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Purification and primary structure determination of a galactose-specific lectin from *Vatairea guianensis* Aublet seeds that exhibits vasorelaxant effect

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ABSTRACT

Vatairea guianensis seeds, a typical plant from the Brazilian Amazon region that belongs to the Dalbergieae tribe, possess a lectin that was isolated by precipitation with solid ammonium sulfate followed by guar gum affinity chromatography. This lectin was named VGL. The *V. guianensis* lectin strongly agglutinated rabbit erythrocytes and was inhibited by D-galactose and D-galactose-derived sugars, especially N-acetyl-D-galactosamine. VGL has been shown to be a stable protein, maintaining its hemagglutinating activity after incubation at a wide range of temperature and pH values and after incubation with ethylenediamine tetraacetic acid (EDTA). In a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, the purified VGL exhibited an electrophoretic profile consisting of a major 30–32 kDa double band, which is termed the alpha-chain, and two minor components of 18 and 15 kDa, which are referred to as the beta- and gamma-chains, respectively. An analysis using electrospray ionization mass spectrometry also indicated that purified VGL contains a mixture of chains with molecular weights of 28,437 ± 2, 14,952 ± 2 and 12,332 ± 2. The complete amino acid sequence of VGL, as determined using tandem mass spectrometry, consists of 239 amino acid residues. VGL is a glycoprotein exhibiting high similarity in primary structure to other lectins from evolutionarily related plants, such as *Vatairea macrocarpa* lectin and lectins belonging to the Sophoreae tribe. VGL exhibits vasorelaxant activity in contracted rat aortas, an effect that is strictly dependent on the endothelium and involves nitric oxide and the lectin domain.

1. Introduction

The structural variability and complexity of cell surface glycans allows them to function as signaling molecules, recognition molecules 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, metastasis, host–pathogen interaction during infection and disease development [1,2].

Lectins are defined as a structurally heterogeneous group of proteins or glycoproteins that possess at least one non-catalytic domain that binds reversibly to a specific mono- or oligosaccharide [3]. These proteins are ubiquitous in nature and are found in all types of living organisms. By deciphering the codes present in the glycan structure, lectins have been used for decades as models in the study of the molecular basis of protein–carbohydrate interaction and specificity [4,5].

Lectins manifest a diversity of activities that are important in practical applications, such as plant defense [6], biomedical diagnostics of a broad spectrum of diseases [7], antitumor and antiviral drugs [8,9] and drug delivery systems [10]. Besides their biological activities, lectins are attracting increasing interest in biotechnology, and the continuous investigation of new lectins may yield new biotechnological tools.

The most studied group of carbohydrate-binding proteins consists of lectins purified from species of the Leguminosae family [11]. Among the studies of the lectins from Leguminosae, many are focused on members of the Phaseoleae and Vicieae tribes, subfamily Papilionoideae, and investigations of lectins from the others tribes are scarce. Lectins from the tribe Dalbergieae have rarely

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been investigated, despite the wide distribution of some representatives of this group. At present, within the tribe Dalbergieae, only the seed lectins from *Pterocarpus anglolensis* [12], *Lonchocarpus sericeus* [13], *Lonchocarpus capassa* [14] and *Vatairea macrocarpa* [15] have been isolated and biochemically characterized.

Among the lectins belonging to tribe Dalbergieae, the *V. macrocarpa* lectin (VML) is the best characterized with regard to its biological properties. These properties include respiratory stimuli in *Rhizobium* sp., which induce nodules and fix atmospheric nitrogen in legume roots [16], induction of leukocyte infiltration in rat paw edema [17], neutrophil migration via an indirect mechanism that involves the release of cytokines such as TNF-α by macrophages [18,19] and an increase in renal vascular resistance, glomerular filtration rate and urinary flow [20].

Many lectins of the Diocleinae subtribe elicit relaxation in endothelized rat aortas, which involves the participation of endothelium relaxant factors, of which nitric oxide (NO) is a common factor [21–23]. However, plant lectins isolated from the subtribe Dalbergieae have rarely been explored in these models.

In the present paper, we report the purification, partial characterization and complete amino acid sequence of a new galactose-specific lectin from *Vatairea guianensis* seeds, which is a typical plant from the Brazilian Amazon region, and we describe its activity in the contractile response of isolated rat aortas.

2. Materials and methods

2.1. Drugs and reagents

Acrylamide, N′,N′-methylenebisacrylamide, Coomassie brilliant blue (G-250 and R-250), tetramethylethylenediamine (TEMED), bovine serum albumin (BSA), chymotrypsin, guar gum, N-nitro L-arginine methyl ester (L-NAME), phenylephrine (Phe), D-galactose and acetylcholine (ACh) were purchased from Sigma (St. Louis, MO). Sodium dodecyl sulfate (SDS) and 2-mercaptoethanol were purchased from Merck, Darmstadt, Germany. Trypsin was purchased from Promega (Madison, WI, USA). All other chemicals were of analytical grade.

2.2. Animals

Wistar rats (250–300 g) and adult white rabbits (New Zealand) were maintained with free access to food and water and controlled environmental conditions (12/12 h light/dark cycle, temperature of 25 °C). The experimental protocols were approved by the Animal Care and Use Committee of the State University of Ceará (UECE-No. 10130208-8/40) Fortaleza-CE, Brazil, in accordance with international guidelines (NIH publication no. 85-23, revised 1985).

2.3. Lectin purification

Mature seeds from *V. guianensis* (collected at Manaus, Amazonas-Brazil) were ground into a fine powder using a coffee mill. The powder was incubated at a 1:10 ratio (w/v) in 100 mM glycine-HCl buffer, pH 2.6, containing 150 mM NaCl at room temperature with continuous stirring for 3 h before centrifugation at 10,000 × *g* for 20 min at 4 °C. The supernatant (crude extract) was precipitated with ammonium sulfate (0–60% saturation, F0/60) and centrifuged, and the pellet was resuspended and dialyzed in 150 mM NaCl. The F0/60 was applied to a guar gum column (10 cm × 2 cm) equilibrated with same solution. After removing the unbound material, the lectin was eluted with 100 mM D-galactose in an equilibrium solution. The elution was monitored at 280 nm and 3 mL fractions were collected manually and tested for haemagglutinating activity on trypsinized rabbit erythrocytes. The active fractions were pooled, dialysed extensively against distilled water and freeze-dried.

2.4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The purification process of the *V. guianensis* lectin was monitored using SDS-PAGE, as described by Laemmli [24] in 0.75 mm vertical gel slabs of 12% polyacrylamide separation gel consisting of 0.33 M Tris–HCl, pH 8.8, ammonium persulfate (100 mg/mL), tetramethylethylenediamine (TEMED) and 1% SDS buffer, and 4% polyacrylamide stacking gel consisting of 0.2 M Tris–HCl, pH 6.8, ammonium persulfate (100 mg/mL), tetramethylethylenediamine (TEMED), and 4% SDS buffer, using a Mini-Protean II apparatus (Bio-Rad; Milan, Italy). Samples (2.0 mg/mL) were dissolved in 0.065 M Tris–HCl, pH 6.8, 1% SDS buffer, 0,02% bromophenol blue and 10% glycerol in the absence or presence of β-mercaptoethanol (2%), and then incubated at 100 °C for 5 min. The electrophoresis was conducted at a constant current of 25 mA for 1 h 30 min. The protein bands were visualized by staining with Coomassie Brilliant Blue R-250. The molecular markers were phophorylase b (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa).

2.5. Hemagglutination activity and inhibition assays

Hemagglutination assays were performed in microtitration plates as previously described by Moreira & Perrone [25] using native and enzyme-treated rabbit erythrocytes. Inhibition tests were carried out according to standard procedure [26] using monosaccharides and dissaccharides (D-glucose, D-mannose, D-galactose, L-fucose, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, lactose and sucrose), N-glycoproteins (fibrinogen, ovoalbumin, human serotransferrin, desialylated human serotransferrin, bovine lactotransferrin and desialylated human lactotransferrin) and O-glycoproteins (bovine fetuin, bovine asialofetuin). The saccharides were purchased from Sigma–Aldrich, and the glycoproteins were isolated by the Laboratoire de Chimie Biologique et Unité Mixte de Recherche (Université des Sciences et Technologies de Lille, France). The hemagglutination inhibition titer was recorded as the highest dilution of the inhibitor that inhibited the agglutination produced by 4 hemagglutinating units of lectin after a 12-h incubation period.

2.6. Protein content

The protein content was determined as described by Bradford [27] using bovine serum albumin (BSA) as the standard protein. The absorbance at 280 nm was also used to estimate protein concentration in the chromatographic fractions.

2.7. Carbohydrate content

The total carbohydrate content of the purified lectin was determined using the phenol–sulfuric acid method [28] with glucose as a standard. The carbohydrate composition was determined using the heptafluorobutyrate derivatives of *O*-methyl-glycosides, which were obtained after methanolysis in 0.5 M methanol–HCl for 24 h at 80 °C, and lysine was used as an internal standard. The samples were analyzed using a gas chromatography instrument (GC-14A, Shimadzu) equipped with a Ross injector and a 25 m capillary column (25QC3/BP1; 0.5 μm film phase; SGE France SARL; Villeneuve St. Georges(France) as described by Zanetta et al. [29]. The results of the carbohydrate composition analysis are expressed as the number of monosaccharide residues per mole. The mass of the oligosaccharide structure was calculated using the GlycanMass tool (http://web.expasy.org/glycanmass/) [30].

2.8. Effect of pH, divalent cations and temperature on lectin haemagglutinating activity

The stability of the lectin at different pH values was determined after dialysis of the lectin solution (0.5 mg/mL of *V. guianensis* lectin in 150 mM NaCl) against different pH (4.0–10.0) buffers 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). The influence of divalent cations was determined after dialysis of the lectin solution against 100 mM EDTA (containing 150 mM NaCl) for 24 h at 4 °C. The activity recovered was determined after the addition of different concentrations (0–10 mM) of divalent metals ($CaCl_2$ and $MnCl_2$). The effect of different temperatures was determined by incubating the *V. guianensis* lectin (0.5 mg/mL) for 60 min at 10–100 °C with 10 °C increments.

2.9. Molecular mass analysis

The isotopic average molecular mass of *V. guianensis* lectin was determined by electro spray ionization using a hybrid quadrupole/ion mobility separator/orthogonal acceleration–time of flight mass spectrometer (Synapt HDMS System-Waters Corp., Milford, USA). Protein suspension (10 pmol/μL) was infused into the system at a flow rate of 1 μL/min. The capillary voltage and the cone voltage were set at 3 kV and 40 V, respectively. The source temperature was maintained at 100 °C and nitrogen was used as a drying gas (flow rate of 150 L/h). The acquisition of data was performed by Mass Lynx 4.0 software and the multiply charge spectra were deconvoluted using maximum entropy techniques [31]. In addition, the native molecular mass of lectin was estimated by gel filtration in an TSK G3000 SWXL column coupled to a of high performance liquid chromatography system (Akta purifier, Amersham Biosciences). The column was equilibrated with 50 mM Tris–HCl buffer, pH 7.6, containing 500 mM NaCl and calibrated with standard proteins: β-amylase (200 kDa), *Canavalia brasiliensis* lectin (100 kDa), BSA (66 kDa) and trypsin inhibitor (21.5 kDa).

2.10. Protein digestion and tandem mass spectrometry analysis

The protein digestion was performed as previously described by Shevchenko et al. [32]. For this, the protein was submitted to SDS-PAGE and the Coomassie stained bands were excised and bleached in a solution of 50 mM ammonium bicarbonate in 50% acetonitrile. The bands were then dehydrated in 100% acetonitrile and dried in a speedvac (LabConco). The gel was rehydrated with a solution of 50 mM

Table 1
Purification of the lectin from *Vatairea guianensis* seeds.

Fraction	[a]Total protein (mg/mL)	[b]Total HU	[c]Specific activity (HU/mg)	Purification (fold)
Crude extract	8.8	2048	232.7	1
Fraction 0–60%	10.5	4096	390.1	1.68
PII (guar gum)	0.86	4096	4762.8	20.5

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

ammonium bicarbonate containing trypsin (Promega) or chymotrypsin (Sigma) (1:50 (w/w); enzyme:substrate ratio) at 37 °C overnight. The peptides were then extracted in a solution of 50% acetonitrile with 5% formic acid and were concentrated in speedvac. The peptides were separated on a C18 chromatography column (75 μm × 100 mm) using a nanoAcquity™ system and eluted with acetonitrile gradient (10–85%), containing 0.1% formic acid. 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 80 °C and capillary voltage of 3.5 kV. The instrument was calibrated with double protonated ion of glucofibrinopeptide B (*m*/*z* 785.84). The LC–MS/MS experiment was performed according to the DDA (data dependent acquisition) function, selecting the MS/MS doubly or triply charged precursor ions for the experiments, which were fragmented by collision-induced dissociation (CID) using a ramp collision energy that varied according to the charge state of the precursor ion. The data were processed and analyzed using Proteinlynx (Waters), and the search parameter was the fragmentation pattern of the peptides. The CID spectra were interpreted manually. The primary sequence alignments were performed using ESPript 2.2 [33]. The identity score was determined using ClustalW and the theoretical pI was determined using ProtParam [30].

2.11. Effect in the contractile response of isolated rat aorta

The rats were sacrificed by stunning, and the thoracic aorta was quickly removed and cleaned of adhering fat and connective tissue. The ring segments (3–5 mm) were mounted for tension recordings (2 g) in a 10 mL organ bath filled with a modified tyrode solution containing 136 mM NaCl, 5 mM KCl, 0.98 mM $MgCl_2$, 2 mM $CaCl_2$, 0.36 mM NaH_2PO_4, 11.9 mM $NaHCO_3$, and 5.5 mM glucose. The rings were equilibrated at 37 °C with 95% O_2 and 5% CO_2 (pH 7.4) for 45 min. The contractile response (isometric tension, in g) was measured using a force transducer coupled to a preamplifier and a computerized data acquisition system (PowerLab, Chart 4.2, ADInstruments). In all experiments, the aortic rings were challenged after equilibration with KCl (60 mM) to ensure that the proper contractile conditions were obtained.

A cumulative concentration curve of the *V. guianensis* lectin (1–100 μg/mL) was prepared at the contraction plateau induced by phenylephrine (0.1 μM) or at the aorta basal tonus in either the intact or denuded endothelium. The remotion of the endothelium was assessed by mechanical rubbing of the aorta intimal surface, and the intact endothelium was considered for the relaxant responses to acetylcholine that were greater than 75% of the phenylephrine-induced tone [34]. The control group received an equivalent volume of tyrode. To investigate the participation of nitric oxide in the *V. guianensis* lectin relaxation, L-NAME (100 μM), an inhibitor of nitric oxide synthase (NOS), was added to the tissues with an intact endothelium 30 min before the addition of phenylephrine. The participation of the lectin domain was assessed by incubating (1 h, 37 °C) the *V. guianensis* lectin at the most active concentration with its binding sugar D-galactose (0.1 M) before addition into the tissue to allow for interaction between the lectin and the sugar. The *V. guianensis* lectin or galactose were also incubated in separate solutions as controls.

2.12. Statistical analysis

The results are expressed as the mean ± S.E.M. of 5–6 animals per group. The differences ($p < 0.05$) were analyzed using ANOVA and Student's *t*-test. The IC50 values were calculated by interpolation from semi-logarithmic plots.

3. Results

Crude protein extracts of *V. guianensis* seeds possess relatively high levels of hemagglutinating activity against rabbit blood cells, either native or treated with proteolytic enzymes. The lectin from *V. guianensis* seeds has been purified by precipitation with solid ammonium sulfate followed by a single step using an affinity chromatography column (Fig. 1A). For this purification, the crude soluble protein extract obtained from *V. guianensis* seeds was initially precipitated by the addition of solid ammonium sulfate (0–60%), and the protein fraction obtained exhibited strong hemagglutinating activity. The active fraction was later applied to a guar gum affinity chromatography column, and after elution of the unbound material (peak I), the lectin (peak II) was recovered by elution with 0.1 M galactose in the equilibrium solution. Peak II contained all of the hemagglutinating activity and consisted of purified lectin, which was named VGL. This purification procedure resulted in a high purification of 20.5-fold (Table 1).

The glycan-recognizing specificity of VGL was investigated via hemagglutination-inhibition assays using several different carbohydrates and glycoproteins (Table 2). The hemagglutinanting activity was fully inhibited by D-galactose and D-galactose-derivative sugars. Among the glycoproteins tested, bovine asialofetuin and bovine lactotransferrin exhibited a potent inhibitory effect at a minimum concentration of 330 μg/mL.

The SDS-polyacrylamide gel electrophoresis of the affinity-purified VGL, both in the presence and absence of β-mercaptoethanol, exhibited an electrophoretic profile consisting of a major 30–32 kDa double band, termed the alpha-chain, and two minor components of 18 and 15 kDa, referred to as the beta- and gamma-chains, respectively (Fig. 1B). The electrospray ionization mass spectrometry analysis also indicated that the purified VGL contained a mixture of chains with molecular weights of 28,437 ± 2; 14,952 ± 2 and 12,332 ± 2 (data not shown). Moreover, the analytical gel size-exclusion chromatography of VGL under non-denaturing conditions exhibited a single sharp symmetrical peak (data not shown) with a molecular mass of approximately 120 kDa.

The monosaccharide analysis indicated that the purified lectin contained 3.6% carbohydrates by weight, and as determined by a gas chromatography analysis, it is mainly composed of Man, GlcNAc, Xyl and Fuc in molar proportions of approximately 3:2:1:1.

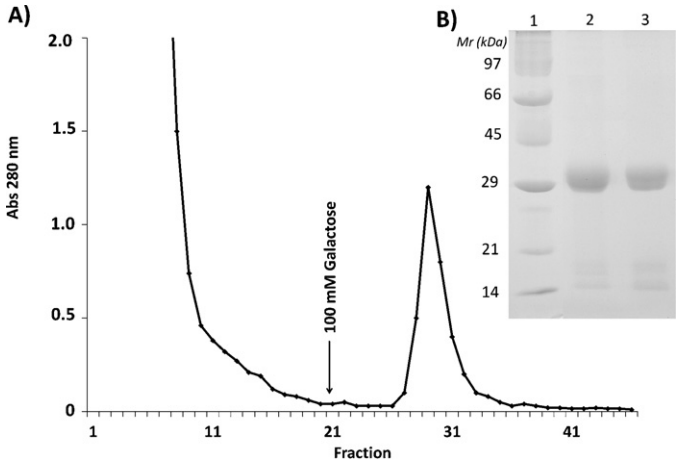

Fig. 1. (A) Elution profile of the guar gum affinity chromatography. Approximately 10 mL of the 0–60% fraction was applied to the guar gum column (10 cm × 2 cm) that was equilibrated with 150 mM NaCl. The lectin was eluted with 100 mM D-galactose in the buffer described above at a flow rate of 1 mL/min. Fractions (approximately 2.5 mL) were collected and monitored for protein content by measuring the absorbance at 280 nm. (B) SDS-PAGE. Lane 1: molecular mass markers (phosphorylase b, 97 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa; trypsin inhibitor, 20.1 kDa; and α-lactalbumin, 14.4 kDa); Lane 2: VGL (20 μg); Lane 3: VGL (20 μg) + β-mercaptoethanol.

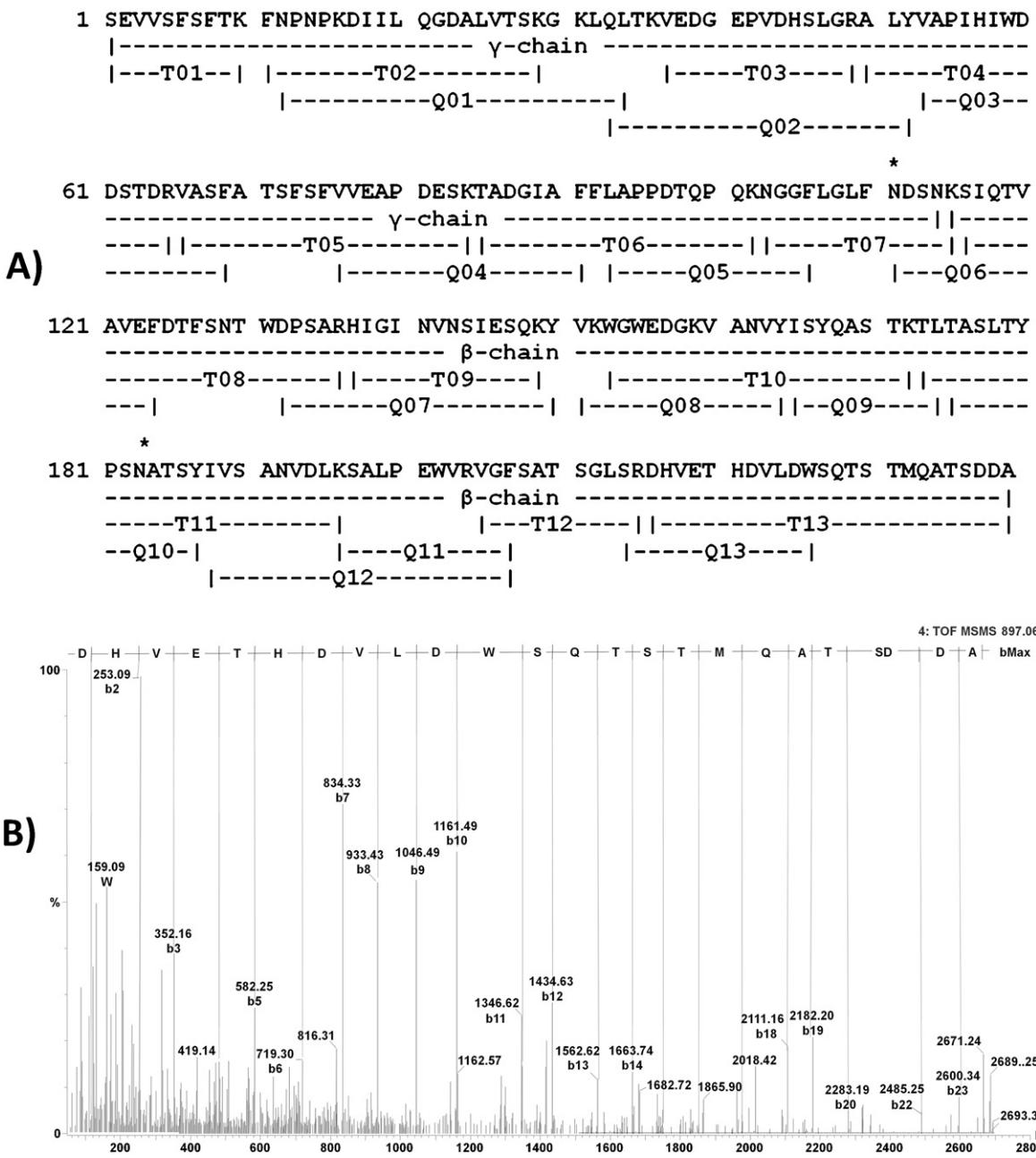

Fig. 2. (A) Amino acid sequence of VGL assembled from sequences of overlapping degradation products generated by cleavage with trypsin (T-) and chymotrypsin (Q-). The amino acid sequences of the α (1–239), γ (1–114) and β (115–239) chains are highlighted. The asterisks represent the glycosylation sites. (B) Collision-induced dissociation of the triply charged ion at m/z = 897.06 corresponding to the T13 peptide of VGL (See Table 3). The sequence-specific b ions used for the structure determination are indicated.

The calculated mass for the oligosaccharide structure of VGL was 1188.42 Da.

The complete VGL amino acid sequence was obtained from the overlapping regions of the digested peptides that were sequenced using tandem mass spectrometry, in which 27 peptides were sequenced to result in a total of 239 amino acid residues (Fig. 2A). Table 3 displays all of the sequenced peptides and their respective molecular masses. The theoretical pI calculated for VGL based on the final sequence is 4.71. The protein sequence data reported in this paper are deposited in the UniProt Knowledgebase under the accession number P86893.

VGL was shown to be highly thermostable (Fig. 3A) because its hemagglutinating activity was maintained after incubation of the lectin at 70 °C for 1 h, and this activity was completely lost only at higher temperatures (100 °C). The activity of VGL was also maintained over a wide pH variation, and the stability was more apparent at pH 6.0–8.0 (Fig. 3B). The hemagglutinating activity of the native lectin was not affected by either sequential dialysis (with EDTA followed by NaCl) or the addition of Ca^{2+} and Mn^{2+} to the dialyzed lectin.

The mechanical activity evaluation of a rat aorta demonstrated that phenylephrine induced tonic contractions in the aorta with an amplitude of 1.705 ± 0.239 g in an endothelized aorta and 1.543 ± 0.093 g in an endothelium-denuded aorta. The cumulative addition of VGL significantly relaxed the endothelized aorta in a concentration-dependent manner; this effect was initiated at 1 μg/mL VGL was maximal at 100 μg/mL, indicating an in inhibition of 61.98 ± 6.99% relative to the 100% inhibition of the phenylephrine-induced contraction. However, no relaxant effect was observed in the endothelium-denuded tissue (Fig. 5A). In

Table 2
Inhibitory effects of saccharides and glycoproteins on the hemagglutinating activity of VGL.

Sugar	MIC[a]
Saccharides	mM
D-glucose	NI[b]
D-galactose	8.2
D-mannose	NI
D-fucose	NI
N-Acetyl-D-glucosamine	NI
N-Acetyl-D-galactosamine	1.0
Lactose	2.0
Sucrose	NI
Glycoproteins	μg/mL
Fibrinogen	NI
Ovoalbumin	NI
Human serotransferrin	NI
Bovine lactotransferrin	330
Bovine fetuin	NI
Bovine asialofetuin	330

[a] Minimum inhibitory concentration.
[b] Sugar not inhibitory until a concentration of 100 mM for saccharides or 5000 μg/mL for glycoproteins.

addition, VGL exhibited no significant effect on the tissue basal tone (Fig. 5B). Prior incubation of endothelized aorta with L-NAME blocked the VGL relaxant effect and increased the IC50 value of VGL from 45.44 ± 2.57 μg/mL to 111.69 ± 2.46 μg/mL in the presence of the NO inhibitor (Fig. 5C). Similarly, prior incubation of the endothelized aorta with galactose partially inhibited the VGL relaxant response by 42.88 ± 5.73%. However, galactose itself exhibited no significant effect on the phenylephrine-induced contractions (Fig. 5D). In each protocol, VGL did not alter the tissue responsiveness because at the end of each experiment, the KCl-contractile response appeared similar to the initial tone.

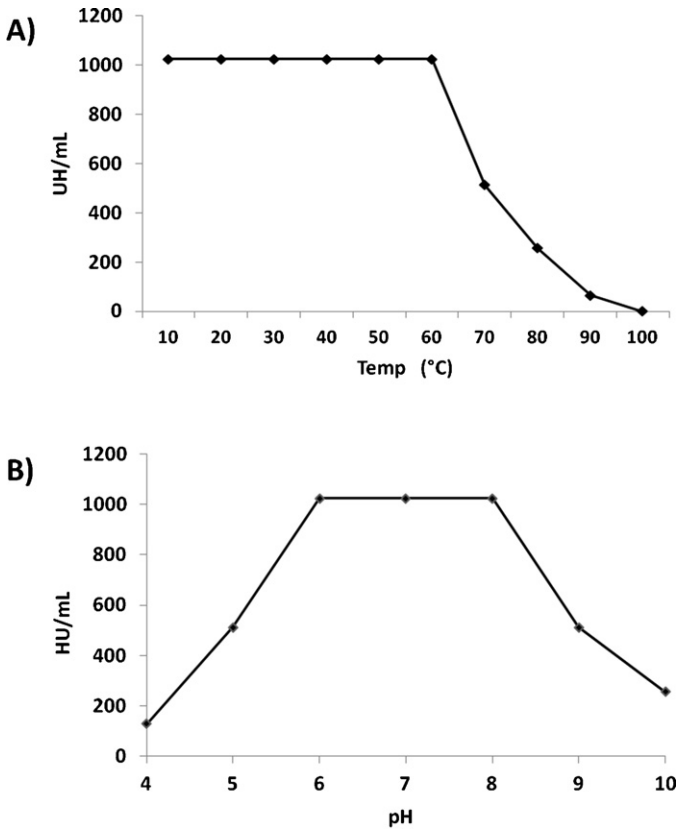

Fig. 3. Physicochemical properties of the lectin from *V. guianensis* seeds. The effects of temperature (A) and pH (B) on the hemagglutinating activity of VGL.

4. Discussion

V. guianensis seeds, a typical plant from the Brazilian Amazon region that belongs to the Dalbergieae tribe, possess a lectin, which was isolated by precipitation with solid ammonium sulfate followed by guar gum affinity chromatography and was named VGL. This *V. guianensis* lectin strongly agglutinated rabbit erythrocytes and was fully inhibited by D-galactose and D-galactose-derived sugars. Similar carbohydrate-binding specificity has also been demonstrated by other lectins isolated from a species

Table 3
Amino acid peptide sequences of VGL obtained using tandem mass spectrometry and their respective molecular masses.

Peptide	Experimental mass (Da)	Theoretical mass (Da)	Sequence
T1	1129.4844	1129.5656	SEVVSFSFTK
T2	2069.1824	2069.1157	FNPNPKDIILQGDALVTSK
T3	1408.5244	1408.6582	VEDGEPVDHSLGR
T4	1870.7644	1870.9214	ALYVAPLHLWDDSTDR
T5	2016.9644	2016.9644	VASFATSFSFVVEAPDESK
T6	1915.7644	1915.9676	TADGIAFFLAPPDTQPQK
T7	1381.5844	1381.6626	NGGFLGLFNDSNK
T7′	2552.9443	2553.0825	NGGFLGLFNDSNK + N-linked Glycan
T8	2370.0366	2370.1128	SIQTVAVEFDTFSNTWDPSAR
T9	1437.7566	1437.7576	HIGLNVNSLESQK
T10	2301.1042	2301.1064	WGWEDGKVANVYISYQASTK
T11	2528.2844	2528.3010	TLTASLTYPSNATSYIVSANVDLK
T12	1080.5044	1080.5564	VGFSATSGLSR
T13	2688.1865	2688.1575	DHVETHDVLNWSETSTMQATSDDA
Q01	2461.3564	2461.3904	NPNPKDIILQGDALVTSKGKLQL
Q02	2226.0366	2226.1276	QLTKVEDGEPVDHSLGRALY
Q03	1927.7266	1927.9115	VAPIHIWDDSTDRVASF
Q04	1647.7644	1647.7992	VVEAPDESKTADGIAF
Q05	1468.6244	1468.7310	LAPPDTQPQKNGGF
Q06	1913.8844	1913.9006	NDSNKSIQTVAVEFDTF
Q07	2127.0964	2127.0708	DPSARHIGINVNSIESQKY
Q08	1649.7444	1649.8202	VKWGWEDGKVANVY
Q09	1110.5044	1110.5920	ISYQASTKTL
Q10	2546.0143	2546.0504	TASLTYPSNATSY + N-linked Glycan
Q11	1387.6843	1387.7612	KSALPEWVRVGF
Q12	2199.1643	2199.2051	IVSANVDLKSALPEWVRVGF
Q13	1607.7244	1607.7328	SRDHVETHDVLDW

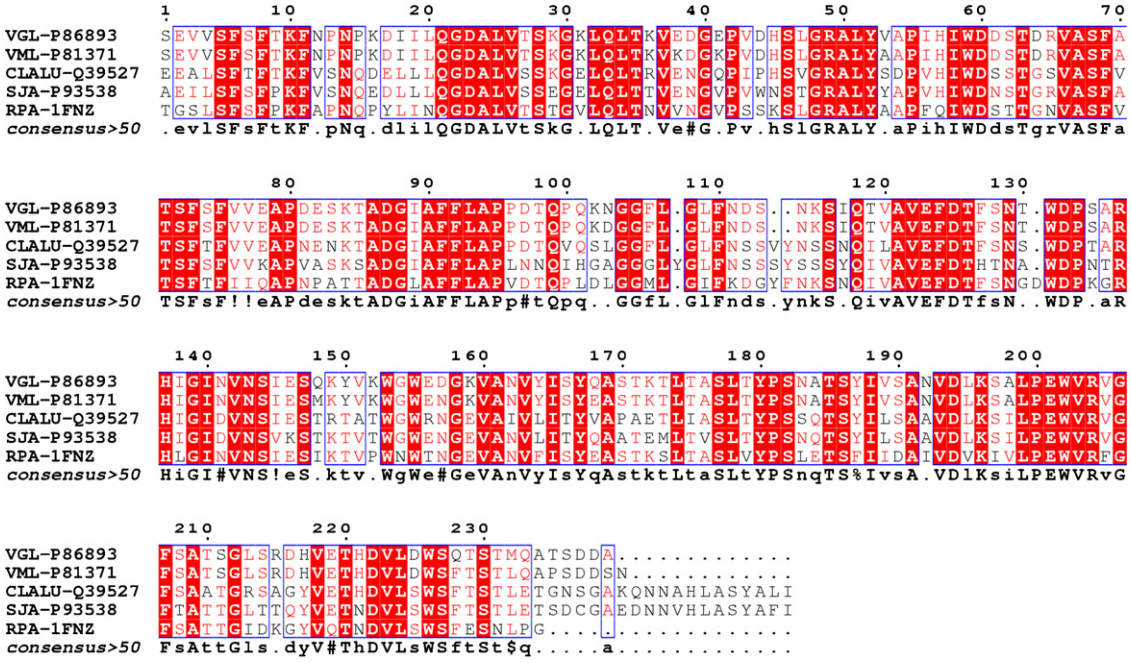

Fig. 4. Multiple alignment among the amino acid sequences of the *Vatairea guianensis* lectin (VGL), the *Vatairea macrocarpa* lectin (VML), the *Cladrastis kentukea* lectin (CLALU), the *Sophora japonica* lectin (SJA) and the *Robinia pseudoacacia* lectin (RPA). The numbers after the acronyms represent the databank codes.

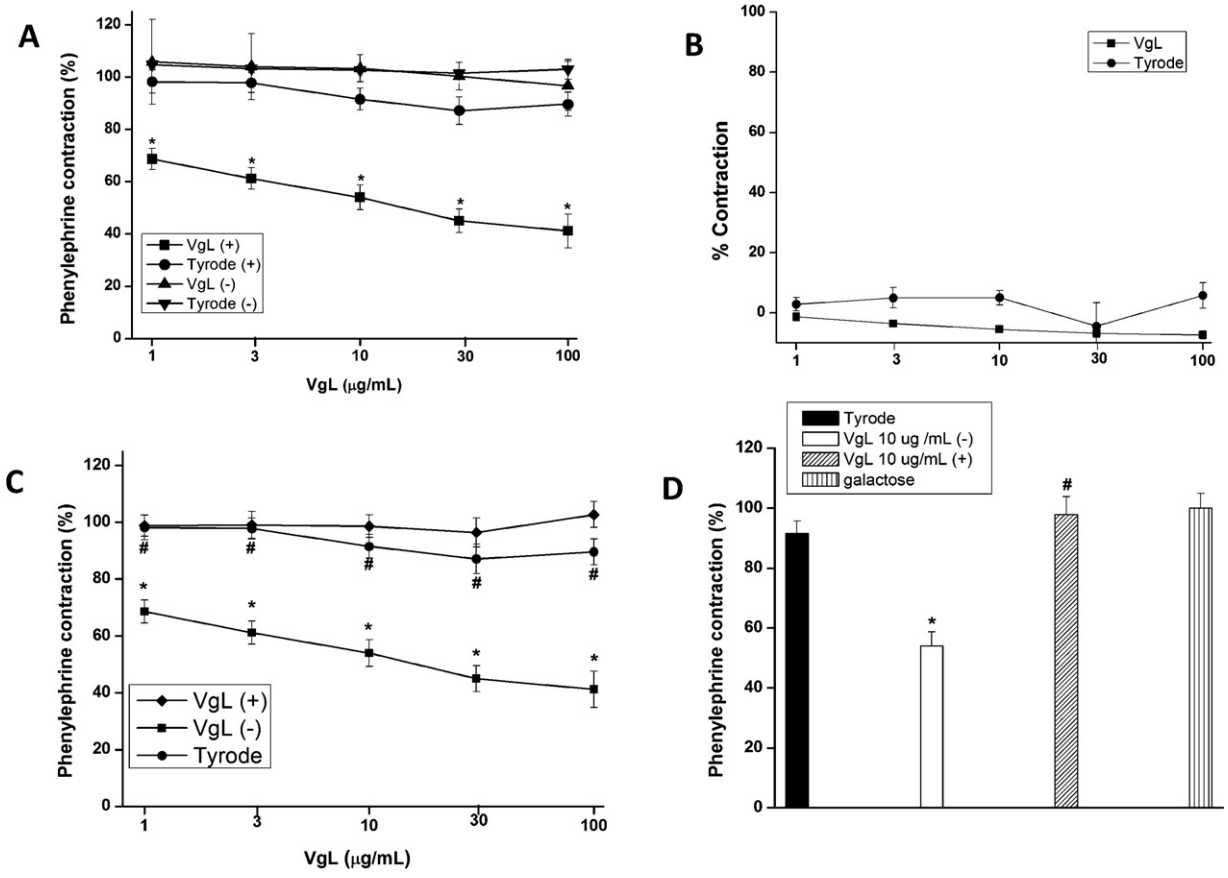

Fig. 5. The *V. guianensis* lectin induces endothelium-dependent relaxation of an isolated rat aorta, which involves NO and the lectin domain. Tissues pre-contracted with phenylephrine (0.1 μM): tyrode or VGL (1–100 μg/mL) (A) with (+) or without (−) endothelium, (B) at basal tonus. Endothelized tissues: VGL (10 μg/mL) (C) with (+) or without (−) L-NAME (100 μM), (D) with (+) or without (−) galactose (0.1 M) or galactose only. Mean ± S.E.M (n = 5–6). $^{*}p < 0.05$ vs. tyrode. $^{\#}p < 0.05$ vs. VGL.

of the same genus, the *V. macrocarpa* lectin [15]. Furthermore, it was observed that VGL has a high affinity for N-acetyl-D-galactosamine over galactose, indicating that the N-acetamido substituent at the C-2 position is favored by the establishment of additional interactions with the lectin binding site [35]. A similar pattern was observed for many other D-galactose/N-acetyl-D-galactosamine-binding lectins, such as the *Bauhinia purpurea* lectin [36], the *Moluccella laevis* lectin [37] and the *Luetzelburgia auriculata* lectin [38] that exhibit greater affinity for N-acetyl-D-galactosamine than for D-galactose.

Among the glycoproteins tested, only bovine lactotransferrin and bovine asialofetuin exhibited an inhibitory effect on the hemagglutinating activity of VGL, whereas bovine fetuin was not able to inhibit the lectin activity. According to Green et al. [39], bovine fetuin contains a great number of N-acetyllactosamine-type glycans that differ in the number of peripheral branches (17% biantennary and 83% triantennary glycans), the extent of sialylation, the N-acetylneuraminic acid linkage (α-2,3 vs. α-2,6) and the linkage (β-1,4 vs. β-1,3) of the galactose residues. The presence of N-acetylneuraminic acid appears to prevent the interaction between the lectin and the bovine fetuin.

The hemagglutinating activity of VGL was maintained even after incubation of the lectin at a wide range of pH values and temperatures or in the presence of EDTA, Ca^{2+} and Mn^{2+}. These data suggest that VGL, unlike other leguminous lectins [40,41], does not need metal ions for its full activity or that the metal ions are tightly bound to the molecule. The hemagglutinating activity of the *L. auriculata* lectin, which is an evolutionarily related species belonging to the Sophoreae tribe, was also unaffected by EDTA [38].

The complete sequence of the VGL alpha-chain was determined using tandem mass spectrometry and consists of 239 amino acid residues. VGL has a high degree of similarity with VML (95%) (SwissProt accession code: P81371), differing only in eleven residues (VML/VGL): 38(K/E), 41(K/E), 53(A/V), 103(D/N), 148(M/Q), 157(D/N), 168(E/Q), 228(F/Q), 232(L/M), 235(P/T) and 229(S/A) (Fig. 4). Fig. 2B shows the CID spectra of the C-terminal peptide (T13) in which sequence-specific b ions were used for the structure determination. To obtain the sequence of this peptide, the $[M+3H]^{3+}$ 897.06 precursor ion was selected in quadrupole mode and then fragmented using different ramps of collision energy at the Trap analyzer (27–38 V and 31–42 V) to obtain a complete CID spectrum because a higher collision energy promotes the formation of b ions fragments, whereas a lower collision energy favors the formation of y ions [42].

VGL also possesses similarity with other galactose/N-acetylgalactosamine-specific lectins from evolutionarily related plants belonging to the Sophoreae tribe, such as the *Cladrastis kentukea* lectin (68%) (TrEMBL accession code: Q39527) and the *Sophora japonica* lectin (66%) (SwissProt accession code: P93538) (Fig. 4). These results corroborate the idea of evolutionary proximity between the Dalbergieae and Sophoreae tribes [38]. In addition, VGL also exhibits sequence similarity with the *Robinia pseudoacacia* lectin (62%) (PDB accession code: 1FNZ). Rabijns and coworkers [43] solved the three-dimensional structure of the *R. pseudoacacia* lectin in complex with N-acetyl-D-galactosamine and determined the primary amino acid residues involved in the carbohydrate- (Asp87, Gly105, Apn131, Ile216 and Asp217) and metal-binding (Asp127, Phe129, Asn13 and Asp135) sites. A comparison of both lectins using a sequence alignment with ESPript 2.1 [33] attested that VGL contains the same residues present in the metal-binding site of the *R. pseudoacacia* lectin. In relation to the carbohydrate-binding site, VGL possesses one replacement at position 217 (D/S) (Fig. 4).

In addition to the high similarity of the primary structure, VGL is also structurally similar to the *V. macrocarpa* lectin in other aspects. For instance, similarly to VML [15,44], VGL exhibits an electrophoretic profile composed of a major 32–34 kDa double subunit (full-length α-subunit) and two minor polypeptides, the β-chain (22 kDa) and the γ-chain (13 kDa), resulting from posttranslational processing of the full-length α-subunit. Moreover, VML is N-glycosylated at asparagine residues at positions 111 and 183 with one major glycan structure, Manα1-6[(Manα1-3)(Xylβ1-2)]Manβ1-4-GlcNAcβ1-4(Fucα1-3)GlcNAc, which is a typical plant N-glycan that is also found in other plant lectins, such as those of *R. pseudoacacia* [45], *Erythrina corallodendron* [46] and *E. variegata* [47] seeds. Our results, which were obtained from a gas chromatography analysis of the heptafluorobutyrate derivatives of *O*-methyl-glycosides, suggest that VGL possesses a similar carbohydrate composition. Furthermore, the position of the N-glycosylation sites in VGL were confirmed in the protein digestion and tandem mass spectrometry analysis by the presence of two doubly charged ions at *m*/*z* 1277.47 and 1274.01, which correspond to the T07′ (^{103}NGGFLGLFNDSNK^{114}) and Q10 (^{175}TASLTYPSNATSY^{187}) ions plus the calculated mass of one N-linked glycan structure (1171.42 Da) (Table 3). Therefore, VGL is glycosylated at sites corresponding to those of VML. Our results suggest that VGL and VML undergo a similar mechanism of post-translational processing.

Based on the results of mass spectrometric analyses, Calvete and colleagues [44] suggested a mechanism for the proteolytic processing of the *V. macrocarpa* lectin. VML contains a mixture of doubly (28,525 Da) and singly (27,354 Da) glycosylated alpha-chains. Deglycosylation of Asn-111 correlates with the proteolytic cleavage of the Asn-114-Lys-115 bond, yielding nonglycosylated gamma- (residues 1–114, 12,304 Da) and glycosylated beta- (residues 115–240, 14,957 Da) chains. Several beta-chain molecules are further deglycosylated and N-terminally processed, yielding products with molecular masses of 13,783 Da and 13,670 Da. The electrospray ionization mass spectrometry analysis indicated that VGL also contained a mixture of chains with molecular weights of 28,437 ± 2, 14,952 ± 2 and 12,332 ± 2. The calculated average molecular mass for the primary structure of VGL (26,096 Da) plus the calculated mass of two N-linked glycan structures (1171.42) is 28,438.7 Da, which is notably close to the mass observed using electrospray ionization mass spectrometry. The 12,332 Da component corresponds exactly to the mass calculated for residues 1–114 (gamma-chain), and the o 14,952 Da component corresponds exactly to the mass calculated for residues 115–239 (beta-chain) plus the calculated mass of one N-linked glycan structure.

The analytic gel size-exclusion chromatography showed that VGL, under non-denaturing conditions, exhibits a molecular mass of approximately 120 kDa. These results suggest that the *V. guianensis* lectin in solution is a tetrameric protein with monomers composed of a mixture of both single-chain and double-chain molecules. Similarly, the *V. macrocarpa* lectin [44], the *S. japonica* leaf and bark isolectins [48,49] and the *L. auriculata* lectin [37] possess a tetrameric structure. In contrast, the galactose-specific lectins isolated from the genus *Erythrina* have been reported to be dimeric proteins [46,47,50].

Additionally, this study reported the vasorelaxant activity of the lectin from *V. guianensis*, which is the first demonstration of this activity in lectins belonging to the Dalbergieae tribe. The relaxant effects had previously been demonstrated only for lectins from the Diocleinae subtribe [21–23]. The VGL relaxant effect on the phenylephrine-induced contraction was shown to be completely dependent on the presence of the endothelium and involves the lectin domain. Similarly, Diocleinae lectins relaxed the aorta in a strictly endothelium-dependent manner involving the participation of NO. Indeed, in vascular smooth muscles, NO is the primary mediator of endothelium-dependent relaxation [34]. In addition, the relaxant responses of Diocleinae lectins were reversed by the

binding of sugars to the lectin [21–23]. Accordingly, in the present study, the relaxant effect of VGL was completely blocked by L-NAME, an inhibitor of the nitric oxide synthase enzyme, and also by galactose, which is a sugar that can bind to VGL. Furthermore, VGL did not cause aorta toxicity because the tissue responsiveness and its basal tonus were not altered, showing no effect on the normal physiology of the muscle. Thus, VGL can be used as an important tool in the study of diseases in which the vessels are maintained in their contracted state, such as hypertension.

5. Conclusion

The results reported here clearly demonstrate the purification and characterization of a new galactose-specific lectin from *V. guianensis* seeds, which possesses a high similarity with other lectins from evolutionarily related plants, such as VML and lectins belonging to the Sophoreae tribe. VGL exhibits vasorelaxant activity in contracted rat aortas, an effect that is strictly dependent on the endothelium and involves NO and the lectin domain.

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