 1 h under temperatures that varied from 30 °C to 60 °C. At highest temperatures (70 °C–90 °C), the activity was abruptly reduced, and at 100 °C, it was completely lost (Fig. 2A). CNA hemagglutinating activity was maintained over a relatively wide pH variation, and showed more stability between pH 6.0 and 7.0 (Fig. 2B). Dialysis against EDTA decreased CNA hemagglutination activity, while the addition of divalent cations (Ca^{+2} and Mg^{+2}) partially recovered the activity (Fig. 2C).

3.4. Molecular mass determination, carbohydrate content and N-terminal analysis

SDS-PAGE showed a single band of apparent molecular mass approximating 28 kDa in the presence or absence of β-mercaptoethanol, demonstrating the absence of disulfide bridges (Fig. 3A). Furthermore, CNA was stained by periodic acid Schiff's reagent (Fig. 3A), demonstrating that the lectin is a glycoprotein. According to the phenol-sulfuric acid method, CNA is composed of about 3.3% of carbohydrates.

In agreement with SDS-PAGE results, the analysis of CNA by electrospray ionization mass spectrometry indicated a predominant ion of 27,406 ± 2 Da and other less abundant ions possibly corresponding to isoforms and glycoforms of the protein (Fig. 3B). When applied directly onto an automated protein sequencer, CNA (0.3 nmol) revealed a 20-amino acid sequence of the N-terminus: SDELSFTFNLFVPNQQDLIF. The sequence showed significant similarity with other lectins isolated from the *Sophoreae* and *Genisteae* tribes (Fig. 4).

For native lectin, analytical gel size-exclusion chromatography of CNA showed a single sharp symmetrical peak, ensuring lectin purity with molecular mass of approximately 85 kDa (data not shown).

3.5. Artemia lethality test

CNA showed no lethality against *Artemia* sp. nauplii at any concentration tested, indicating that CNA is innocuous.

3.6. Evaluation of relaxant effects

Phenylephrine induced stable tonic contractions of 0.40 ± 0.05 g in aortic ring preparations possessing an intact endothelium (n = 5) and 0.65 ± 0.12 g in those in which endothelium had been mechanically removed (n = 4). In preparations with preserved endothelium, CNA (IC_{50} = 20.33 ± 9.48 µg/mL) elicited relaxation at 30 and 100 µg/ml in 57.00 ± 17.74% and 78.8 ± 17.19%, respectively. However, in preparations of denuded endothelium, cumulative addition of CNA was not able to induce relaxant effect (Fig. 5A–B). In addition, L-NAME and ODQ completely blocked lectin effect (Fig. 5C). Incubation of CNA with its specific binding sugar D-galactose completely inhibited the relaxant effect of CNA,

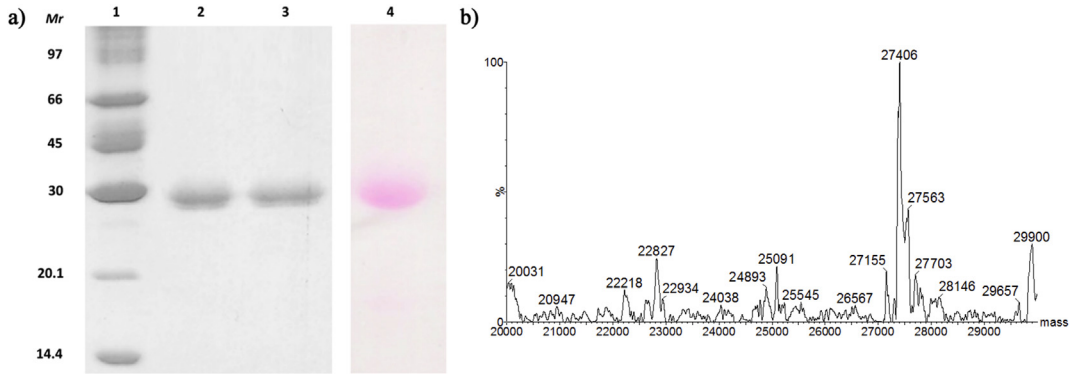

Fig. 3. A) Electrophoretic profile (SDS-PAGE) of CNA. Lane 1: Molecular mass markers: phosphorylase b, 97 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 30 kDa; α-lactalbumin, 14.4 kDa; Lane 2: retained fraction of guar gum column (CNA); Lane 3: CNA + β-mercaptoethanol; Lane 4: CNA stained with periodic acid–Schiff's reagent. **B)** Spectrum acquired by mass spectrometry with a 10 pmol/μL sample and deconvoluted using the MaxEnt 1 program available on MassLynx 4.1 software package from Waters.

demonstrating the participation of lectin domain (Fig. 5D), which suggests lectin interaction with glycans present in endothelial cells.

3.7. Lectin immobilization on chitosan beads

CNA was successfully immobilized on chitosan beads (1.95 mg of CNA immobilized) and was capable of strongly binding to PSM that was eluted with buffer 0.1 M sodium acetate, pH 4.0, containing 0.5 M NaCl (Fig. 6). These data demonstrated that the carbohydrate recognition domains (CRD) remain active after immobilization and that they account for the interaction of CNA-chitosan with PSM. CNA-chitosan column was able to efficiently isolate PSM (0.465 mg), displaying high potential for glycoproteomics studies.

4. Discussion

Species from the genus *Clathrotropis* (*Sophoreae* tribe) may be found in Venezuela, Colombia and Brazil. Some authors have described the presence of alkaloid in the genus *Clathrotropis* [26,47,48]; however, no study has reported lectin activity in species of this genus.

The crude extract of *C. nitida* seeds showed strong hemagglutinating activity against rabbit erythrocytes, both native and treated with proteolytic enzymes; however, it showed low activity against human erythrocytes. Hemagglutinating results of this study agree with those of other *Sophoreae* lectins, such as the lectins of *L. auriculata*, *Sophora flavescens* and *S. alopecuroides* [30,32,36].

CNA hemagglutinating activity was inhibited by galactosides. Previous studies demonstrated that legume lectins have greater specificity for carbohydrates that possess substituents with high hydrophobicity in carbons 1 and 2 [9,39,41,44,52]. In the case of CNA the methyl group present at carbon 1 of methyl-α-D-galactopyranoside and methyl-β-D-galactopyranoside did not change its specificity compared to D-galactose. On the other hand, N-acetyl-D-galactosamine, 4-nitrophenyl-α-D-galactopyranoside and 4-nitrophenyl-β-D-galactopyranoside increased the specificity of the lectin present in the extract. These data suggest additional interactions with some hydrophobic groups at the carbohydrate-binding site, such as acetyl and 4-nitrophenyl, increase lectin affinity for carbohydrates. Among the glycoproteins tested, only PSM inhibited the hemagglutinating activity of CNA. PSM is a glycoprotein rich in D-galactose and N-acetyl-D-galactosamine residues [8], which explains this effect.

Fig. 4. Alignment of CNA N-terminal sequence, as obtained by Edman degradation, with lectins from the tribes *Sophoreae* (*Maackia amurensis* and *Sophora japonica*) and *Genisteae* (*Ulex europaeus*).

The new lectin (CNA) was purified by one-step affinity chromatography in a galactomannan column, which has shown high efficiency in the purification of galactose-specific leguminous lectins [2,53,55]. The physicochemical characterization of CNA demonstrated a stability profile close to that of other leguminous lectins [5,37].

Several lectins from the *Sophoreae* tribe and tribes evolutionarily close, such as *Dalbergieae*, present a major eletrophoretic band and low molecular mass peptides, as a result of post-translational processing. For example, *L. auriculata* lectin (*Sophoreae* tribe) shows a major band of 29 kDa and two minor bands around 15 kDa. *B. mildbraedii* lectin (*Sophoreae* tribe) shows a major band of 26 kDa and three more minor bands of 16, 14 and 12 kDa [6,36]. On the other hand, CNA showed an electrophoretic profile consisting of a broad band of 28 kDa without the presence of other components, as also observed in other lectins from the genus *Sophora* and *Platymiscium floribundum* lectin (*Dalbergieae* tribe) [25,30,32,39]. In agreement with SDS-PAGE, electrospray mass spectrometry analysis of CNA indicated a molecular mass of 27,406 ± 2 Da and other less abundant ions, possibly corresponding to isoforms and glycoforms of the protein, similar to the results observed for the lectin from *Dialium guineense* [9]. The N-terminal sequence of CNA showed 75% identity with the N-terminal of *Maackia amurensis* lectin (GenBank accession code: AAB39934.1), 74% with *S. japonica* lectin (GenBank accession code: AAB51442), both belonging to the *Sophoreae* tribe, and this sequence also presented 65% identity with *Ulex europaeus* lectin (GenBank accession code: AAG16779.1) of the *Genisteae* tribe. The result obtained by gel filtration chromatography suggests that the lectin is a homotrimer. However, most known legume lectins are homodimers or homotetramers, with the tetramers being dimers of dimers [15].

The carbohydrate content of CNA (3.3%) is close to that of other lectins from the *Sophoreae* tribe, such as *L. auriculata* lectin having 3.2% of carbohydrates, *S. alopecuroides* lectin having 2.89% of carbohydrates, as well as lectins of seeds, leaves and barks from *S. japonica* that show between 3 and 10% of carbohydrates

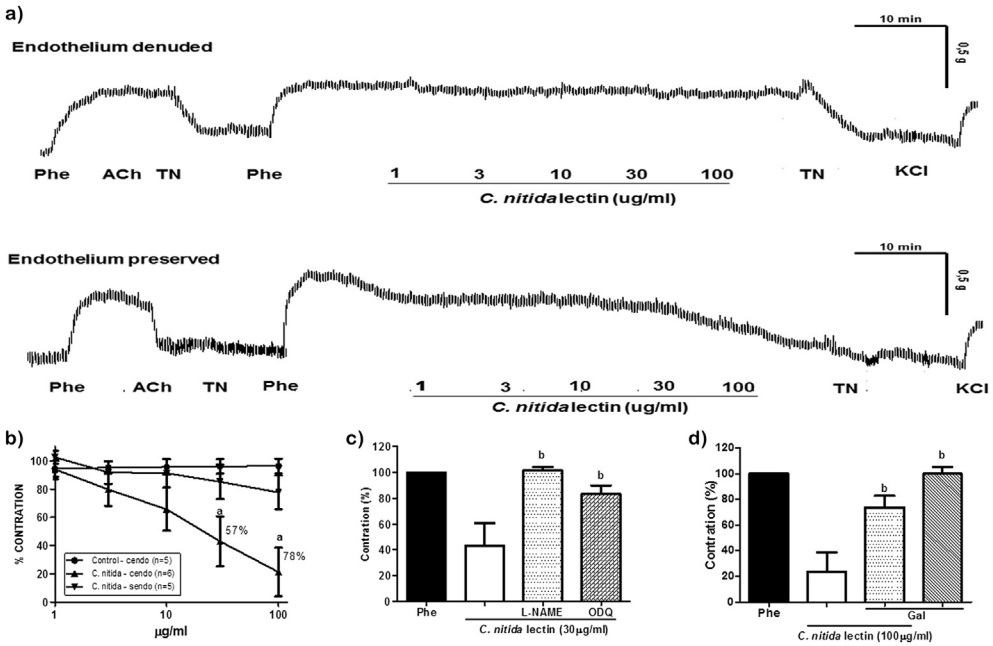

Fig. 5. CNA induces endothelium-dependent relaxation in isolated rat aorta, which involves NO and the lectin domain. **A)** Typical traces showing CNA effect in endothelium intact or denuded aorta; **B)** Data comparing the responses of CNA (1–100 µg/mL) to control (100% of phenylephrine (Phe) contraction) in endothelialized and denuded aorta; **C)** Reversion of CNA (30 µg/mL) relaxant responses by L-NAME and ODQ. **D)** Reversion of CNA (100 µg/mL) relaxant responses by galactose. Mean ± S.E.M. (n = 4–6); [a]p<0.05 compared to Phe; [b]p < 0.05 compared to CNA TN: washing with Tyrode's solution. Gal:galactose.

[25,36,43]. Moreover, it is well known that several leguminous lectins are glycoproteins, such as lectins of the tribes *Swartzieae*, *Sophoreae* and *Dalbergiae* that present glycosylation [17,19,36,53].

Although few studies have reported on the toxicity of lectins by the use of *Artemia* lethality test [16,40], this test has been applied as a preliminary toxicity assay commonly performed before other bioassays. Some lectins have been found to exhibit toxic and cytotoxic activities using this assay, such as lectins from *Diocleinae* subtribe and three lectins isolated from *Cassia fistula* that showed high toxicity [4,50]. On the other hand, similar to CNA, the lectin of *Sebastiania jacobinensis* exhibited no toxicity against *Artemia* sp. nauplii [58].

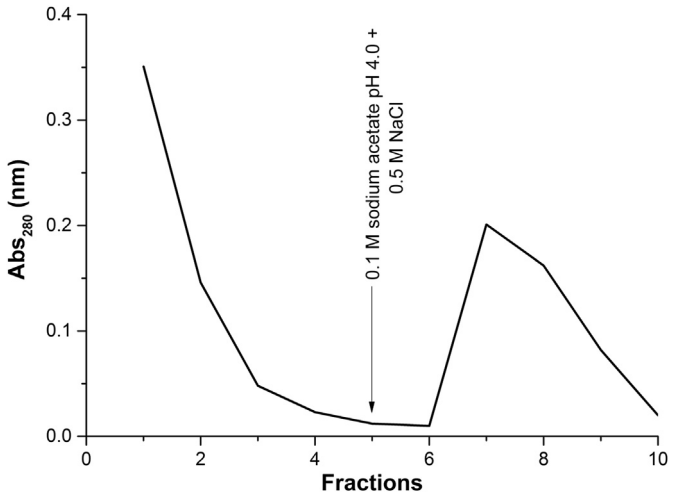

Fig. 6. Chromatography of PSM on CNA-Chitosan column. The column (0.8 × 2.7 cm) was equilibrated with 50 mM Tris–HCl buffer, pH 7.6, containing 0.5 M NaCl. PSM (1.2 mg) was applied to the column. The lectin affinity support was washed with the same buffer. Elution was carried out at 25 °C at a flow rate of 3 mL/min. Arrows demonstrate the points at which eluent was added. Fractions of 1.0 mL were collected.

Other *Leguminosae* lectins, especially those from the *Diocleinae* subtribe, present relaxant effects in rat aorta precontracted with phenylephrine via NO and the lectin domain, both in *Dioclea* and *Canavalia* genus [7,12,27]. However, this is the first study reporting on the vasorelaxant effect of a lectin from the *Clathrotropis* genus. The relaxation elicited by CNA was found to be strictly dependent on the intact endothelium and highlighted the importance of lectin-carbohydrate binding sites. The lectin of *Vatairea guianensis*, also possessing binding specificity for galactose, had its relaxant effect reduced by galactose [53]. To our knowledge no studies have reported on the vasorelaxant effect of lectins from the *Sophoreae* tribe.

NO is the principal mediator of endothelium-dependent relaxation in vascular smooth muscles [21]. After its synthesis by NOS, NO diffuses to adjacent smooth muscle cells and acts through activation of soluble guanylate cyclase, thereby increasing intracellular GMPc concentration and promoting relaxation [11]. Thus, the role of NO can be determined if the relaxant responses are inhibited by several substituted L-arginine analogues [34]. In order to verify the participation of the enzyme and the NO/GMPc pathway, we incubated preparations with L-NAME, a non-selective inhibitor of NOS, and ODQ, an inhibitor of the NO-sensitive guanylate cyclase [22]. NO seems to be responsible for vasorelaxant effect of CNA. In fact, our results showed that L-NAME and ODQ blocked the vasorelaxant effects of CNA.

Lectin affinity chromatography is based on separation of sample through affinity chromatography columns containing immobilized lectins [33]. According to Gemeiner et al. (2008); assays based on multilectin affinity chromatography coupled with mass spectrometry show promise for lectin-based diagnostic methods [35]. immobilized a GalNac-specific lectin from *Dolichos biflorus* in Sepharose 6B, and by lectin-affinity chromatography, normal leukocytes were separated from leukemia T-cell lines. Moreover, using a combination of lectin affinity chromatography and lectin microarray [60], were able to differentiate pancreatic cancer from chronic pancreatitis. Thus, in the future, the immobilization of CNA in chitosan matrix may be an important diagnostic tool.

5. Conclusions

In summary, we purified and characterized a novel galactose-binding lectin from seeds of *C. nitida* (named CNA). In comparison with other legume lectins, CNA showed some differences in its specificity to carbohydrates and in its structure. The purified lectin exhibited no toxicity to *Artemia* sp. nauplii, demonstrated vaso-relaxant effect in aortic rings, and was successfully immobilized on chitosan beads, demonstrating that CNA can be applied as biotechnological tool. This is the first report of the purification, characterization and application of a lectin from the *Clathrotropis* genus.

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