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Purification and physicochemical characterization of a cotyledonary lectin from *Luetzelburgia auriculata*

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Abstract

A lectin was purified from the cotyledons of *Luetzelburgia auriculata* (Fr. All) Ducke by affinity chromatography on agarose-*N*-acetyl-D-galactosamine. The lectin is a potent agglutinin for rabbit erythrocytes, reacts with human red cells, but is inactive against cow, sheep, and goat erythrocytes. Hemagglutination of rabbit erythrocytes was inhibited by either 0.39 mM *N*-acetyl-neuraminic acid or *N*-acetyl-D-galactosamin, 12.5 mM D-lactose or D-melibiose, 50 mM D-galactose or raffinose. Its hemagglutinating activity was lost at 80 °C, 5 min, and the activation energy required for denaturation was 104.75 kJ mol^{-1}. Chromatography on Sephadex G-100, at pH 7.6, showed that at this hydrogenic ionic concentration the native lectin was a homotetramer (123.5 kDa). By denaturing SDS-PAGE, LAA seemed to be composed of a mixture of 29 and 15 kDa polypeptide subunits. At acidic and basic pHs it assumed different conformations, as demonstrated by exclusion chromatography on Superdex 200 HR 10/30. The N-terminal sequence of the 29 kDa band was SEVVSFSFTKFNPNQKDII and the 15 kDa band contained a mixture of SEVVSFSFTKFNPNQKDII and KFNQIVAVEEDTDXESQPQ sequences, indicating that these bands may represent full-length and its endogenous fragments, respectively. The lectin is a glycoprotein having 3.2% neutral carbohydrate, with a pI of 5.8, containing high levels of Asp + Asn and Glu + Gln and hydroxy amino acids, and low amount or absence of sulfur amino acids. Its absorption spectrum showed a maximum at 280 nm and a $\varepsilon^{1\%}_{1cm}$ of 5.2. Its CD spectrum was characterized by minima near 228 nm, maxima near 196 nm and a negative to positive crossover at 210 nm. The secondary structure content was 6% α-helix, 8% parallel β-sheet, 38% antiparallel β-sheet, 17% β-turn, 31% unordered and others contribution, and 1% RMS (root mean square). In the fluorescence spectroscopy, excitation of the lectin solution at 280 nm gave an emission spectrum in the 285–445 nm range. The wavelength maximum emission was in 334.5 nm, typical for tryptophan residues buried inside the protein.
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Keywords: Luetzelburgia auriculata; Leguminoseae; Purification; Characterization; Lectin

Abbreviations: BCIP, 5-bromo-chloro-3-indolyl phosphate; BSA, bovine serum albumin; EDTA, Ethylenediaminetetraacetic acid; IEF, isoelectric focusing; LAA, *Luetzelburgia auriculata* lectin; NBT, nitro blue tetrazolium; PHT, phenylthiohydantoin; PVDF, polyvinylidene difluoride.

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1. Introduction

Lectins constitute a class of proteins which possess at least one noncatalytic domain capable of specific recognition and reversible binding to carbohydrate (Peumans and Van Damme, 1995). They are ubiquitously distributed in nature and most abundant in the *Plantae* kingdom, where they can be found in seeds, leaves, barks, bulbs, rhizomes, roots and tubers depending on the plant species (Diaz et al., 1990; Hankins et al., 1988; Van Damme et al., 2000; Wright et al., 1999; Zhu et al., 1996). However, the majority of the studies on lectins have been carried out on legume species (Kocourek, 1986; Lakhtin, 1994) particularly in their seeds where they comprise up to 15% of the total protein. As a result of these studies, many plant lectins have become a very popular class of proteins because of their obvious potential in aiding researchers in other areas of the life sciences. Regarding their endogenous cellular functions, plant lectins appear to exert multiple physiological roles. Besides the proposed involvement of some lectins in the binding of nitrogen-fixing bacteria to legume roots (Diaz et al., 1989; Hirsch, 1999; Jayaraman and Das, 1998), it has been hypothesized that plant lectins are involved with chemical defense mechanisms developed by plants to overcome pathogen attack and herbivory (Gatehouse et al., 1995; Oka et al., 1997). According to Peumans and Van Damme (1995) there are several molecular, biochemical, cellular, physiological, and evolutionary arguments that are compatible with this idea such as: (a) the binding of plant lectins to glycoconjugates of other organisms; (b) their marked stability under unfavorable conditions, and (c) the preferential association of lectins with those parts of the plant most susceptible to attack by foreign organisms. For instance, several lectins such as those of rice, wheat germ, stinging nettle rhizomes, and seeds from *Phaseolus vulgaris* and *Griffonia simplicifolia* have insecticidal or antifungal activity (Does et al., 1999;Van Parijs et al., 1992; Zhu et al., 1996).

Luetzelburgia auriculata (*Fabaceae: Papilionoideae*) is a tree native to northeastern Brazil. Although its seeds are rich in proteins and lipids they are not consumed by animals and humans owing to high toxicity as reported by native people. Preliminary studies with *L. auriculata* plantlets showed the presence of hemagglutinating activity against rabbit and human erythrocytes in the extracts from cotyledons, embryonic axe, leaves, epicotyls, hypocotyls and roots. Furthermore we have observed that upon seed imbibition at pH 6.0, the lectin is released to the medium suggesting that it has a protective function in the early stage of germination when the seeds are particularly vulnerable to pathogen attack. In this paper we report the purification and physicochemical characterization of the cotyledonary lectin from *L. auriculata* as a prerequisite to further study this lectin with respect to its physiological role in the plant.

2. Results and discussion

2.1. Lectin purification

Crude protein extracts of *L. auriculata* cotyledons contain relatively high levels of hemagglutinating activity against rabbit erythrocytes (Table 1). Fractionation of the crude extract by precipitation with ammonium sulfate (40–60% saturation) increased specific activity 18.4-fold. The lectin present in the ammonium sulfate fraction ($F_{40/60}$) was bound to the agarose-*N*-acetyl-D-galactosamine matrix (Fig. 1) and eluted in an apparently pure form by 0.2 M D-galactose. The purified lectin represented a 46.4-fold increase in hemagglutinating activity over the crude extract, with overall activity- and protein recovery of approximately 36.5 and 0.8%, respectively (Table 1). This purified lectin will hereafter simply be referred to as LAA (*L. auriculata* agglutinin).

Table 1
Purification[a] of *Luetzelburgia auriculata* cotyledonary lectin

Fraction	Total protein (mg)	Specific activity (HU[d] mg protein^{-1})	Amount of lectin[b] (μg ml^{-1})	Purification[c] -fold
Crude extract[e]	1416.20±68.47	4.35±0.33	230.76±17.16	1
$F_{40–60}$	269.18±21.08	79.97±6.45	12.56±0.97	18.4
Purified lectin[f]	11.16±0.24	201.72±3.60	4.96±0.09	46.4

[a] Purification stages are described under Experimental.
[b] Minimal concentration of protein able to cause visible agglutination of a 2% suspension of rabbit erythrocytes.
[c] Purification index was calculated as the ratio between the minimal concentration of crude extract able to cause visible agglutination of rabbit erythrocytes and that of the protein fraction obtained at each purification step.
[d] HU, hemagglutinating unit.
[e] Crude extract from 10 g of cotyledons.
[f] The lectin was obtained by affinity chromatography on agarose-*N*-acetyl-D-galactosamine.

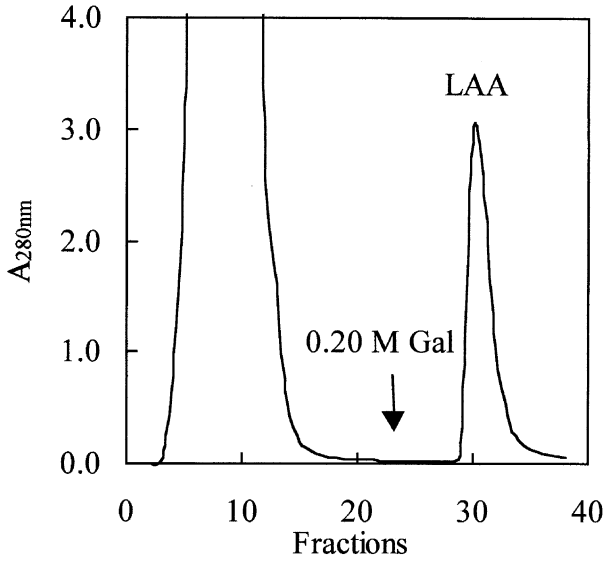

Fig. 1. Agarose-*N*-acetyl-D-galactosamine affinity chromatography. The 40–60% ammonium sulfate fraction was applied to the agarose-*N*-acetyl-D-galactosamine column (2.5 × 5.0 cm) equilibrated with 0.05 M sodium acetate buffer (pH 6.0) containing 0.15 M NaCl. The lectin was eluted with 0.2 M D-galactose included in the above buffer at a flow rate of 30 ml h^{-1}. Fractions (2.5 ml) were collected and monitored for protein content at 280 nm.

2.2. Hemagglutinating activity

LAA was a potent agglutinin of native rabbit erythrocytes (4.96 μg ml^{-1} minimal concentration) and of A^+ (5.52 μg ml^{-1}), B^+ (15.62 μg ml^{-1}), and O^+ (62.5 μg ml^{-1}) human blood cells. The activity against rabbit erythrocytes occurred immediately after the cells were added to the assay medium containing the lectin. Thus, according to Peumans and Van Damme (1995), LAA is a hololectin since it has at least two carbohydrate binding sites in the native molecule. However, the activity of LAA against native cow, sheep, and goat blood cells could not be detected. This difference in the agglutination may be due to the nature of the glycoproteins protruding on the cell surface of these cells which are weakly or not recognized by the lectin. Full hemagglutinating activity was maintained after EDTA treatment (data not shown) suggesting that LAA does not need metal ions to be fully active or that they are tightly bound to the molecule. Hemagglutination of rabbit erythrocytes by a 9.92 μg ml^{-1} (2 hummaglutinating units, see Experimental) solution of purified LAA was inhibited by 0.39 mM *N*-acetyl-neuraminic acid or *N*-acetyl-D-galactosamine, 12.5 mM D-lactose or D-melibiose, and 50 mM D-galactose or raffinose. However, hemagglutination was not inhibited even by 100 mM D-glucose, *N*-acetyl-D-glucosamine, D-mannose or sucrose. From this inhibition study it was observed that there is a much greater affinity (128 times) of the purified lectin for *N*-acetyl-D-galactosamine over D-galactose indicating that a more hydrophobic substituent at C-2 is favored by apparently allowing the establishment of additional interactions with hydrophobic regions of the binding site of the lectin (Ramos et al., 1996). Similar behavior was observed for the lectin from *Moluccella laevis* seeds for which *N*-acetyl-D-galactosamine was 300–600 times more inhibitory than D-galactose (Lis et al., 1988).

2.3. Amino acid composition and carbohydrate content

LAA contained large amounts of Asp + Asn and Glu + Gln and hydroxylated amino acids and very low content of sulfur amino acids (Table 2). Native LAA was found to be a glycoprotein with 3.2% covalently linked carbohydrate (Table 2).

2.4. Native molecular mass

Analytical gel size-exclusion chromatography of LAA on Sephadex G-100, at pH 7.6, under non-denaturing

Table 2
Molecular and physicochemical properties of the cotyledonary lectin from *L. auriculata*

Properties[a]		Amino acid composition (nM %)[b]		
Molecular mass				
native	123.5 kDa[c]	Asx 13.79±0.32	Val 8.45±0.20	Lys 5.10±0.13
Subunits	15; 29 kDa	Glx 9.99±0.58	Met 0.00±0.00	Pro 5.51±0.13
Carbohydrate (%)	3.2	Trp 2.07±0.02	Ile 4.11±0.09	Arg 1.58±0.40
Isoelectric point	5.8	Ala 6.22±1.07	Leu 6.82±0.13	Ser 12.32±0.28
$\varepsilon^{1\%}$ (280 nm)[d]	5.2	Tyr 3.47±0.13	Gly 4.79±0.90	Phe 6.52±0.18
$\Delta G'$[e]	104.75 kJ.mol^{-1}	Cys 0.53±0.07	His 1.46±0.18	Thr 7.28±0.23

N-terminal sequences: 29 kDa band: SEVVSFSFTKFNPNQKDII;
15 kDa band: mixture of SEVVSFSFTKFNPNQKDII and KFNQIVAVEEDTDXESQPQ

[a] Molecular and physicochemical properties were obtained as described in the Method section.
[b] Mean±SD of three independent analyses.
[c] Obtained through gel filtration chromatography on Sephadex G-100.
[d] Extinction coefficient (280 nm, 1% lectin, 1 cm cuvette).
[e] The activation energy required for denaturation of LAA.

conditions, showed a single, sharp and symmetrical peak (data not shown), with molecular mass of about 123.5 kDa. This molecular mass is very similar to that (122–130 kDa) of *Vatairea macrocarpa* lectin (VML) calculated by analytical ultracentrifugation (Calvete et al., 1998). Both *L. auriculata* and *V. macrocarpa* species belong to the *Fabaceae* family, sub-family *Papilionoideae* (Custódio, 1991). However *L. auriculata* is a species of the tribe *Sophoreae* whereas *V. macrocarpa* belongs to the *Dalbergieae* which evolved from *Sophoreae*.

2.5. Effect of pH on the lectin structure

LAA was found to be tetrameric at pH 7.6, dimeric at pH 5.0 and monomeric at pH 3.0 (Fig. 2). According to Cavada et al. (1998) size-exclusion chromatography of *V. macrocarpa* lectin indicated that, at neutral pH, it is predominantly dimeric (70 kDa), although tetramers (115 kDa) and larger aggregates (300 kDa) were also present. However, the quaternary structure of the *V. macrocarpa* lectin was not pH-dependent when investigated by analytical ultracentrifugation equilibrium sedimentation (Calvete et al., 1998). This discrepancy between these above results produced by the same group is difficult to explain. However, it cannot be excluded that the results reported by Cavada et al. (1998) were obtained using a *V. macrocarpa* lectin preparation that had not a high degree of purity. Nevertheless, differences in the association of subunits of

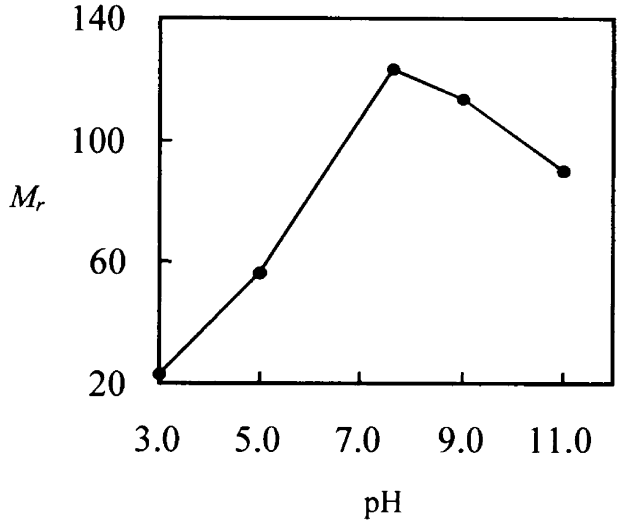

Fig. 2. Molecular mass of the cotyledonary *L. auriculata* lectin at different pHs, calculated after exclusion chomatography on Superdex 200 HR 10/30. Buffers at 0.05 M concentration and containing 0.15 M NaCl were: glycine–HCl (pH 3.0); sodium acetate (pH 5.0); Tris–HCl (pH 7.6); glycine–NaOH (pH 9.0); and sodium phosphate (pH 11.0). The lectin (3 mg) was dissolved with 1 ml of the appropriate buffer and 200 μl applied onto the column at 30 ml h^{-1} flow rate. The column was previously calibrated with proteins of known molecular mass at all pH analyzed. Fractions (1 ml) were collected and monitored for protein content at 280 nm.

legume lectins had been reported even for species phylogenetically related. The lectin from *Canavalia ensiformis* seeds (ConA) behaves as a tetramer at pH above 7.0 (Kalb and Lusting, 1968) and as a dimer at pH below 5.0 (Agrawal and Goldstein, 1968) whereas the molecular mass of the lectin from *Canavalia brasiliensis* seeds (ConBr) continuously increases as the pH rises (Grangeiro et al., 1997). Although LAA and *V. macrocarpa* lectin showed extensive similarities regarding some of their physicochemical properties, association of their polypeptide subunits to form the quaternary structure seems to be different under similar hydrogen ionic conditions. It is worthwhile to mention that at neutral pH, when LAA assumes a tetrameric form, it was capable of inhibiting the growth of some phytopathogenic fungi and binding reversibly to *S. cerevisiae* cells, suggesting that it could interact with the cell wall and/or plasma membrane of various micro-organisms (unpublished data). However, it is also possibile that monomeric or dimeric forms of LAA bind to cell structure and exert their effects upon the micro-organisms being the unique exigency the presence of at least one carbohydrate binding site per monomer able to recognize a cell receptor. For instance, hevein a 9–10 kDa lectin present in the latex of the rubber-tree (*Hevea brasiliensis*) behaves as a monomeric protein and exhibited antifungal properties to various fungi (Van Parisjs et al., 1991).

2.6. Denaturing polyacrylamide gel electrophoresis and N-terminal sequence

SDS-PAGE of LAA, both in the presence and absence of β-mercaptoethanol, showed a major band of 29 kDa, and two minor ones around 15 kDa, which are not linked by a disulfide bridge (Fig. 3; Table 2). The N-terminal amino acid sequence (SEVVSFSFTKFNPNQKDII) of the 29 kDa band showed 94% identity to that of the *V. macrocarpa* lectin (SEVVSFSFTKFNPNPKDII) (Calvete et al., 1998) and high homology with the lectins from species belonging to the *Sophoreae* tribe such as *Maackia amurensis*, *Sophora japonica* and *Cladrastis lutea* (Altschul et al., 1997). However, the 15 kDa double band gave two distinct N-terminal sequences. One of them was identical to that of the intact subunit (29 kDa) and the other one was KFNQIVAVEEDTDXESQPQ, which when aligned with various other internal polypeptide sequences showed some degree of homology with several legume lectins (Table 3). Since this 15 kDa-double band excised from the PVDF sheet was not further fractionated, the two amino acid sequences obtained might comprise the N- and C-terminal fragments of the LAA 29 kDa main subunit. It has been reported that several lectins from legumes are cleaved in situ by endopeptidases producing a mixture of intact

Table 3
Comparison of the N-terminal sequence of the 15 kDa band of *Luetzelturgia auriculata* lectin (LAA) with internal polypeptides of seed lectins of *Vatairea macrocarpa* (Vm), *Maackia amurensis* (Maa), *Ulex europeus* (Uea), *Griffonia simplicifolia* (GS-IV), *Robinia pseudoacacia* (Rp), *Sophora japonica* (Sj) and *Erythrina variegata* (Ev)

Lectin	N-terminal sequence																			
LAA 15 kDa	1	K	F	N	Q	I	V	A	V	E	E	D	T	D	X	E	S	Q	P	Q
Vm (β-chain)	116	K	S	V	Q	T	V	A	V	E	F	D	T							
Maa	121			N	Q	I	V	A	V	E		D	T							
Uea	120			N	Q	I	I	A	V	E		D	T							
GS-IV	123			N	Q	V	V	A	V	E		D	T							
Rp	150			N	Q	I	V	A	V	E		D	T							
Sj	158				Q	I	I	A	V	D		D	T							
Ev	122				Q	T	L	A	V	E		D	T							

and cleaved subunits (Lakhtin, 1994). For instance, the lectin from *V. macrocarpa* seeds is composed of a major 32–34 kDa double subunit (full-length α-subunit) which is posttranslationally processed to give two minor polypeptides, the β-chain (22 kDa) and the γ-chain (13 kDa) corresponding to the C- and N-terminal fragments, respectively (Calvete et al., 1998; Cavada et al., 1998). Taken into account the molecular mass, number of subunits, cross-reactivity of the anti-LAA polyclonal antibody with the protein bands of *V. macrocarpa* lectin in Western blot assays (data not shown), and N-terminal sequence similarities of LAA and *V. macrocarpa* lectin, it is suggested that LAA exists as a homotetramer of 123.5 kDa composed of the 29 kDa intact chain and its derived fragments.

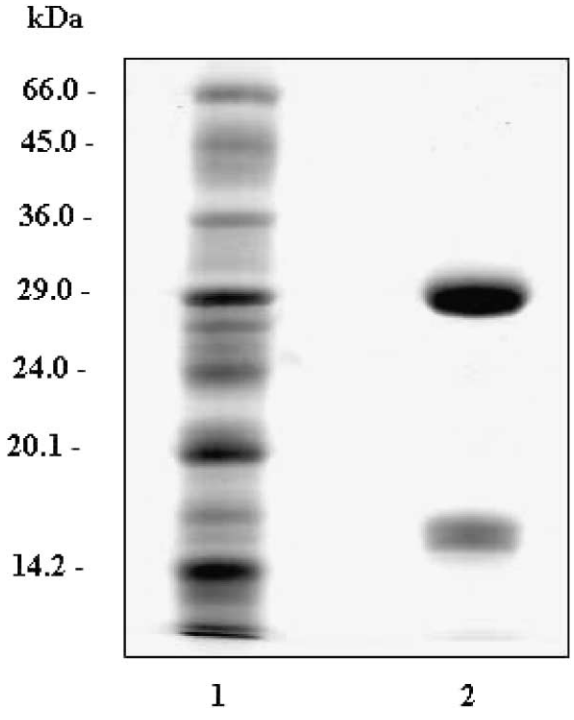

Fig. 3. Denaturing polyacrylamide gel electrophoresis (SDS-PAGE) of the lectin from *L. auriculata* cotyledons (lane 2) at 10 μg per well. Molecular markers (lane 1).

2.7. pI

The pI of 5.8 (Table 2) is in agreement with the observation that when buffered with 0.05 M sodium acetate (pH 4.0) and loaded on a CM-cellulose column, it was fully adsorbed and eluted as a single peak at 0.52 M NaCl concentration (data not shown). Acidic values of pI is common for several galactose-specific lectins such as those from various *Erythrina* species (Bhattacharyya et al., 1986), *Maclura pomifera* and *S. japonica* (Hankins et al., 1988).

2.8. Heat stability

LAA was relatively heat stable preserving 100% hemagglutinating activity when treated at 60 °C for 25 min (data not shown). However, at 80 °C the activity was fully abolished within 5 min. The activation energy of denaturation ($\Delta G'$) for LAA was estimated to be 104.75 kJ mol^{-1}. This value is comparable to those obtained for the galactose-specific lectins from *Erythrina velutina* forma *aurantiaca*, 104.08 kJ mol^{-1} (Moraes et al., 1996), *Artocarpus incisa*, 99.81 kJ mol^{-1} (Monteiro, 1998) and *V. macrocarpa*, 108.81 kJ mol^{-1} (Cavada et al., 1998) treated under similar conditions.

2.9. Spectroscopic characteristics

The absorption spectrum of LAA (figure not shown) showed a maximum at 280 nm and a extinction coefficient ($\varepsilon^{1\%}_{1cm}$) of 5.2 which is very close to that ($\varepsilon^{1\%}_{1cm} = 5.6$) reported for *V. macrocarpa* lectin. In the presence of the inhibiting sugar D-galactose, but not D-glucose which is not inhibitory, an increase in the extinction coefficient ($\varepsilon^{1\%}_{1cm} = 8.6$) was observed indicating that tryptophan and/or tyrosine residues are constituents of the carbohydrate binding site of the lectin or they reside in the vicinity of this binding site.

The CD spectrum of LAA was characterised by minima near 220 nm, maxima near 196 nm and a negative to positive crossover at 207.6 nm (Fig. 4). The

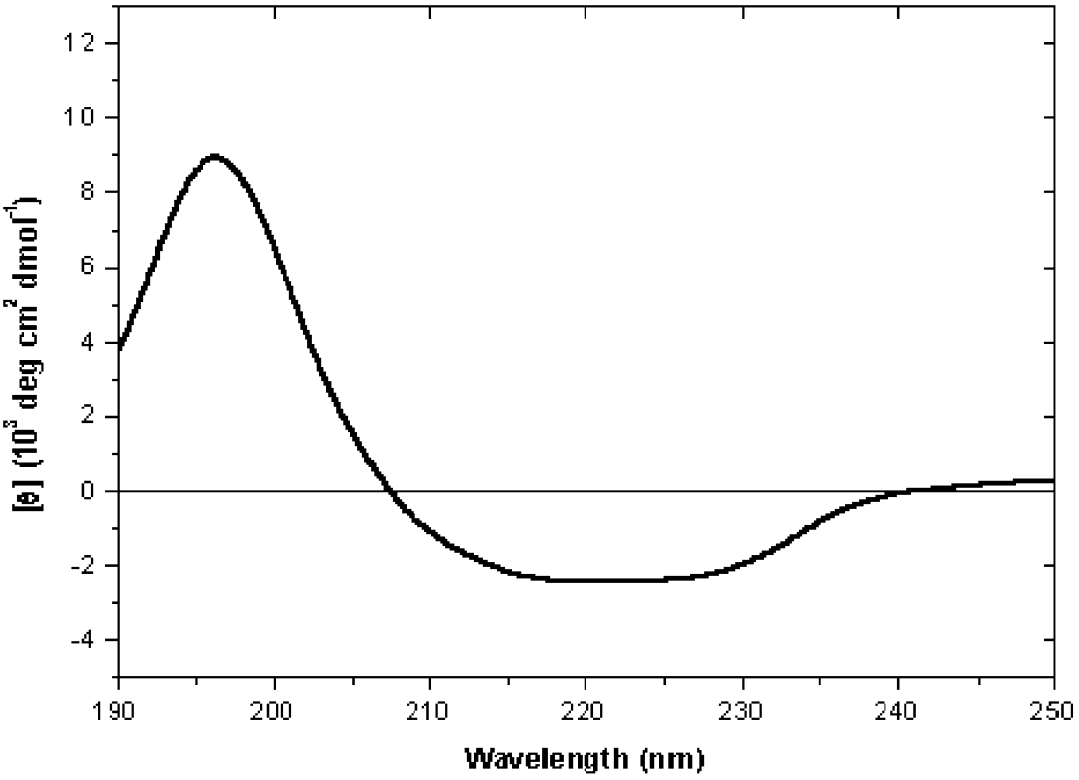

Fig. 4. CD spectrum of the cotyledonary lectin from *Luetzelturgia auriculata* in water.

shape of this spectrum is characteristic of a non-helical protein. The secondary structure content was estimated using the SELCON program and the following results were obtained: 6% α-helix; 8% parallel β-sheet; 38% antiparallel β-sheet; 17% β-turn; 31% unordered and others contributions; and 1% RMS (root-mean-square). Using a database of 33 references as standards, a very good correlation between predicted and calculated curves (1% RMS) was obtained. Among all the methods of determining protein conformation from CD spectra, the SELCON program is highly recommended for the estimation of the secondary structure of proteins in solution. In addition, the results of CD presented in this article are totally compatible with those of the literature data, when compared with lectins from *Erythrina corallodendron*, *Griffonia simplicifolia* and *Ulex europaeus* isolectin II, with known X-ray structures (Adar et al., 1998; Audette et al., 2000; Elgavish and Shaanan, 2001). All of these lectins are constituted by β-sheets, turns and random structures.

In the fluorescence spectroscopy, excitation of the lectin solution at 280 nm gave an emission spectrum in the 285–445 nm range (figure not shown). The wavelength maximum emission, λ_{max}^{emis} 334.5 nm, is typical for tryptophan residues buried inside the protein (Burstein et al., 1973; Eftink, 1991). These data confirm that the tertiary structure of LAA, and not merely its secondary structure, was correctly folded.

3. Concluding remarks

To our knowledge, the cotyledonary lectin of *L. auriculata* is the first agglutinin purified from this genus within the tribe *Sophoreae*. It showed remarkable similarities with the lectin from *V. macrocarpa* seeds and N-terminal homology with several other legume lectins, particularly with those from the tribe *Sophoreae* which are at the root of the *Papilionoideae* phylogenetic tree. These data suggest that during evolution many of their characteristics were conserved in accordance with an important physiological role for this class of protein. For instance, we have shown in our laboratory that LAA is released to the surrounding medium upon seed imbibition. Furthermore we observed that LAA displays inhibitory activity against several phytopathogenic fungi (unpublished results). As already observed for other plant lectins, these data suggest that LAA might be involved in the defense against soil microorganism during germination when the seed is most susceptible to pathogen attack.

4. Experimental

4.1. Materials

Sugars were obtained from Merck or from Sigma and acrylamide, bis-acrylamide and agarose-*N*-acetyl-D-

galactosamine from Sigma. Sephadex G-100 and Superdex 200 HR 10/30 were purchased from Pharmacia-LKB, Uppsala, Sweden. The other reagents were of analytical grade. Rabbit blood was obtained by puncture of the marginal ear vein of healthy animals. Human red blood cells were obtained from healthy donors at Centro de Hemoterapia da Universidade Federal do Ceará, Brazil. Cow, chicken and sheep blood cells were collected from animals at the Agronomy School of the Universidade Federal do Ceará, Brazil. *Luetzelburgia auriculata* (Fr. All) Ducke seeds were collected from plants grown in the state of Ceará, Brazil. Polyclonal antibodies against *L. auriculata* lectin were raised in New Zealand white rabbit according to Rios et al. (1996).

4.2. Protein determination

The method described by Bradford (1976) was used with bovine serum albumin (BSA) as standard. Absorbance at 280 nm (Ultrospec III spectrophotometer, Pharmacia LKB) was also used to determine protein content of column eluates.

4.3. Hemagglutination and inhibition assay

The assays were carried out essentially as described by Moreira and Perrone (1977), employing human, rabbit, cow, chicken, and sheep red blood cells. The hemagglutination titer is defined as the minimal amount (μg) of protein per ml able to induce visible erythrocyte agglutination and denoted as one hemagglutinating unit (HU). Carbohydrate-binding specificity of the lectin was assessed by the ability of sugars to inhibit agglutination of rabbit erythrocytes measured by preparing two-fold serial dilutions of the sugar solutions (0.1 M initial concentration) in 0.15 M NaCl. The lectin was added to each tube at a concentration of 9.92 μg ml^{-1}, corresponding to a 2 HU. The lowest sugar concentration giving full inhibition of agglutination was determined. The effect of EDTA on hemagglutinating activity was investigated by dissolving (2 mg) lectin in sodium acetate buffer (1 ml, 0.05 M, pH 6.0) containing 0.15 M NaCl and 0.025, 0.05, 0.10, 0.15, 0.20 or 0.25 M EDTA, respectively, and serially diluted with the corresponding solution. The hemagglutinating titer of each preparation was determined as described above.

4.4. Purification of the lectin

Mature seeds were dehulled and the cotyledons separated from the axes. Cotyledons were ground in a coffee grinder with the resulting flour treated with petroleum ether. Defatted flour (10 g) was extracted with 100 ml of 0.05 M sodium acetate buffer (pH 6.0) containing 0.15 M NaCl, for 4 h at room temp under constant stirring. After centrifugation at 20 000 *g*, 5 °C, 20 min (Sorvall RC-5B refrigerated centrifuge), the supernatant, denoted crude extract, was fractionated by saturation to 0–40% and 40–60% with solid ammonium sulfate. The precipitates were resuspended with the extracting buffer, dialyzed against distilled water until free of NH_4^+ ions, and lyophilized. The 40–60% fraction ($F_{40/60}$) was dissolved in 0.05 M sodium acetate buffer (pH 6.0) containing 0.15 M NaCl, the precipitate removed by centrifugation as above and the clear supernatant applied to an agarose-*N*-acetyl-D-galactosamine column. After elution of the unbound proteins from the column with the starting buffer, the lectin was eluted with 0.20 M galactose in the same buffer. Alternatively the lectin was purified using guar gum affinity chromatography following the same procedure used above.

4.5. Ion-exchange chromatography

L. auriculata lectin (10 mg) dissolved in 1 ml of 0.05 M sodium acetate buffer (pH 4.0) was applied to a CM-cellulose column (2.1 × 17 cm) equilibrated with the same buffer. After elution with 130 ml 0.05 M sodium acetate buffer, a linear gradient of increasing NaCl concentration (from 0 to 1 M in 100 ml) in the same buffer was applied, and the desorbed fraction collected and monitored at 280 nm.

4.6. Physicochemical properties

4.6.1. Molecular mass determination

The apparent molecular mass of the purified lectin was estimated using Sephadex G-100 column (1.2 × 75 cm) chromatographic analysis. The column was equilibrated with 0.05 M Tris–HCl buffer (pH 7.6) containing 0.15 M NaCl and calibrated with proteins of known molecular mass (BSA, 66 kDa; carbonic anhydrase, 29 kDa; and cytochrome c, 14 kDa). The lyophilized lectin (2.5 mg) was dissolved in 0.5 ml of equilibrating buffer and centrifuged at 15 000 *g*, 4 °C, for 10 min (Eppendorf refrigerated bench-top centrifuge 5417R). The resulting supernatant was filtered through a Pro-X™ filter unit (0.22 μm hydrophilic cellulose acetate membrane, Ltda Manufacturing Corp.) and applied to the column at 24 ml h^{-1} flow rate. The eluate absorbances were taken at 280 nm.

4.6.2. Effect of pH on molecular mass

The influence of pH on molecular mass of the lectin was assessed by exclusion chromatography on Superdex 200 HR 10/30 equilibrated with the following buffers at 0.05 M concentration and containing 0.15 M NaCl: glycine–HCl (pH 3.0); sodium acetate (pH 5.0); Tris–HCl (pH 7.6); glycine–NaOH (pH 9.0); and sodium phosphate (pH 11.0). The lectin (3 mg) was dissolved with 1 ml of the appropriate buffer, centrifuged as above, filtered through a Pro-X™ filter unit and

applied (200 μl) onto the column at 30 ml h^{-1} flow rate. The column was previously calibrated with the same molecular marker proteins described above at all pH analyzed. Fractions (1 ml) were collected and monitored for protein content at 280 nm.

4.6.3. Polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE)

The apparent subunit molecular mass of the lectin was determined on SDS-PAGE according to Laemmli (1970). Protein bands were stained with Coomassie Brilliant Blue R-250. The apparent molecular masses of the lectin subunits were estimated using bovine serum albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), soybean trypsin inhibitor (20.1), and lactalbumin (14.2) as standards (Sigma).

4.6.4. Heat stability

Heat stability was determined by incubating the lectin [1 mg ml^{-1} 0.05 M sodium acetate buffer (pH 6.0) containing 0.15 M NaCl] at 60, 65, 70, 75 and 80 °C for periods up to 60 min. The samples were cooled and hemagglutinating activity was determined as described above. The activation energy of the denaturing process ($\Delta G'$) was calculated using the Arrhenius equation (Dawes, 1972): $\Delta G' = RT\ln(kT/k_1h)$, where $\Delta G'$ is the standard free energy change, R is the gas constant (8.315 J K^{-1} mol^{-1}), T is the absolute temperature (K), k is the Boltzman constant (1.381×10^{-23} J K^{-1}), h is the Planck constant (6.626×10^{-34} J s), and k_1 is the velocity of reaction given by $k_1 = (\ln A/A_o)$ s^{-1}. A_o and A are initial and residual hemagglutinating activities, respectively.

4.6.5. Amino acid analysis

The native lectin (1 mg) was hydrolyzed in 1 ml 6 M HCl plus 1% phenol (w/v), in sealed glass tube under N_2, at 110 °C for 22 h. After hydrolysis, HCl and phenol were removed by evaporation and the residue analyzed in a BIOCHROM 20 (Pharmacia - LKB) amino acid analyzer. Tryptophan was determined according to the method described by Goodwin and Morton (1946). In addition the amino acid composition of the L. auriculata lectin bands extracted from the gel after SDS-PAGE were also determined.

4.6.6. Carbohydrate content

The neutral sugar content of the *L. auriculata* lectin was estimated by the method of Dubois et al. (1956) using D-glucose as standard. To remove non-covalently bound sugar, the lectin (1 mg ml^{-1}) was dissolved and dialyzed against 1 M acetic acid for 24 h at 4 °C (4 changes, 100 times the sample volume) followed by dialysis against 0.05 M sodium acetate buffer (pH 6.0) containing 0.15 M NaCl. The presence of covalently bound carbohydrate was also confirmed by specific staining of the protein bands after SDS-PAGE using the Glycoprotein Detection Kit (Sigma).

4.6.7. Analytical isoelectric focusing (IEF)

IEF was performed on precast polyacrylamide gels (FastGel, Pharmacia, Sweden) in the pH range of 3–9. The pH gradient in these gels was determined from the results of simultaneous runs performed with a wide-range isoelectric-focusing protein calibration kit (Pharmalyte 3-10). The stained gels were traced onto graph paper and the position of the bands plotted *vs* the known isoelectric point of the proteins in the calibration kit.

4.6.8. N-terminal sequence analysis

The N-terminal amino acid sequence of the purified lectin was determined on a Shimadzu PPSQ-10 Automated Protein Sequencer performing Edman degradation. Sequences were determined from lectin blotted on PVDF (Immobilon P; Millipore, Bedford, MA) after SDS-PAGE. PHT-amino acids were detected at 269 nm after separation on a reversed phase C_{18} column (4.6 × 2.5 mm) under isocratic conditions, according to the manufacturer's instructions. The sequence was compared to amino acid databases. The sequences selected were submitted to automatic alignment, which was performed by using the NCBI-BLAST search system.

4.6.9. Immunodetection of proteins

After SDS-PAGE, proteins were transferred to PVDF membranes (Towbin et al., 1979) with a semidry transfer unit (Multiphor II, Pharmacia) with 0.025 M Tris base (pH 9.4), 0.198 M glycine, containing 20% MeOH (w/v), for 1.5 h at 0.8 mA cm^{-1}. The transfer efficiency was checked by staining the membrane with 2% Ponceau S (w/v). The blots were blocked for 1 h, at room temperature, in 0.1 M sodium phosphate buffer (pH 7.6) containing 0.5 M NaCl and 2% nonfat milk powder (w/v) and incubated overnight with the primary antiserum (diluted 1:1000) against the *L. auriculata* lectin included in the blocking buffer. After membranes had been washed (4 times, 10 min each) with the phosphate buffer without milk, the blots were incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma Immuno Chemicals) (1:2000) in the blocking buffer for 2 h at room temperature and washed as above. Color development was performed with Sigma Fast™ BCIP/NBT tablets insoluble alkaline phosphatase substrate, according to the manufacturer's directions.

4.6.10. Spectroscopic measurements

Absorption spectra were recorded using a Pharmacia-LKB Ultrospec III spectrophotometer, with 1 mg lectin dissolved in 1 ml water (Milli-Q grade) in the presence or absence of 0.10 M D-galactose at 210–330 nm range.

Circular dichroism (CD) measurements were performed using a Jasco J-720 spectropolarimeter over a wavelength range of 185–260 nm, under constant N_2 purging according to the manufacturer's instructions (Jasco). Measurements were made on lectin samples at a 0.085 mg ml^{-1} protein concentration in deionized water, in quartz cuvettes of l mm path length. Spectra were typically recorded as an average of 64 scans, with the CD spectra obtained on a mdegree ellipticity (θ) scale. They were transformed using the mean weight residue and concentration prior to secondary structure analysis. Analysis of the CD spectra in terms of secondary structure content (deconvolution) was performed using the Selcon-2 method (Self Consistent Method), with 33 proteins in the data bank, developed by Sreerama and Woody (1993). Fluorescence measurements were performed at 22 °C on a Perkin-Elmer spectrofluorometer. Samples were excited at 280 nm and the emission was monitored in the 290–450 nm range. Quartz cuvettes (1 cm path length) of l ml volume were used for the measurements. The protein concentration used in these experiments was 0.085 mg ml^{-1}, in deionised water, so that the optical density at 280 nm was always 0.08.

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