Isolation and characterization of novel lectins from C*anavalia ensiformis* DC and *Dioclea grandiflora* Mart. ex Benth. seeds

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Received: 19/07/2005, Accepted: 03/10/2005

Two lectins were isolated from *Canavalia ensiformis* and *Dioclea grandiflora* seeds. Gel filtration produced a fraction corresponding to Con A or *D. grandiflora* lectin while erythroagglutination assays revealed a distinct fraction presenting a lectin that agglutinates human red blood cells (RBCs) but not rabbit RBCs. Hydrophobic interaction chromatography showed that the latter fraction yielded a protein that readily agglutinates human erythrocytes; the lectin was also purified by affinity chromatography on Lac-Sepharose showing similar properties to that of the Phenyl-Sepharose-purified lectin. Despite minor differences (carbohydrate content or $A^{1\%}_{1cm}$), the two lectins showed similar molecular properties in that they consisted of two non-covalently linked monomers having a M_r of 29-30 kDa and their pI values indicated that both lectins were slightly acidic proteins. The *C. ensiformis* lectin (CEL-II) and *D. grandiflora* lectin (DGL-II) specifically recognised the H-type 2 blood group (α-L-Fuc (1-2)–β-D-Gal (1-4)-β-D-GlcNAc–O-R), while binding to H-type 1, H-type 3, H-type 4, Le^a or Le^y was weaker. Carbohydrate inhibition of erythroagglutination showed that simple sugars were weakly recognised by the lectins, if at all. The N-terminal region presented a unique sequence hitherto found only in some Diocleinae lectins (designated type II). The overall results confirmed the existence of a second distinct lectin type, phylogenetically close to Diocleinae species. The data indicate a functional similarity among lectins of this type which possesses distinctive characteristics differentiating them from "classical" Man/Glc lectins.
Key words: Diocleinae, Leguminoseae, characterization, lectins.

Isolamento e caracterização de novas lectinas de sementes de *Canavalia ensiformis* DC e *Dioclea grandiflora* Mart. ex Benth.: Isolaram-se lectinas de sementes de *Canavalia ensiformis* e *Dioclea grandiflora*. Coluna de filtração em gel produziu uma fração correspondente Con A ou lectina de *D. grandiflora*, enquanto ensaios de eritroaglutinação revelaram uma fração distinta, com uma lectina que aglutinou glóbulos vermelhos humanos, mas não de coelho. Cromatografia de interação hidrofóbica mostrou que a fração contendo a última lectina rendeu uma proteína que prontamente aglutinou eritrócitos humanos. Essa lectina também foi purificada por cromatografia de afinidade em coluna Lac-Sepharose e mostrou propriedades similares àquela purificada da coluna de interação hidrofóbica. Apesar de pequenas diferenças (conteúdo de carboidratos ou $A^{1\%}_{1cm}$), ambas lectinas mostraram propriedades moleculares semelhantes, consistindo em dois monômeros não-covalentemente ligados, com M_r de 29-30 kDa, e com valores de pI que as indicavam como proteínas pouco acídicas. As lectinas de *C. ensiformis* (CEL-II) e *D. grandiflora* (DGL-II) reconheceram especificamente o grupo sanguíneo "H-type 2" (α-L-Fuc (1-2)–β-D-Gal (1-4)-β-D-GlcNAc–O-R), com ligação mais fraca com os grupos "H-type 1, H-type 3, H-type 4, Le^a ou Le^y. Inibição de eritroaglutinação por carboidratos mostrou que açúcares simples foram fracamente reconhecidos pelas lectinas. A região N-terminal apresentou um seqüência única observada apenas em algumas lectinas de Diocleinae (denominadas tipo II). Os resultados confirmam a existência de um segundo tipo distinto de lectina, filogeneticamente próxima a espécies de Diocleinae. Os dados indicam uma similaridade funcional entre as lectinas desse tipo, que possuem características que as distinguem das clássicas lectinas Man/Glc.
Palavras chave: Diocleinae, Leguminoseae, caracterização, lectinas.

INTRODUCTION

The specific carbohydrate recognition properties of lectins makes them important tools in glycobiology and, although their physiological role still remains unknown, they appear to mediate protein–cell and cell–cell interactions (Sharon and Lis, 1989).

The vast majority of lectins have been isolated from Leguminosae, although they are also present in bacterial and animal cells (Liener et al., 1986). ConA is the most extensively studied lectin, being present in *Canavalia ensiformis*, a species from the Diocleinae subtribe. This subtribe is widely distributed throughout the neotropics and, in recent years, several species from the genus Dioclea have been shown to possess a lectin closely related to ConA. The better characterized lectins are those from *D. grandiflora* (Moreira et al., 1983, Richardson et al., 1984), *D. lehmanni* Diels (Pérez et al., 1990, Pérez et al., 1991), *D. sericea* Kunth (Sierra and Pérez, 1999) and *D. altissima* Rock (Moreira et al., 1997) seeds. Lectins from *Cratylia molllis* Mart. ex Benth. (Correia and Coelho, 1995), *D. virgata* (Rich) Amshoff (Sousa Cavada et al., 1996a), *D. rostrata* Benth. (Sousa Cavada et al., 1996b) and *D. violacea* Mart. ex Benth. (Moreira et al., 1996) seeds have been partially characterized. All of them belong to the Man/Glc group and have very similar physicochemical properties and structural features.

Pérez (1998) has shown that a second lectin is present in the seeds of *D. lehmanni* (DLL-II), having distinct structural and functional properties. It is localised in mature cotyledon protein bodies where the lectin analogous to ConA (DLL-I) is also present (Melgarejo and Pérez, 1997). The aim of the present investigation was to determine the presence and properties of lectins similar to DLL-II in the related *C. ensiformis* and *D. grandiflora* species.

MATERIAL AND METHODS

Materials: *C. ensiformis* seeds were collected near San Juan de Arama, Meta (Colombia), and *D. grandiflora* seeds were collected in Crato, Ceará (Brazil) and kindly provided by Dr R. Moreira (Universidade Federal do Ceará, Brazil). Fresh human blood was obtained from the University's clinical laboratory. Animal erythrocytes were supplied by the Veterinary Faculty's haematology laboratory. Pharmacia and BioRad supplied the chromatographic supports. ConA-Sepharose and proteins were all obtained from Sigma. The neoglycoconjugates were a kind gift from Dr J. Le Pendu (INSERM, Nantes). Sugars and commercial products were of the highest available purity. The reagents for sequence determination were all sequencer grade. The rest of the reagents were analytical grade.

Lectin purification: Defatted flour of *C. ensiformis* or *D. grandiflora* seeds (10 g in small-scale experiments and 150-200 g in medium-scale experiments) was extracted (1:10, w/v) with 1 % NaCl, 5 mM PMSF, 2.5 mM EDTA, 2.5 mM EGTA and 0.4 μM Leupeptin at pH 7.0. The procedure followed was essentially as that described by Peña et al. (1988), using three consecutive extractions. The extracts were pooled and precipitated overnight at 4ºC with ammonium sulphate 30-70 % saturation for *D. grandiflora* and 50-70 % saturation for *C. ensiformis*. After centrifugation at 39,000 g_n for 20 min at 4ºC, the pellet was suspended in water and dialysed (x3) against 50 mM ammonium bicarbonate pH 8 and freeze-dried. The freeze-dried material (0.4 g) was fractionated on Sephacryl S-200 (2.5 x 148 cm) using the same procedure as described for the *D. lehmanni* lectin (DLL-II) (Pérez, 1998). Three fractions (I-III) were obtained after Sephacryl S-200 chromatography of which II and III exhibited erythroagglutination. These fractions were dialysed and freeze-dried as described above. The freeze-dried fraction II (40-50 mg) were applied to a Phenyl-Sepharose 4B column equilibrated with 20 mM phosphate buffer pH 7.2 containing 150 mM NaCl (PBS) and eluted by a 0-50 % linear acetonitrile (MeCN) gradient. In some instances, the MeCN gradient was replaced by elution with deionised H_2O. Alternatively, a Lac-Sepharose support was used as affinity matrix, according to Almanza et al. (2004). A crude extract or fraction II from Sephacryl S-200 was applied to the support and the lectin eluted with 0.2 M Lac-PBS, dialysed and freeze-dried as described above.

General methods: The methods cited by Pérez (1984) were used for determining agglutination and carbohydrate inhibition as well as for protein, neutral sugars, Trp content and extinction coefficients. The specific titre was defined as being the haemagglutination titre divided by the protein concentration ($mg.mL^{-1}$) of the assay solution. This assay was carried out with O+ erythrocytes. Amino acid composition was determined using an Applied Biosystems 421 Amino Acid Analyzer. The minimal inhibitory concentration assays were carried out with 30-150 mM lactose, melezitose and sucrose with 25 μL pure lectin and A^+ erythrocytes. SDS-PAGE was performed using a 15 % separating gel and 4 % stacking gel, according to Laemmli (1970). The lectin's glycoprotein nature was assessed using the periodate-Schiff and ConA-peroxidase (Faye and Chrispeels, 1985) methods.

Lectin binding to neoglycoconjugates: The assay method was similar to that described by Almanza et al. (2004). The neoglycoconjugates (100 µL), diluted to 0.68 µg.mL^{-1}, were coated on microtitration plates (NUNC, F16 Maxisorp) by incubating for 3 h at 37°C and then overnight at room temperature. Each plate was then washed once with PBS-20 % Tween and twice with PBS. After blocking the free sites with 200 µL 3 % BSA-PBS for 1 h at 37°C, 100 µL biotinylated lectin (0.5 mg.mL^{-1}) were added serially diluted in 3 % BSA-PBS and incubated for 2 h at 37°C. Each plate was then washed once with PBS-20 % Tween and twice with PBS. Streptavidin-peroxidase (100 µL at 1 mg.mL^{-1}, diluted 1:500 in 3 % BSA-PBS) was added and incubated for 1h at 37°C. H_2O_2-2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) was added after five washes (the first one with PBS-20 % Tween and the rest with PBS) and Abs_{405} was read in a Bio-Rad ELISA autoreader.

Molecular weight: Native protein M_r was determined using a Biogel P150 column (1.5 x 110 cm) equilibrated with 10 mM phosphate buffer pH 7.4. BSA (66.2 kDa), α-amylase (50 kDa), ovalbumin (45.0 kDa), myoglobin (17.2 kDa) and lysozyme (14.3 kDa) were used as standards. Subunit Mr was determined by SDS-PAGE, according to Laemmli (1970); the protein standards were the same as above.

pI: The pI was determined in non-denaturing conditions, according to Bollag and Edelstein (1991), using a 3.5-10 pH gradient.

N-terminal amino acid sequence: The protein was subjected to SDS-PAGE followed by Western blot transfer to a PVDF membrane (Matsudaira, 1987). The protein band was cut out and its sequence determined with an Applied Biosystems 470A Protein Sequencer (18 cycles).

Protease V8 digestion: The protein was digested according to Cleveland et al. (1977) and the peptide digest was subjected to electrophoresis according to Schagger and von Jagow (1987). The resulting bands were transferred to a PVDF membrane (Matsudaira, 1987) and sequenced with an Applied Biosystems 470A Protein Sequencer.

RESULTS

Lectin purification: Fractionation of *C. ensiformis* extracts by Sephacryl S-200 (figure 1A) yielded three fractions.

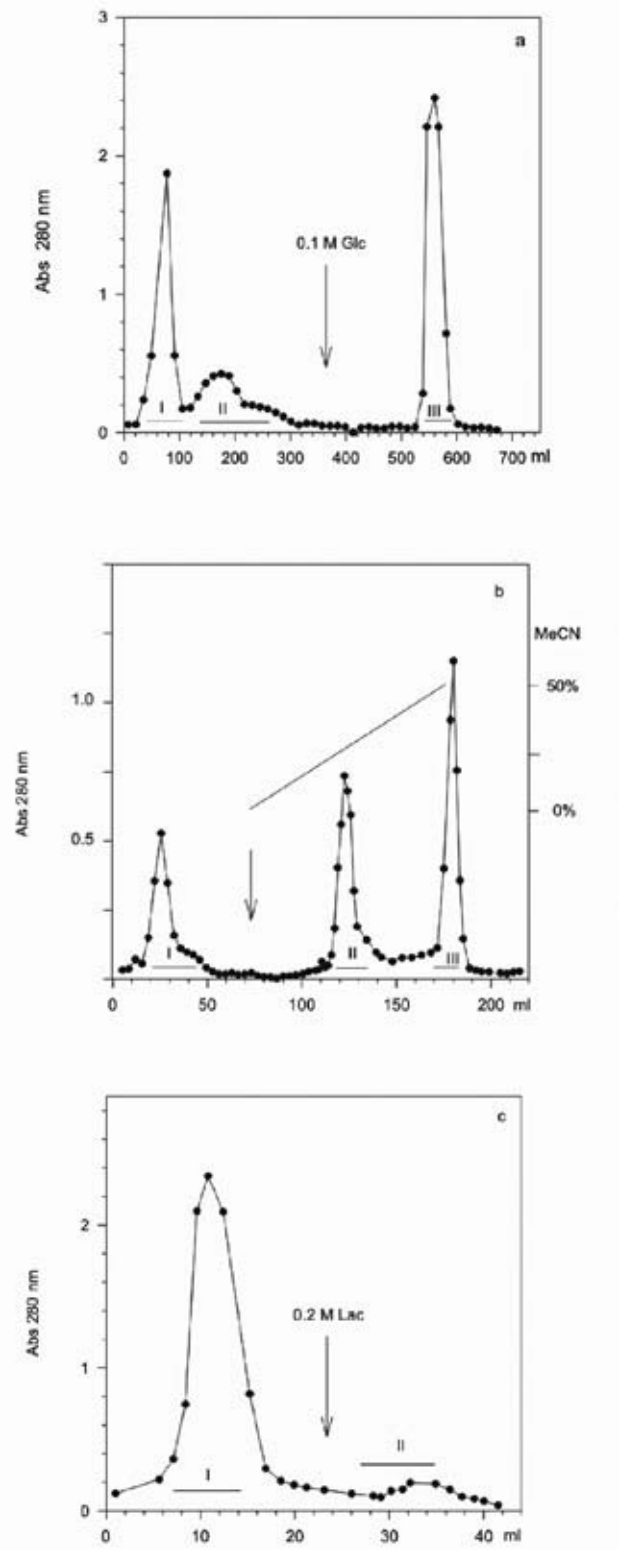

Figure 1. *Canavalia ensiformis* type-II lectin purification. (a) Gel filtration of $(NH_4)_2SO_4$ 50-70 % saturation precipitate on Sephacryl S-200. (b) Hydrophobic chromatography of fraction II (eluted from Sephacryl S-200) on Phenyl-Sepharose 4B. (c) Affinity chromatography of crude extract on Lac-Sepharose 4 B.

Fraction III corresponded to Con A since it showed the same erythroagglutination characteristics (rabbit positive, human negative), carbohydrate inhibition pattern and dextran gel-binding properties as those shown by Con A (Agrawal and Goldstein, 1967; Olson and Liener, 1967). Erythroagglutination assays of fraction II revealed the presence of a lectin that agglutinated human red blood cells (RBCs) but not rabbit RBCs. This fact explains why this lectin has been hitherto undetected in *C. ensiformi*s extracts as current procedures only employ rabbit RBCs. Similar behaviour has been observed by Pérez (1998) and Sierra and Pérez (1999) with *D. lehmanni* and *D. sericea* type-II lectins. Fraction II was therefore chromatographed on a Phenyl-Sepharose column and a MeCN gradient used to elute the retained fraction. The elution profile (figure 1B) showed three peaks, of which only II was able to agglutinate human erythrocytes; the third peak (devoid of erythroagglutination activity and characteristically turbid) was eluted at the very end of the gradient; MeCN was occasionally and advantageously replaced by deionised H_2O with similar results, the only difference being the smaller size of the eluted peaks (results not shown). The H_2O-eluted protein was able to agglutinate human RBCs and preliminary experiments (SDS-PAGE, carbohydrate inhibition of erythroagglutination) showed that it possessed the same properties as the MeCN-eluted protein. Protein elution by H_2O probably occurred due to the weakening of hydrophobic interactions between the lectin and the support; a similar situation has been described during the purification of Phaseolus (Ochoa and Kristiansen, 1978) and Arthocarpus lectins (Moreira and Oliveira, 1983).

An alternative way of purifying the lectin is suggested by the similarity between *Galactia lindenii* lectin and Dioclea lectins observed by Almanza et al. (2004). As the former was usually purified by affinity chromatography on Lac-Sepharose, crude extracts from *C. ensiformis* or fractions II from Sephacryl S-200 were run on this support. The elution profile showed a distinct fraction eluted by 0.2 M Lac-PBS (figure 1C, fraction II) which presented similar behaviour to the Phenyl-Sepharose purified lectin after dialysis. Most characterization assays were done on the latter. Quantifying lectin activity during the several purification steps showed that the specific *C. ensiformis* lectin II (CEL-II) titre increased as its purification proceeded (table 1). The purification achieved after the hydrophobic chromatography step was 25.7 fold, which was higher than that obtained with DLL–II (9.6 fold) (Pérez, 1998).

Similar results were obtained with *D. grandiflora* extracts which yielded a fraction (figure 2A, fraction II) after Sephacryl S-200 chromatography that was able to agglutinate human RBCs and a fraction eluted by 0.2 M Glc (figure 2A, fraction III) which was unable to agglutinate human RBCs and corresponded to the DGL-I lectin described from *D. grandiflora* (Moreira et al., 1983). An erythroagglutinating peak (figure 2B, fraction II), named DGL-II, eluted with the MeCN gradient when fraction II was run on Phenyl-Sepharose, while elution with H_2O yielded a similar profile (results not shown) with an erythroagglutinating protein fraction II. Affinity chromatography on Lac-Sepharose allowed the lectin to be recovered (figure 2C, fraction II). PAGE-SDS of CEL-II and DGL-II revealed a single M_r 29-30 kDa band (figure 3). Protein amount and specific titre were monitored throughout the several purification steps (table 2), where a 33.5 fold purification was achieved.

Table 1. Purification of lectin II from *C. ensiformis.*

Purification step	Volume (mL)	Protein (mg.mL^{-1})	Total Protein (mg)	Specific Titre [a]	Purification (fold)
1. First saline extract	83	17.69	1468.3	0.45	
2. Second saline extract	96	7.26	967	0.28	
3.Third saline extract	103	1.73	178.2	0.58	
Pooled saline extracts	282	7.56	2131.9	1.06	1.0
4. 50-70% $(NH_4)_2SO_4$	29	9.75	282.8	1.64	1.55
5. Affinity Chromatography Peak II [b]	190	0.641	121.8	3.12	2.94
6.Hydrophobic chromat [c]	36	0.294	10.6	27.21	25.67

[a] The specific titre is defined as the haemagglutination titre divided by the protein concentration (mg.mL^{-1}) of the assayed solution. This assay was done with O+ erythrocytes; [b] 240.29 mg of freeze-dried globulin fraction applied to the column; [c] 42.84 mg of freeze-dried fraction II applied to the Phenyl-Sepharose column.

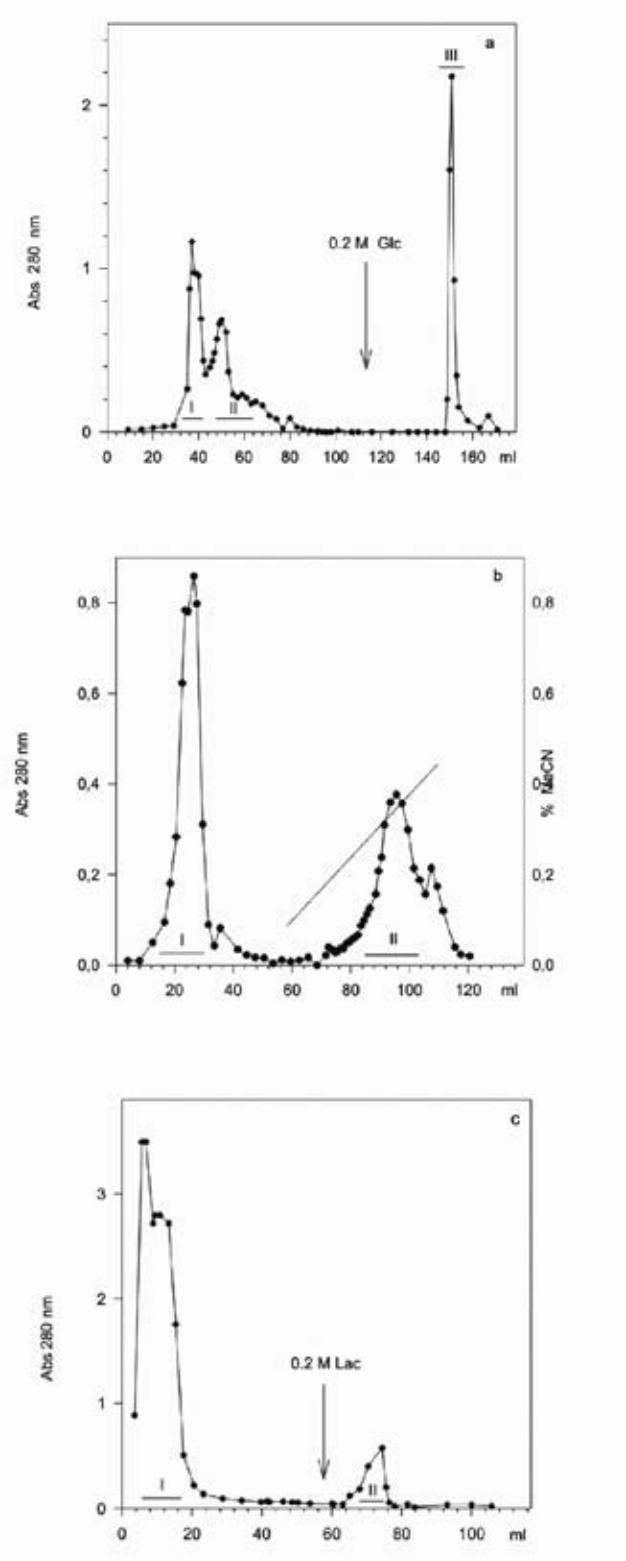

Figure 2. *Dioclea grandiflora* type-II lectin purification. (a) Gel filtration of $(NH_4)_2SO_4$ 30-70 % saturation precipitate on Sephacryl S-200. (b) Hydrophobic chromatography of fraction II (eluted from Sephacryl S-200) on Phenyl-Sepharose 4B. (c) Affinity chromatography of crude extract on Lac-Sepharose 4 B.

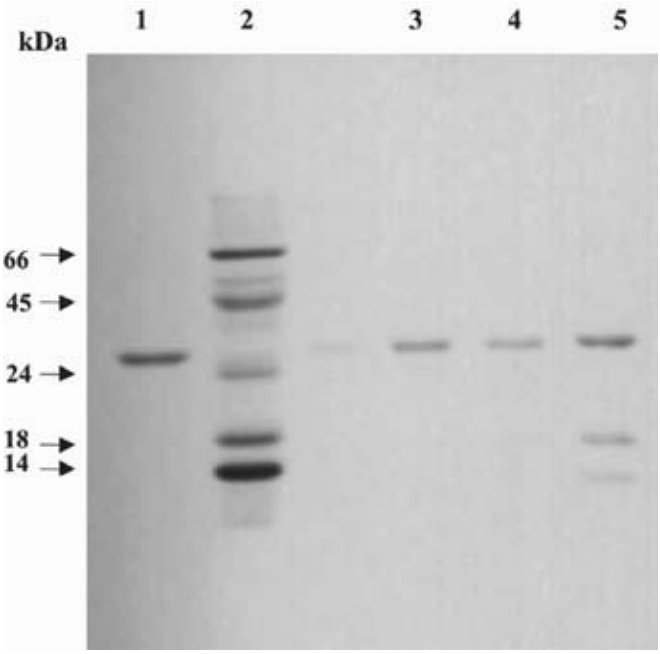

Figure 3. SDS-PAGE of CEL-II and DGL-II. Lane 1, DGL-II; lane 2, molecular weight standards; lane 3, CEL-II ; lane 4, DLL-II; lane 5, *Dioclea grandiflora* lectin (I).

Erythroagglutination and carbohydrate inhibition: Human A+, A_1+, B+ or 0+ RBC were agglutinated to the same extent by CEL-II and DGL-II, presenting distinct behaviour to that of ConA (*C. ensiformis* type I lectin) or DGL, which characteristically agglutinate rabbit erythrocytes with higher specific titres than those shown for human RBCs (Pérez, 1998).

Amongst the tested carbohydrates (*c.a.* 30), CEL-II and DGL-II were only inhibited by di/trisaccharides having no apparent structural relationship, contrary to that established for ConA and DGL–I which recognise Glc/Man (table 3). Although weak, melezitose inhibition was clearly stronger for DGL-II than for DLL-II. The relatively high sugar concentrations needed to inhibit erythroagglutination indicates that type II lectins probably recognise more complex structures.

Lectin binding to neoglycoconjugates: CEL-II bound the H-type 2-BSA neoglycoconjugate 10 times more strongly than H-type 1 and about 100 times more strongly than H-type 3 or H-type 4 (figure 4A) in our experimental conditions. Binding to Le^a and Le^y was equally weak. DGL-II behaved similarly to CEL-II, showing stronger binding to H-type 2-BSA than to the other assayed neoglycoconjugates (figure 4B). H-type 2 binding specificity was also observed with *D. lehmanni* lectin-II (figure 4C).

Table 2. Purification of lectin II from *D. grandiflora*

Purification step	Volume (ml)	Protein (mg.mL^{-1})	Total Protein (mg))	Specific Titre [a]	Purification (fold)
1.First saline extract	90	24.28	2185.20	2.64	
2. Second saline extract	95	5.79	550.1	0.69	
3. Third saline extract	94	1.64	154.2	0.61	
Pooled saline extracts	279	10.32	2879.3	0.78	1.0
4. 30-70% $(NH_4)_2SO_4$	30	21.56	646.8	2.97	3.81
5.Affinity Chromatography Peak II [b]	125	0.609	76.13	3.28	4.21
6.Hydrophobic chromat. [c]	14	0.612	8.57	26.14	33.51

[a] The specific titre is defined as the haemagglutination titre divided by the protein concentration (mg.mL^{-1}) of the assayed solution. This assay was done with O+ erythrocytes; [b] 292 mg of freeze-dried globulin fraction applied to the column; [c] 39.87 mg of freeze-dried fraction II applied to the column.

Table 3. Erythroagglutination inhibition by carbohydrate of DGL-II and CEL-II

Sugar (mM)	DGL-II	CEL-II	DLL-II[a]
Lactose	90	75	92
Melezitose	25	50	100
Saccharose	40	40	112
D - Mannose	- [b]	- [b]	- [b]
D - Glucose	- [b]	- [b]	- [b]

[a] Data taken from Pérez (1998); [b] 200 mM.

Molecular properties: The lectin subunit molecular weight (determined by SDS-PAGE) was close to 29-30 kDa (figure 3), being practically the same as that presented by DLL-II and DSL-II (table 4). The electrophoretic pattern was clearly different from that exhibited by type-I lectins where three polypeptide chains (α = 29-30 kDa, β= 16-17 kDa and γ= 12-13 kDa) are always present and arise as products from post-transcriptional processing of the mature chain (Bowles et al., 1986). Gel filtration revealed that the two lectins had similar molecular weights in their native state, being close to those shown by DLL-II and DSL-II (Table 4). Taking subunit MW into account, it is likely that the lectins were in dimeric form. It has been demonstrated (Moreira et al., 1983; Goldstein and Poretz, 1986) that tetrameric forms prevail in type I lectins, which are in rapid equilibrium with dimers and monomers whose amount depends on pH or solution ionic strength.

Determination of the pI showed that DGL-II and CEL-II were slightly acidic proteins (table 4) whereas ConA and DGL-I were neutral or clearly basic proteins. The several close bands observed (results not shown) were most likely due to isoforms which also appeared to be present in DSL-II. As opposed to type I lectins, CEL-II and DGL-II revealed the presence of associated carbohydrate which was also present in previously analysed type II Diocleinae lectins (table 4).

Amino acid composition: Table 5 shows the amino acid composition of the two lectins. DGL-II showed high amounts of acidic and hydroxy amino acids as well as Gly and Ala; sulphur amino acids were very scarce. A similar amino acid distribution was found for CEL-II, while DLL-II and DSL-II are shown for comparison. The extinction coefficient values found for the type–II lectins (table 4) were similar to those expected from their Trp and Tyr content.

The calculated M_r of CEL-II (24,311) on the basis of amino acid composition was close to the M_r calculated from SDS-PAGE data, taking the carbohydrate content into account (28,626). In the case of DGL-II, calculations showed similar agreement (24,393 *cf* 28,391). The resulting differences in both cases can be accounted for by the well-established M_r deviations exhibited by glycoproteins when analysed by electrophoresis. M_r over-estimation was evidenced by comparing the value determined for DSL-II by SDS-PAGE to that obtained by MALDI-TOF MS (table 4).

N-terminal sequence: DGL-II and CEL-II N-terminal sequences are shown in table 6. DLL-II, DSL-II, GLL, Con A and DGL-I N-terminal sequences are included for comparison. All residues could not be determined due to the limited amount of CEL-II. However, the available information allowed us to align its sequence with that of DGL-II and those of other lectins. The DGL-II N-terminal sequence showed a very high similarity to DLL-II, as only 6 out of 49 residues were not identical, including a two-residue gap and the substitution at position 29 (NxD).

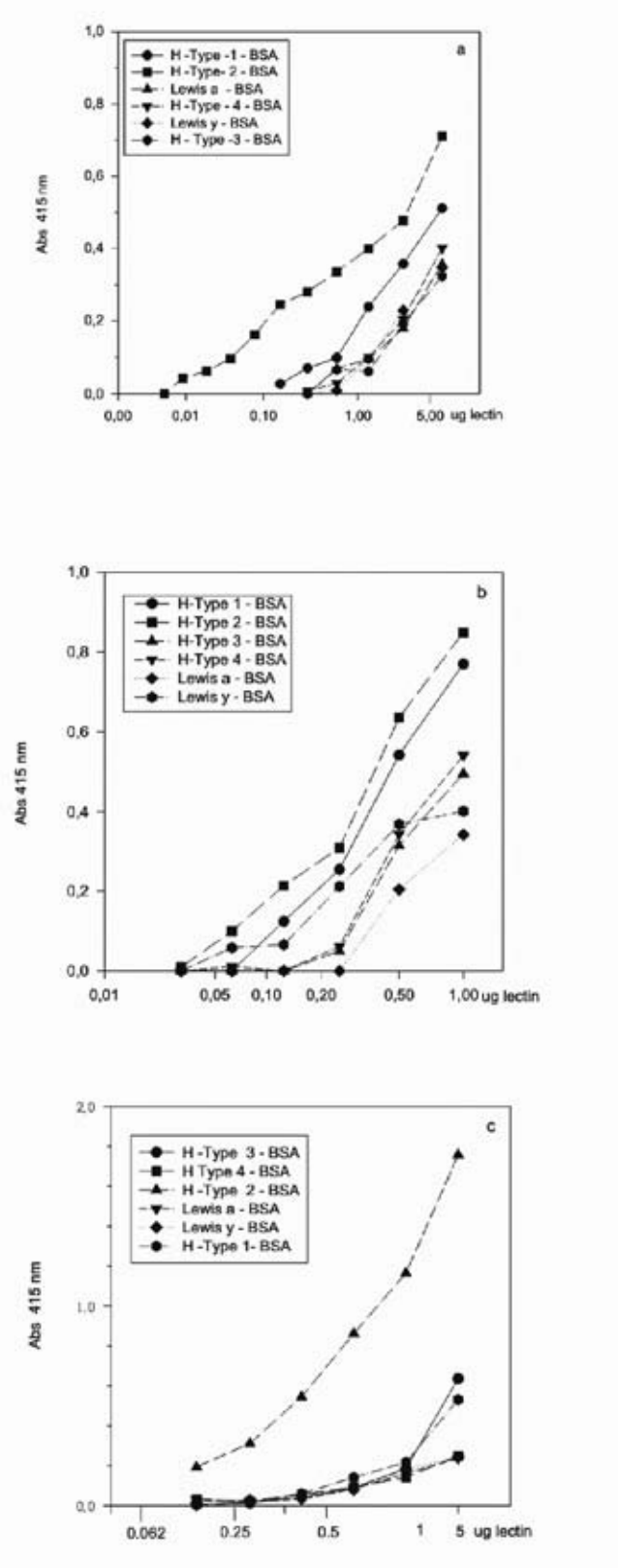

Figure 4. Lectin binding to H-type neoglycoconjugates. (a) *Canavalia ensiformis* type-II lectin. (b) *Dioclea grandiflora* type-II lectin. (c) *Dioclea lehmanni* type-II lectin.

Taking the CEL-II determined residues into account (21), all were identical to corresponding positions in DGL-II and a remarkable similarity was observed with the hitherto described Dioclea type-II lectins. It is likely that the seven undetermined residues (X) were similar to those of DGL-II. It is noteworthy that the SSSS sequence was present in all type II lectins from the Dioclea genus as well as in *G. lindenii* lectin. DGL-II and CEL-II N-terminal sequences were clearly different from those of Con A and DGL-I lectins.

DGL-II digestion with protease V8 yielded (amongst recovered peptides) +GAVYF, VSNNGILALT and FDDYSN+W++P peptides. The first one probably corresponds to the N-terminal end; the second peptide (residues 26-35) confirmed the NGILA sequence which appeared to be present only in DGL-II. The third peptide was homologous to *D. lehmanni* type-II lectin residues 124-130 (Pérez, unpublished results).

DISCUSSION

A close examination of Dioclea and Canavalia extract elution profiles on dextran supports (G-50 /G-75), commonly employed for isolating Glc/Man specific lectins (Moreira et al., 1993, 1996, 1997; Correia and Coelho, 1995; Sousa Cavada et al., 1996a; 1996b), shows (in all cases) a distinct unretained second peak. Type-II lectins are characteristically present in this fraction and have probably been overlooked as no erythroagglutination assays have been carried out with human RBCs. It should be recalled that no agglutination of human RBCs has been observed with this lectin since ConA was isolated (Sumner and Howell, 1936).

An estimation of the amount of lectin recovered showed lower proportions of CEL-II and DGL-II than DLL-II, these being 1.84 $mg.g^{-1}$ flour (Pérez, 1998) or GLL, 5.88 $mg.g^{-1}$ flour (Almanza et al., 2004), suggesting different expression levels.

Despite minor differences (carbohydrate content or $A^{1\%}_{1cm}$) the two lectins showed similar molecular properties, i.e. non-covalently linked homodimers with 57-58 kDa M_r, 29-30 kDa M_r subunit and a 5.1-5.4 pI.

The high similarity shown by the Diocleinae lectins in the N-terminal region prompted us to search for homology/ similarity with lectins from several Fabaceae and other non-leguminous plants; no similarity with any N-terminal lectin sequence was found, suggesting that, contrary to common belief, legume lectins have at least two distinctive N-terminal regions. The possibility that this region corresponds to a signal peptide was excluded as no similarity was found when lectin

Table 4. Molecular properties of lectins from Diocleinae species

	D. grandiflora		*C. ensiformis*		*D. lehmanni*	*D. sericea*
	Lectin II.[a]	Lectin I [b]	Lectin II.[a]	Con A [c]	Lectin II [d]	Lectin II [e]
M_r (kDa) Native protein	58.9	100	57.5	106[f]	58.4	53,273 [g] 57.3
M_r (kDa) Subunits	29-30	25-26; 13-14; 8-9	29-30	26.5; 14.0; 12.5 [f]	29-30	26,582 [g] 30
pI	5.4, 5.3, 5.1	8.6;8.7; 8.8;8.9;9.0	5.4;5.3;5.2	7.1	6.55	5.72, 5.38
Neutral sugars (%)	2.1	-	1.3	-	4.1	1.5
$A^{1\%}_{1cm}$	21.8	12.0	17.8	11.4	23.95	N.D.

[a]This work; [b]Moreira et al. (1983); [c]Agrawal and Goldstein (1968); [d]Pérez (1998); [e]Sierra and Pérez (1999); [f]Goldstein and Poretz (1986); [g]Determined by MALDI-TOFMS

Table 5. Amino acid composition of DGL-II and CEL-II

	D. grandiflora Residues/mol	*C. ensiformis* Residues/mol	*D. lehmanni* [a] Residues/mol	*D. sericea* [b] Residues/mol
Asx	23	25	38	33
Thr	14	13	21	19
Ser	22	31	32	35
Glx	23	30	21	13
Pro	14	0	12	9
Gly	43	58	22	33
Ala	27	27	24	31
Cys	0	2	0[c]	0[c]
Val	16	15	25	22
Met	1	1	3[d]	3[d]
Ile	10	9	16	14
Leu	20	14	21	16
Tyr	4	4	9	7
Phe	7	5	9	9
Lys	9	7	13	9
His	4	4	1	2
Arg	6	5	8	4
Trp[e]	2	3	4	6

[a] Pérez (1998) ; [b] Sierra and Pérez (1999) ; [c] Determined as $CySO_3$; [d] Determined as $MetSO_2$; [e] Determined spectrophotometrically. Calculations are based on a M_r 29,000 with 2.1 % carbohydrate for *D. grandiflora* and 1.3 % carbohydrate for *C. ensiformis* lectins.

precursor sequences were examined. The first eleven type-II lectin residues of Dioclea species are mainly hydrophobic amino acids, although this region has less marked hydrophobicity for the *G. lindenii* lectin. It is noteworthy that the SSSS sequence is present in all Dioclea genus type-II lectins as well as in *G. lindenii* lectin. We propose that this sequence is characteristic of type II Diocleinae lectins as it has not been found in any of the type-I lectins studied thus far.

Interestingly, Diocleinae type-II lectins (such as those found in the Galactia genus) specifically recognise and bind tightly to the H type-2 determinant (LePendu et al., 1986; Almanza et al., 2004). This fact, together with the structural relationships revealed by highly similar N-terminal regions, led us to test CEL-II and DGL-II binding to the H type-2 determinant. The relative order of binding to the neoglycoconjugates showed that the two lectins behaved similarly to *G. lindenii* lectin and *D. lehmanni* lectin.

Table 6. N-terminal sequence of D. g*randiflora, C. ensiformis, D. lehmanni, D. sericea* and *G. lindenii* lectins

10	20	30	40
1. AGAVYFXFTK	FTTSSSSLTL	QGSAEVSNNG	ILALTNLKNP TNKVGRALY
2. + GAV YF+FTK	FTTS+S+LTL	+G+AEV+N	
3. AGA VYFYFTK	FTTSSSSLTL	QGSAEVSNDH	K--LTNLKNP TNKVGRALY
4. AGA VYI RI TF	IT I SSSVLTL		
5. AKV T SI KYTS	IS - SSXGKPL	QGNA	
6. ADTIVA VELD	TYPNTDIGDP	DYPHIGIDIK	SVRSKKTAKW NMQDGKVGT
7. ADT I VA VELN	SYPNTDIGDP	NYPHIGIDIK	SIRSKSTARW NMQTGKVGT

1. *D. grandiflora* lectin II; 2. *C. ensiformis* lectin II; 3. *D. lehmanni* lectin II (Pérez, 1998) ; 4. *D. sericea* lectin II (Sierra and Pérez, 1999) ; 5. *G. lindenii* lectin (Almanza et al., 2004); 6. *C. ensiformis* lectin (ConA) (Becker et al., 1975); 7. *D. grandiflora* lectin I (Richardson et al., 1984) ; X = unidentified residue.

The overall results confirmed the existence of a second distinct lectin type in phylogenetically close Diocleinae species, as has been proposed by Almanza et al. (2004), and suggest a functional similarity amongst this type of lectin. These types of lectins possess distinctive characteristics that differentiate them from the "classical" Man/Glc lectins: a) Ability to strongly agglutinate human RBCs; b) specific interaction with Lac-Sepharose supports; c) recognition of H-type 2 trisaccharide; and d) N terminal sequences.

The evidence of a second different seed lectin in several Diocleinae species (including *C. ensiformis* and *D. grandiflora*) give rise to several questions. Are the two lectins localised in the same cellular compartment as occurs in *D. lehmanni* seeds (Melgarejo and Pérez, 1997)? Is there some sort of post-transcriptional processing as has been described for ConA (Herman et al., 1985; Bowles et al., 1986)? Is there a relationship between the expression levels of each lectin type? The still unresolved question concerning the physiological role of seed lectins seems to become more complex as distinct lectins occur simultaneously in a given species.

Acknowledgements: We wish to thank Dr J. LePendu (Nantes, INSERM) for his kind gift of neoglycoconjugates, Dr J.L. Fernández (ICN, Universidad Nacional) for species identification, Dr. L. Falquet (Swiss Institute of Bioinformatics) for his help and advice on multiple sequence alignments, Drs I. Hara-Nishimura and M. Nishimura (National Institute for Basic Biology, Japan) for the facilities provided to one of us (L.M.M.) and N-terminal sequence determinations. Financial support was provided by the Universidad Nacional's Chemistry Department and COLCIENCIAS.

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