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Lectins from seeds of *Crotalaria pallida* (smooth rattlebox)

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Abstract

A lectin from the seeds of *Crotalaria pallida* (CPL), with an apparent molecular mass of 30 kDa, determined by SDS–polyacrylamide gel electrophoresis, showed human type A and B erythrocytes agglutination activity, which is inhibited by raffinose and galactose. The lectin requirement for divalent cation was demonstrated with EDTA/EGTA blocking hemagglutination activity. Although the N-terminal amino acid sequence of CPL is identical to another lectin from *Crotalaria striata*, which is taxonomically synonymous to *Crotalaria pallida*, these lectins differ in amino acid composition and hemagglutination properties. © 2002 Published by Elsevier Science Ltd.

Keywords: *Crotalaria pallida*; Smooth rattlebox; Leguminosae; Galactose-specific lectin; Amino acid composition; N-terminal sequence

1. Introduction

Lectins are carbohydrate-binding proteins widely distributed in many plants, as well as in animals and other organisms (Lis and Sharon, 1986). Generally, lectins are very abundant in plants seeds, especially in the Leguminosae and Graminaceae seeds. In some plants, lectins are absent in immature seeds. However, most of the plants begin synthesizing lectins some time after anthesis (Pusztai, 1991).

Lectins from leguminous plants constitute a large family of homologous proteins displaying remarkable divergence in their carbohydrate specificity. These carbohydrate-binding molecules have been classified on the basis of their blood group specificity, and subsequently on the potency with which a monosaccharide inhibits their hemagglutination activity (Sharon and Lis, 1990).

The hemagglutination activity observed for almost all of the lectins does not directly reflect their physiological function. Lectins might serve as defensive agents against the attack by beetles, insects and other predators, including some mammalian species (Sengupta et al., 1997). Many plant lectins are often found to be potent natural immunomodulators (Oudrhiri et al., 1985), influencing human lymphocytes proliferation (Ghosh et al., 1999).

Only two lectins from *Crotalaria* seeds have hitherto been isolated and characterized, namely seed lectins from *Crotalaria juncea* (Ersson et al., 1973) and *Crotalaria striata* (Khang et al., 1990).

Studying the *Crotalaria* genus, we have found changes in the taxonomical status of some species in this genus. According to the International Legume DATABASE & Information Service (http://www.biodiversity.soton.ac.uk/legumeweb), 11 species of *Crotalaria* are now considered one single species designated as *Crotalaria pallida* Aiton: *Crotalaria brownei*, *Crotalaria falcata*, *Crotalaria fertilis*, *Crotalaria Hookeri*, *Crotalaria mucronata*, *Crotalaria pallida* Klotzsch, *Crotalaria pisiformis*, *Crotalaria striata*, *Crotalaria striata* var. *acutifolia*, *Crotalaria tinctoria* and *Crotalaria zuccarininana Crotalaria pallida*.

In this work, a lectin from *Crotalaria pallida* (smooth rattlebox) seeds was purified and characterized. Although this lectin is similar to the other seed lectin from *Crotalaria striata*, which is taxonomically synonymous to

Abbreviations: ILDIS; International Legume DATABASE & Information service; DTT; dithiothreitol; EDTA; ethylenedinitrilo tetracetic acid; EGTA; ethylene glycol bis (α-aminoethyl ether) N; N-tetracetic acid; PAGE; polyacrylamide gel electrophoresis; SDS; sodium dodecyl sulfate; TBS; tris buffered saline.

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Crotalaria pallida, each of these lectins posseses different structural and biological properties.

2. Results and discussion

2.1. Purification of CPL

Crotalaria pallida seed lectin was purified in two chromatographic steps, Sephadex G-75 gel filtration, and Protein Pak DEAE 5PW 60 anion exchange column. The arrow in Fig. 1 indicates the fraction having hemagglutination activity. Maximum lectin yield obtained from the purification of 50 g of *Crotalaria pallida* seeds was 45 mg, and the results of a typical purification of CPL are shown in Table 1.

2.2. Electrophoresis

SDS–PAGE analysis of CPL is showed in Fig. 2A. The apparent homogeneity of the lectin under reducing and non-reducing conditions showed that CPL subunit corresponds to a molecular weight of 30 kDa (Fig. 2B). The fact that CPL is about 30 kDa and is not retarded by G-25 column implies that this protein is oligomeric in nature. CPL appears to have carbohydrates covalently bound to its structure, since it gave a positive reaction with Schiff's reagent following treatment with periodate (Fig. 2C). CPL-Schiff's staining was compared with albumin and asialofetuin (positive staining) and trypsin (negative staining).

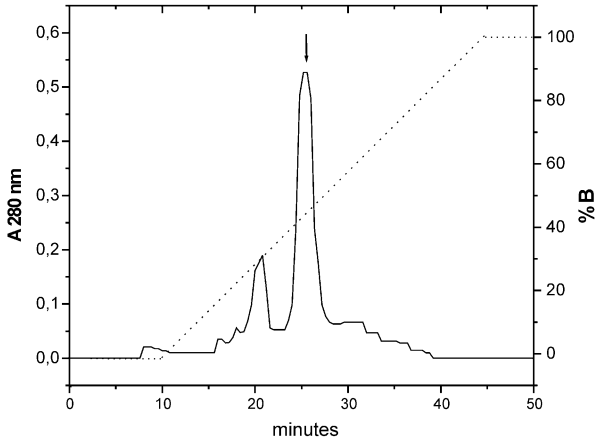

Fig. 1. *Crotalaria pallida* lectin purification by ion exchange column, Protein Pak DEAE 5PW 60 (7.5×75 mm), in 0.05 M Tris–HCl buffer pH 8.0, using a 0–100% linear gradient of solvent B (NaCl 0.5 M). Vertical arrow indicates the lectin (CPL).

2.3. Hemagglutination assays

CPL was found to be blood type specific to types A and B, distinguishing the latter from type O erythrocytes (Table 2). CPL agglutinates human erythrocytes type A, AB and B, being more specific to type A. Trypsinization of erythrocytes led to loss of CPL main specificity to type A. Unlike *Crotalaria striata* lectin (Khang et al., 1990), CPL agglutinated type B red blood cells, similar to *Crotalaria juncea* lectin (Ersson, 1977). CPL requirement for divalent cations was demonstrated with EDTA and EGTA, in a concentration of 24.3 μM, completely inactivating the lectin-induced hemagglutination activity. CPL did not show hemagglutination activity when reduced with DTT and/or carboxymethylated.

CPL-induced agglutination on type A erythrocytes was inhibited by *N*-acetyl-galactosamine, raffinose and galactose, in decreasing order of potency (Table 3). The other sugars, even at a concentration of 150 mM, did not show any inhibitory activity on CPL hemagglutination activity, in agreement with majority of galactose specific legume lectins having a common structural motif that define their binding specificities for Gal/GalNac (Sharma and Surolia, 1997), for example, PNA—peanut lectin (Young et al., 1991) and SBA—soybean agglutinin (Vodkin et al., 1983). *Crotalaria pallida* and *Crotalaria striata* seed lectins did not show variability in the carbohydrate hemagglutination inhibition pattern (Table 3), i.e. both lectins have their hemagglutination activities inhibited by *N*-acetyl-galactosamine and galactose.

2.4. Amino acid composition

CPL contains relatively large amounts of hydrophilic amino acids, such as, Asx, Glx, as well as Gly and Thr

Table 1
Purification of the lectin of *Crotalaria pallida* seeds (50 g)

Fraction	Total protein (mg)	Total activity (titer[a]×volume)	Specific activity (total activity/mg proteins)	Recovery (%)	Yield
Extract	2620	1600	0.610	100	1
Acetone	544.5	900	1.653	56.25	2.71
Sephadex G-75	76.0	320	4.210	20.1	6.90
DEAE 5PW 60	45.0	60	21.050	3.75	34.51

[a] Titer was defined as the reciprocal of the end-point dilution (starting at 1:32) with the hemagglutination with 2% human type A erythrocytes in Tris-buffered saline (TBS).

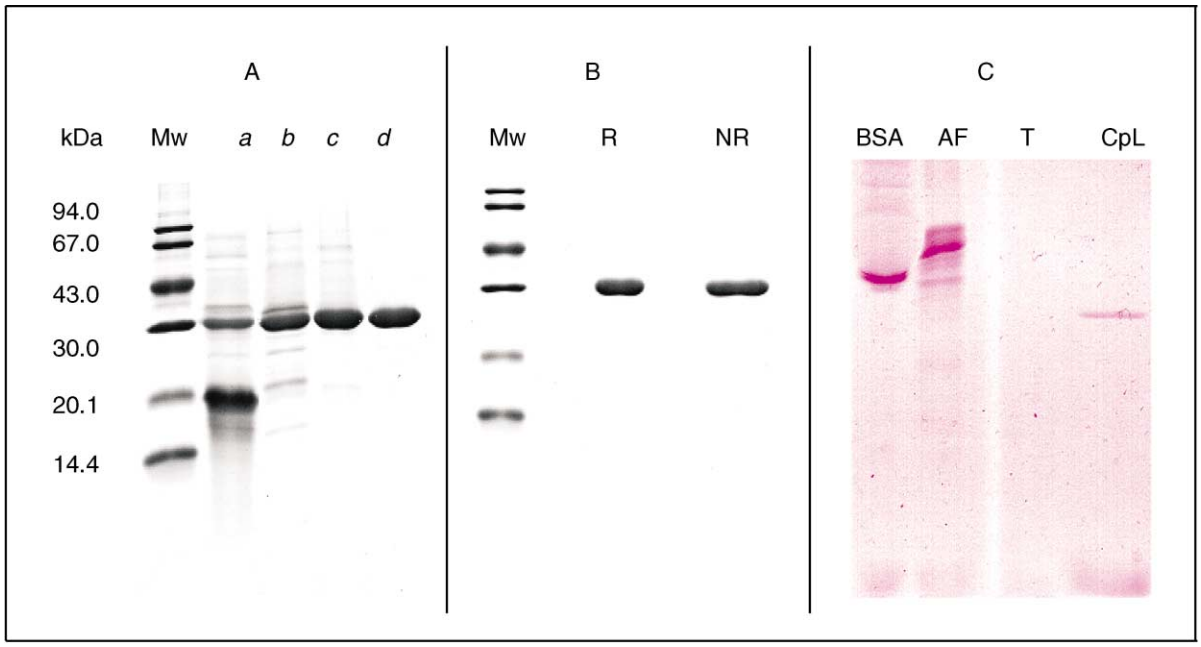

Fig. 2. (A) SDS–PAGE of the purification steps of *Crotalaria pallida* lectin (CPL); *a*—total extract; *b*—acetone precipitation; *c*—G-75 active fraction; *d*—isolated lectin from ion-exchange chromatography. (B) SDS–PAGE analysis of the purified CPL in the presence (R) or absence (NR) of 0.1 M DTT. (C) Non SDS–PAGE of CPL in native conditions stained with Schiff's reagent (PAS), BSA and AF (asialofetuin) were used as positive controls and trypsin (T) as negative control. Molecular mass markers (M_w) are in kDa (94.0—phosphorylase b; 67.0—albumin; 43.0—ovalbumin; 30.0—carbonic anhydrase; 20.1—trypsin inhibitor and 14.4—lactalbumin).

Table 2
Hemagglutination activity of the lectin from *Crotalaria pallida* seeds. Data are given as the minimum concentration in μg/ml that gave visible hemagglutination

Erythrocytes		Intact	Trypsinized
Human	A	7.81	0.46
	B	15.62	0.46
	AB	7.81	0.46
	O	ND	ND
Cow		46.46	2.30
Pig		ND	ND

2% Intact or trypsinized erythrocyte solution was used in this assay. ND—type O human and erythrocytes of pig did not agglutinate in the presence of lectin (up to 68 μg/ml).

Table 3
The inhibitory molecular concentration on hemagglutination activity of the lectin from *Crotalaria pallida* seeds

Sugar	Minimal inhibitory concentration (mM)		
	Crotalaria pallida	*Crotalaria striata*[a]	*Crotalaria juncea*[b]
N-acetyl-galactosamine	12.5	3.1	7.1
D-Raffinose	25.0	NS[c]	5.6
D-Galactose	37.5	12.5	9.3
D-Galactosamine	>150	50	9.4
D-Glucose	>150	>50	NS
D-Glucosamine	>150	>50	NS
D-Maltose	>150	NS	NS
D-Fructose	>150	NS	NS
D-Mannose	>150	NS	NS
D-Lactose	>150	>50	1.2
D-Sucrose	>150	NS	NS

Concentration of CPL was adjusted for 1.0 μg/ml, and 2%—trypsinized erythrocytes human type A solution was used in these assays.
[a] Khang et al., 1990.
[b] Ersson, 1977.
[c] NS, not specified.

residues (Table 4), as observed for VVLB4 (*Vicia villosa* isolectin B4; Osinaga et al., 1997) and *Psophocarpus tetragonolobus* lectin (Higuchi et al., 1986). Together they constituted 43.8% of the total amino acid residues. The Gly content of CPL is 2.3 times larger in *Crotalaria striata* lectin (Table 4). Met residues were not detected in *Crotalaria pallida* and *Crotalaria juncea* lectins, whereas *Crotalaria striata* lectin has one Met residue. The absence of half-cysteines is characteristic of leguminous lectins, and low or null concentrations of Met and His are very common in vegetable proteins (Kortt and Caldwell, 1990).

2.5. N-Terminal amino acid sequence

Crotalaria pallida lectin has a single N-terminal amino acid sequence determined on an automated Edman degradation amino acid sequencer. The N-terminal 16-residue sequence of CPL was determined, and has shown homology (between 87.5 and 100%) with other

Table 4
Amino acid composition (mol/mol)[a] of *Crotalaria pallida* lectin seeds in comparison with the lectins of the seeds of *Crotalaria striata*[b], *Crotalaria juncea*[c], *Psophocarpus tetragonolobus*[d] and *Vicia villosa*[e]

Amino acid	*Crotalaria pallida*	*Crotalaria striata*	*Crotalaria juncea*	*Psophocarpus tetragonolobus*	*Vicia villosa isolectin B4*
Asx	36	24	33	58	34
Glx	27	45	15	50	20
Ser	24	36	27	36	24
Gly	27	63	17	40	13
His	04	07	02	10	05
Arg	06	07	05	10	06
Thr	30	18	21	36	17
Ala	22	24	19	30	14
Pro	ND[f]	13	14	34	12
Tyr	09	08	11	10	08
Val	25	14	19	40	0
Met	0	01	0	02	02
Cys	ND	ND	0	0	0
Ile	10	10	11	20	14
Phe	18	05	16	28	14
Lys	14	09	16	22	10
Trp	ND	03	07	4	02
Total	274	287	233	450	195

[a] Calculated to the nearest integer.
[b] Khang et al., 1990.
[c] Ersson, 1977.
[d] Higuchi et al., 1986.
[e] Osinaga et al., 1997.
[f] ND, not detectable by the procedure employed.

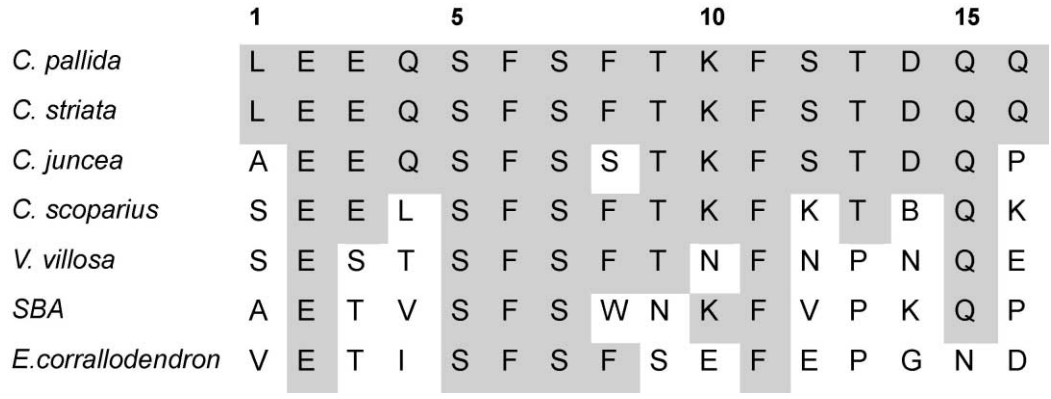

Fig. 3. N-terminal amino acid sequence of the lectin from *Crotalaria pallida* seeds (CPL) aligned with known N-terminal sequences of *Crotalaria striata* (Khang et al., 1990), *Crotalaria juncea* (Ersson, 1977), *Cytisus scoparius* (Konami et al., 1992), *Vicia villosa* (Osinaga et al., 1997) *SBA—Glycine max* (Vodkin et al., 1983) and *Erythrina corallodendron* (Adar et al., 1989) lectins. Homologous sequences are boxed.

lectins from *Crotalaria* seeds and other leguminous seeds (Fig. 3). In particular, CPL has 69% sequence identity with *Cytisus scoparius* (Scotch broom) lectin (Konami et al., 1992), 50% with isolectin B4 from *V. villosa* (Osinaga et al., 1997), 37% with *E. corrallodendron* (Adar et al., 1989) and 44% with soy bean agglutinin (Vodkin et al., 1983). Phe in positions 6 and 11 are also conserved in *Crotalaria pallida* lectin, as in other legume lectins (Fig. 3).

Differences in the amino acid composition and hemagglutination properties of *Crotalaria pallida* and *Crotalaria striata* lectins, suggest the presence of two isolectins, one with high specificity for type A erythrocytes, and other specific for type A and B erythrocytes. Further analysis should be conducted to completely clarify the differences between these two lectins.

There are many examples of seeds containing multiple lectins (Ciopraga et al., 2000; Goldstein and Winter, 1999; Paiva and Coelho, 1992). The African legume shrub *Griffonia simplicifolia* contain several lectins, amongst them, the GS I-B 4 with strict specificity for

α-Gal residues, whereas the closely related lectin GS I-A 4 can also bind to α GalNAc, their amino acid sequences show 89% identity (Lescar et al., 2001). Four isolectins were isolated from seeds of *Talisia esculenta*, and showed similar characteristics such as molecular mass, N-terminal sequence and hemagglutinating activity, but differed in their isoelectric points and in inhibition by carbohydrates (Freire et al., 2001).

Furthermore, differential expression of lectins was observed for seeds of different geographical origins. Seeds of *Mucuna pruriens* collected in India had lectins (Siddhuragu et al., 1996), but in the species collected in Brazil (Udeliba and Carlini, 1998) and Nigeria (Machuka, 2000), lectins were not detectable in hemagglutination tests, suggesting that ecological and climate factors can influence the level and concentrations of lectins expressed in seeds.

3. Experimental

3.1. Materials

Seeds of *C. pallida* were obtained from IAC—Instituto Agronômico de Campinas—Campinas-SP, Brazil. Sugars, Sephadex G-75, albumin, asialofetuin and trypsin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The column Protein Pak DEAE-5PW was acquired from WATERS, electrophoresis reagents were from BioRad, sequencing reagents were from Applied Biosystems, and all other reagents used were of analytical grade.

3.2. Purification of Crotalaria pallida lectin (CPL)

Mature seeds of *Crotalaria pallida* (50 g) were ground, and the proteins were extracted in saline solution and acetone (80%, v/v) fractionation. The lectin was purified by gel filtration on Sephadex G-75 (0.05 M Tris–HCl buffer, pH 8.0, containing 0.15 M NaCl), and HPLC anion exchange on DEAE-5PW column (0.05 M Tris–HCl buffer, pH 8.0 with a linear NaCl gradient of 0–5 mM). After purification, the lectin was dialyzed extensively against distilled water and lyophilized until further characterization.

3.3. Electrophoresis

Apparent molecular mass and subunit composition of the lectin were estimated by 15% SDS–PAGE according to the method of Laemmli (1970). The lectin was subject to SDS–PAGE under reducing (0.1 M DTT) and non-reducing conditions, and was stained with Coomassie Blue R-250. Non-SDS 12.5% polyacrylamide gel was used for carbohydrate content determination using periodic acid-Schiff's reagent as described by Korn and Wright (1973). Albumin and asialofetuin were used as glycosylated controls (positive staining) and trypsin was used as non-glycosylated control.

3.4. Hemagglutination assays

Samples in CTBS (50 μl) were diluted serially (2-fold) on a 96-well microtiter U-bottom plate (Sigma Chemical Co., USA) and mixed with 5 μl of a 2% suspension of human-trypsinized erythrocytes. The plate was left at room temperature for 2 h and the end-point showing minimum hemagglutination was determined. Tests for carbohydrate specifity were performed using sugars diluted two-fold in CTBS. Assays for divalent cation requirements were prepared using EDTA or EGTA in TBS solution. For testing the inhibition of CPL hemagglutination activity by a reducing agent, the lectin was reduced and carboxymethylated as described elsewhere (de Carvalho et al., 2002).

3.5. Amino acid composition

Amino acid analysis was performed in a Pico-Tag amino acid analyzer (Waters System). The purified sample was hydrolyzed with 6 HCl containing 1% phenol (v/v) at 106 °C for 24 h. Hydrolysates reacted with 20 μl fresh derivatization solution (v/v, 7:11:1:1 ethanol:triethylamine:water:phenylisothiocyanate) for 1 h at room temperature. The phenylthiocarbamyl (PTC) amino acids were identified by HPLC, comparing their retention time with those of the standard mixture.

3.6. N-Terminal amino acid sequence

A sample of the isolated lectin (500 pmoles) was used to determine its N-terminal sequence in an automatic Edman sequencer (Applied Biosystems model 477A). Phenylthiohydantoin (PTH) amino acids were identified in a model 120-A PTH-amino acid analyzer, according to the retention times of a 20 PTH-amino acid standards.

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