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Isolation, purification, and physicochemical characterization of a D-galactose-binding lectin from seeds of *Erythrina speciosa*

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Abstract

A lectin was isolated from the saline extract of *Erythrina speciosa* seeds by affinity chromatography on lactose–Sepharose. The lectin content was about 265 mg/100 g dry flour. *E. speciosa* seed lectin (EspecL) agglutinated all human RBC types, showing no human blood group specificity; however a slight preference toward the O blood group was evident. The lectin also agglutinated rabbit, sheep, and mouse blood cells and showed no effect on horse erythrocytes. Lactose was the most potent inhibitor of EspecL hemagglutinating activity (minimal inhibitory concentration (MIC) = 0.25 mM) followed by *N*-acetyllactosamine, MIC = 0.5 mM, and then *p*-nitrophenyl α-galactopyranoside, MIC = 2 mM. The lectin was a glycoprotein with a neutral carbohydrate content of 5.5% and had two p*I* values of 5.8 and 6.1 and $E^{1\%}_{1\,cm}$ of 14.5. The native molecular mass of the lectin detected by hydrodynamic light scattering was 58 kDa and when examined by mass spectroscopy and SDS–PAGE it was found to be composed of two identical subunits of molecular mass of 27.6 kDa. The amino acid composition of the lectin revealed that it was rich in acidic and hydroxyl amino acids, contained a lesser amount of methionine, and totally lacked cysteine. The N-terminal of the lectin shared major similarities with other reported *Erythrina* lectins. The lectin was a metaloprotein that needed both Ca^{2+} and Mn^{2+} ions for its activity. Removal of these metals by EDTA rendered the lectin inactive whereas their addition restored the activity. EspecL was acidic pH sensitive and totally lost its activity when incubated with all pH values between pH 3 and pH 6. Above pH 6 and to pH 9.6 there was no effect on the lectin activity. At 65 °C for more than 90 min the lectin was fairly stable; however, when heated at 70 °C for 10 min it lost more than 80% of its original activity and was totally inactivated at 80 °C for less than 10 min. Fluorescence studies of EspecL indicated that tryptophan residues were present in a highly hydrophobic environment, and binding of lactose to EspecL neither quenched tryptophan fluorescence nor altered λ_{max} position. Treating purified EspecL with NBS an affinity-modifying reagent specific for tryptophan totally inactivated the lectin with total modification of three tryptophan residues. Of these residues only the third modified residue seemed to play a crucial role in the lectin activity. Addition of lactose to the assay medium did not provide protection against NBS modification which indicated that tryptophan might not be directly involved in the binding of haptenic sugar D-galactose. Modification of tyrosine with *N*-acetylimidazole led to a 50% drop in EspecL activity with concomitant acetylation of six tyrosine residues. The secondary structure of EspecL as studied by circular dichroism was found to be a typical β-pleated-sheet structure which is comparable to the CD structure of *Erythrina corallodendron* lectin. Binding of lactose did not alter the EspecL secondary structure as revealed by CD examination.
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Lectins, multivalent cell-agglutinating proteins, by virtue of their exquisite sugar specificities are useful tools in widespread applications for monitoring the expression of cell-surface carbohydrates as well as for the purification and characterization of glycoconjugates [1–3]. Extensive study of sequence homology and 3-D structure of various plant lectins suggests that they are conserved throughout evolution and thus may play, yet unknown, important physiological roles [4,5]. Seeds of

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legumes such as peas and beans have long been known to represent a rich source of lectins [6]. Legume lectins are the best-studied group of lectins and hundreds of these proteins have been isolated and extensively investigated in relation to their chemical, physicochemical, structural, and biological properties. Among the well-studied species of the legume family lectin are *Erythrina* genus lectins which have been well characterized [7–10]. The genus *Erythrina* member occurs widely in the tropical and subtropical regions over the world and comprises about 110 species [11]. The present investigation has been devoted to purify and characterize a D-galactose-binding lectin from seeds of *Erythrina speciosa*. The lectin has been studied with respect to its structure, composition, biological activity, and sugar specificity. These properties were compared to other reports on *Erythrina* lectins.

Materials and methods

Plant materials

Seeds of *E. speciosa* were collected from trees on the campus of the faculty of medicine of Ribeirao Preto, University of Sao Paulo.

Erythrocytes

Typed human blood cells (A, AB, B, and O) were obtained from healthy donors while animal blood cells (rabbit, mouse, sheep, and horse) were obtained from the animal house of the faculty of medicine of Ribeirao Preto, University of Sao Paulo.

Chemicals

Sugars (all of D-configuration), lactose–Sepharose, and ConA–Sepharose affinity matrices were purchased from Sigma Chemical (St. Louis, MO). All other reagents were either of analytical grade or of the highest quality available.

Protein concentration

The protein content of the samples obtained during the purification process was determined by the method of Lowry et al. [12] using bovine serum albumin as the standard. Readings at 280 nm were also used to determine the protein content of the column eluates.

EspecL purification

Season fresh, mature, and good quality seeds of *E. speciosa* were ground to a fine flour in a coffee grinder and the meal (ca. 20 g) was extracted overnight with about 300 mL of 0.145 mM NaCl at 4 °C. The whole extract was filtered through cheesecloth and then centrifuged for 45 min at 5000 rpm at 10 °C. The obtained clear supernatant was dialyzed exhaustively against 0.145 M NaCl for 24 h, precipitated protein during dialysis was removed by centrifugation, and the clear supernatant was applied to a lactose–Sepharose affinity matrix (2×10 cm). After the unbound proteins were washed with 0.145 M NaCl, protein that retained unspecific interactions with carbohydrate were desorbed by 1.0 M NaCl. The column was reequilibrated with 0.145 M NaCl before EspecL was eluted with 0.2 M lactose. Fractions were monitored spectrophotometrically at 280 nm.

Hemagglutination tests

These were performed by making serial dilutions of samples in physiological saline (0.145 M NaCl) using a microtiter plate. To each well 4% erythrocyte suspensions prepared from rabbit blood were used. Hemagglutination unit (HU)[1] was expressed as the reciprocal of the highest dilution showing detectable hemagglutination and the specific activity (HU/mg) was calculated. Rabbit erythrocytes were used for hemagglutination tests throughout unless otherwise stated.

Extinction coefficient estimation

The extinction coefficient of lectin was determined spectrophotometrically using a solution of pure lectin (1 mg/mL) prepared in 0.145 M NaCl.

Carbohydrate content

The neutral sugar content of EspecL was estimated by the phenol–sulfuric acid method using glucose as the standard [13].

Gel electrophoresis and molecular weight determination

Polyacrylamide gel electrophoresis (PAGE) at pH 8.6 was carried out according to Williams and Reisfeld [14]. SDS–PAGE was performed with Laemmli's system [15]. The protein bands were visualized by staining the gels with Coomassie brilliant blue R-250. Glycoproteins were detected by the periodic acid-Schiff's (PAS) staining of gels [16].

Molecular weight of the native protein was calculated from the protein hydrodynamic radius (R_h), which was measured by dynamic light scattering (DLS) using a DynaPro MSTC014 (Protein Solutions, High Wy-

[1] *Abbreviations used:* HU, hemagglutination unit; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PAS, periodic acid-Schiff; DLS, dynamic light scattering; ConA, concanavalin A; NBS, *N*-bromosuccinimide; NAI, *N*-acetylimidaazole; MIC, minimum inhibitory concentration.

combe, Bucks, UK) at a protein concentration of 1 mg/mL. All protein solutions were passed through a 0.22-mm filter and centrifuged at 10,000*g* for 20 min prior to data collection. Data were acquired by accumulating 50 scans of approximately 1.5 s and the particle size distribution was calculated by mass using the software package Dynamics supplied with the instrument.

Affinity chromatography on concanavalin A–Sepharose

Purified EspecL (ca. 1.5 mg) was applied to a column (1 × 5 cm) of ConA–Sepharose that was equilibrated with 0.145 M NaCl. EspecL was recycled at least five times to ensure maximum retention on the affinity resin. The column was then washed initially with 0.145 M NaCl and then followed by 1.0 M NaCl. The absorbed EspecL was eluted from the column with 0.2 M glucose. Elution was followed by monitoring the absorbance of the effluent at 280 nm.

Sugar specificity

The sugar specificity of EspecL was tested by inhibiting the hemagglutinating activity using simple sugars and glycosides. Results were expressed as the minimal concentration of carbohydrates with well-defined structures which effectively inhibited four hemagglutinating dose units of the lectin.

Effect of temperature and pH on EspecL hemagglutinating activity

The heat stability of the hemagglutinating activity of EspecL was determined by incubation of aliquots of lectin solution at different temperatures (50, 60, 70, and 80 °C) for 10, 20, 30, and 90 min and remaining hemagglutinating activity was determined. Effect of pH on EspecL hemagglutinating activity was performed by incubating aliquots of lectin solutions for 1 h with buffers of different pH values ranging from pH 3.0 to pH 9.6. The pH of the lectin solutions was adjusted to pH 7.0 by addition of 0.1 N HCl or 0.1 N NaOH before hemagglutinating activity was examined.

Effect of EDTA, Ca^{2+} and Mn^{2+}

The purified lectin (1 mg/mL) was incubated for 10 h with 50 mM EDTA with continuous shaking. The lectin sample was dialyzed exhaustively against 0.145 M NaCl, and the hemagglutinating activity was assessed before and after addition of 50 mM Ca^{2+} and Mn^{2+} ions.

Analytical isoelectric focusing (pI)

This was performed on precast polyacrylamide gels (FastGel Pharmacia, Sweden) in the pH range of 3–10. The pH gradients in these gels were determined from the results of simultaneous runs performed with a wide-range isoelectric protein calibration kit (Pharmalyte 3-10).

Amino acid analysis

Amino acid analyses were performed using the phenylthiocarbamyl derivative method [17] after acid hydrolysis with 6 N HCl containing 1% phenol in the vapor phase, at 110 °C for 22 h. A mixture containing 2.5 nmol each amino acid was derivatized each day, and 100 pmol was used as standard.

N-terminal determination

The N-terminal determination was performed using a Procise Model 491 sequenator (Perkin–Elmer-Applied Biosystem Division, Foster City, CA) using either gas-phase or pulse-liquid chemistry with on-line identification of phenylthiohydantoin derivatives.

Chemical modification studies

Tryptophan residue modification. This was done using tryptophan-specific reagent *N*-bromosuccinimide (NBS). The lectin solution (1 mg/mL) prepared in acetate buffer, pH 6.0, was titrated with freshly prepared NBS (10 mM). The reagent was added in installments of 10 μL each. After every addition an aliquot was removed and quenched with a 20-μL 50 mM tryptophan solution and the residual activity was determined after removal of excess reagent by dialysis. Lectin sample in the absence of NBS served as control. The NBS-mediated lectin inactivation was also monitored spectrophotometrically, by measuring the decrease in absorbance at 280 nm. The number of modified tryptophan residues was determined using the molar extinction coefficient of 5500 $M^{-1}\,cm^{-1}$ [18].

Tyrosine residue modification. This was carried out using tyrosine-specific reagent *N*-acetylimidazole (NAI). To EspecL solution (1 mg/mL) prepared in Tris–HCl buffer, pH 8.0, 50 mM, installments of NAI reagent (10 mM, 10 μL) were added. After every addition of NAI, an aliquot was removed and quenched with 20 μL, 50 mM L-tyrosine solution and the residual activity was determined after removal of excess reagent. Lectin sample in the absence of reagent served as control. The number of acetylated tyrosine was determined essentially as previously reported [19].

Fluorescence studies

Fluorescence spectra of native and lactose-bound EspecL were recorded on an SLM Amico-8100 spectrofluorimeter at 25 °C. The samples were excited at

295 nm and the emission spectra were recorded between 300 and 400 nm. Quenching of tryptophan fluorescence was measured after titration of 100 μg EspecL with different concentrations of lactose (5–100 mM).

Circular dichroism

The far-ultraviolet circular dichroism spectra of lectins were measured with a Jasco 810 spectropolarimeter using 1-mm path length quartz cuvettes with a 10-nm bandwidth, at a constant temperature maintained at 298 K and a protein concentration of 150 μg/mL. The accumulated average of five protein spectra was corrected by subtraction of the spectra measured from a buffer blank containing the same concentration of buffer in the presence or absence of sugar. The near-ultraviolet circular dichroism spectra were measured using a 5-mm path length quartz cuvette under the same conditions as described above, with a protein concentration of 250 μg/mL. The buffer corrections were performed as described for the far-ultraviolet circular dichroism scan.

Results and discussion

Crude extract of *E. speciosa* seeds contains relatively high hemagglutinating activity that could potentially be inhibited by D-galactose and its derivatives. The purification procedures of this lectin from seed extract of *E. speciosa* are summarized in Table 1. EspecL was extracted from the ground meal of seeds and could be purified in a single step by affinity chromatography on lactose–Sepharose. Loading of crude extract on the affinity column followed by washing of the unbound protein with 0.145 M NaCl and finally with 1.0 M NaCl to detach the proteins that bound by carbohydrate-unspecific forces and subsequent elution of the bound lectin with 0.2 M lactose in 0.145 M NaCl (Fig. 1) led to purification of the lectin with increments in the specific activity of 773 and 66% yield. The obtained lectin was ca. 260 mg/100 g dry weight. This value is in accordance with Moraes et al. results [9]; however, it is higher than all of other reported values for *Erythrina* lectins [7,20].

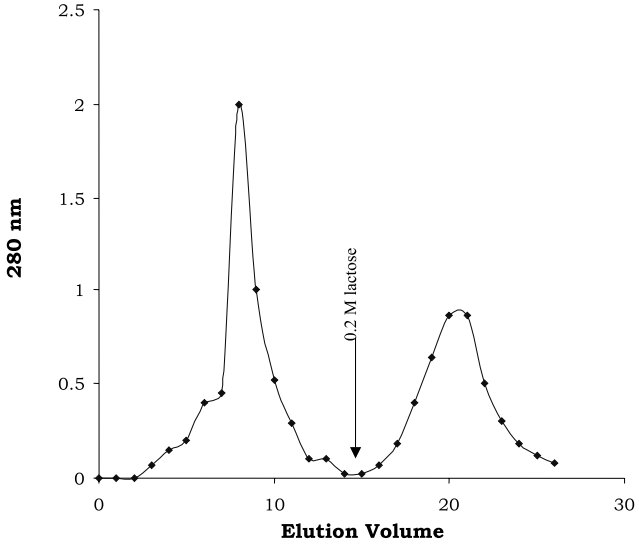

Fig. 1. Affinity chromatography of EspecL on a lactose–Sepharose column. Crude extract of *E. speciosa* seeds was applied to lactose–Sepharose affinity chromatography as described under Materials and methods. Retained EspecL was eluted with 0.2 M lactose. Fractions were monitored for protein at 280 nm.

Characterization of lectin

As a general statement, EspecL shares major structural as well as physicochemical similarities with other reported *Erythrina* lectins; however, it is distinctly unique from other *Erythrina* species lectins in its affinity toward mouse and sheep RBCs, disaccharide affinity, degree of easiness to remove the bound metal ions, and finally its extreme stability to heat denaturation.

Purity examination and molecular weight determination

On native PAGE, the purified EspecL gave a single band suggesting the purity of the preparation (Fig. 2A).

A R_h (protein hydrodynamic radius) of 3.44 nm was determined from the DLS experiments, which assuming a globular conformation corresponds to a particle weight of 58 kDa. This value agrees well with the calculated molecular weight of a dimer, from SDS–PAGE (Fig. 2B) as well as mass spectroscopy data, which revealed a molecular weight of 27.6 kDa per monomer (data not shown).

Table 1
Purification of the lectin from *E. speciosa* seeds

Stage	Volume (mL)	Protein (mg/mL)[a]	Total protein (mg)	Specific activity (U/mg)[b]	Fold purification	% Yield
Saline extract	300	32	9600	2	1	100
Affinity chromatography	20	3	53	773	386	66

[a] Starting from ca. 20 g dry floor.

[b] Specific activity is defined as the hemagglutination unit (HU) divided by the protein concentration (mg/mL) of the assay solution. Rabbit erythrocytes were used for the assay.

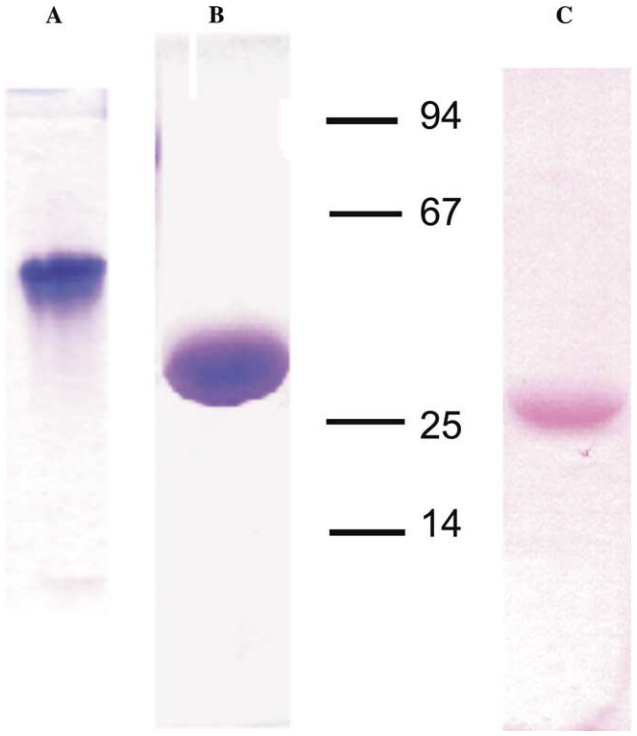

Fig. 2. Purity and subunit molecular weight estimation of EspecL. (A) Native polyacrylamide gel electrophoresis: 10 μg EspecL was loaded and protein was stained by Coomassie brilliant blue. (B) SDS–PAGE: 10 μg EspecL was loaded and the gel was stained with Coomassie brilliant blue. Standard protein markers used were phosphorylase *b* (94 kDa), BSA (67 kDa), trypsinogen (25 kDa), and α-lactalbumin (14 kDa). (C) Glycoprotein staining (PAS staining): 10 μg EspecL was loaded and protein was stained by the periodic acid-Shiff method.

Glycoprotein nature of EspecL

The glycoprotein nature of the EspecL was indicated by the positive phenol–sulfuric acid test as well as glycoprotein PAS staining (Fig. 2C). The estimated content of neutral sugar was 5.5%, which correlates with the general range for carbohydrate content in *Erythrina* lectins, which is in the range of 3–12%.

The glycoprotein nature of EspecL was further confirmed by affinity chromatography on concanavalin A–Sepharose. Almost all of the loaded EspecL was potentially bound to the column and was eluted with 0.2 M glucose as a single major peak (not shown). This experiment, besides being a confirmatory test for the glycoprotein nature of EspecL, is also informative in terms of the carbohydrate structure of the glycan chain of EspecL. Binding of EspecL to ConA with involvement of the later carbohydrate affinity site may indicate the presence of a terminal nonreducing D-mannose or D-glucose in the putative oligosaccharide attached to EspecL; however, since glucose is rarely present in glycoproteins and has not been found in any of the *Erythrina* lectins examined so far [7], the presence of mannose becomes more likely.

pI of EspecL

The p*I* determination of EspecL gave two protein bands, which corresponded to p*I* values of 5.8 and 6.1. This result points to the existence of two isolectins and suggests charge heterogeneity among subunits. The acidic p*Is* may be attributed to the acidic nature of this lectin.

Hemagglutination and sugar specificity

Examination of the hemagglutination ability of EspecL with different blood cell types (human, rabbit, mouse, sheep, and horse) revealed some interesting observations. Purified EspecL was a strong hemagglutinin when tested against different native human blood groups (A, AB, B, and O), the lectin agglutinated all of these blood cells showing no human blood specificity; however, it was distinctly characterized by its slight preference toward the O blood group. EspecL as well was a potent agglutinin for native rabbit erythrocytes (minimum inhibitory concentration (MIC) = 0.8 μg/mL). The preferential agglutination of rabbit RBCs over human blood is a common character of most but not all of *Erythrina* lectins [7,9]. The general preferential agglutination of rabbit RBCs over other animal blood cells may explain the basic motive behind using the former blood cells for routine checking of hemagglutinating activity of lectins. EspecL agglutinated weekly mouse and sheep blood cells, especially the latter. Our results with agglutination of native mouse and sheep RBCs seem to be a novel character for EspecL as no report concerning lectin from other *Erythrina* species has shown agglutination of native sheep and mouse erythrocytes.

Iglesisa et al. [21] reported that purified lectin from *Erythrina cristagalli* did not agglutinate native mouse erythrocytes till a concentration of 1 mg/mL; however, agglutination was enhanced upon treating RBCs with trypsin. A similar result was demonstrated for *Erythrina edulis* [22]. Table 2 gives a summary of the specificity of EspecL toward different blood types. To determine detailed carbohydrate-binding specificity of EspecL, series

Table 2
Agglutination of different blood types by EspecL

Blood type	MECRVA (μg/mL)[a]
Human A	6.25
Human B	6.25
Human AB	6.25
Human O	3.12
Rabbit	0.78
Mouse	25
Sheep	100

[a] Minimum EspecL concentration required for a visible agglutination.

Table 3
Carbohydrate inhibition of agglutination by EspecL

Sugar	MIC (μg/mL)
Galactosamine	100
N-Acetyllactosamine	0.5
Lactose	0.25
Methyl β-galactoside	10
Methyl α-galactoside	5
D-Galactose	10
N-Acetylgalactosamine	5
Melibiose	5
Stachyose	4
p-Nitrophenyl α-galactoside	2
Glucose	NI
Mannose	NI

MIC, minimum inhibitory concentration; NI, sugar not inhibitory until a concentration of 200 mM.

of hemagglutination inhibition assays were conducted using different sugars and their derivatives, and oligosaccharides, and methyl and *p*-nitrophenyl glycosides. The concentrations for inhibitors required for inhibition of hemagglutination are listed in Table 3. Among different galactose derivatives used lactose was the most potent inhibitor (MIC = 0.25 mM) followed by *N*-acetyllactosamine, MIC = 0.5 mM. The better inhibition of *p*-nitrophenyl α-galactoside over galactose indicated that EspecL may possess a hydrophobic-binding pocket in proximity to the lectin sugar affinity site, and that the biding site is the extended type [7].

Effect of pH, temperature, divalent metal, and EDTA on EspecL activity

The results of thermal denaturation of EspecL showed that the lectin remained significantly stable below 65 °C for more than 90 min without losing its hemagglutinating activity. Above 65 °C lectin activity was gradually lost and was totally inactivated at 80 °C for less than 10 min. These results revealed the high thermal stability of EspecL as compared to other *Erythrina* species lectins. However, these results share some resemblance to the results obtained by Moraes et al. [9] with *Erythrina velutina forma aurantiaca* seed lectins.

Examination of EspecL activity toward different pH values showed that the lectin was acidic pH sensitive. The lectin rapidly lost its activity at all pH values below pH 6. On the other hand, lectin activity remained virtually unaltered from pH 6.5 to pH 9.6. EspecL was Ca^{2+} and Mn^{2+}-dependent metaloprotein. Incubating the purified lectin with 50 mM EDTA for 10 h with continuous shaking and after exhaustive dialysis to remove excess EDTA the hemagglutinating activity examination revealed total inactivation of EspecL; however, when Ca^{2+} and Mn^{2+} were added to the assay medium in equimolar concentrations, EspecL activity was fully restored. Addition of Ca^{2+} or Mn^{2+} separately did not restore the activity of EspecL, which indicated that EspecL required both metals for its optimal activity. The loosely bound Ca^{2+} and Mn^{2+} to EspecL and their importance in EspecL hemagglutination activity seem to be interesting as no work with this species of lectin has shown similar results. Gerardo [23], Pena et al. [20], and Bhattachayyra et al. [8] reported that exhaustive dialysis of the lectins from *Erythrina rubrinervia, edulis*, and *indica*, respectively, against metal chelating agents did not bring about any change in the hemagglutination titer. These results led Gerardo to conclude that the metal ions are either firmly bound to the lectin or they are not crucial for its hemagglutinating activity [23]. An attempt to use a harsh demetalization process was done by Iglesias et al. [21] by treating *E. cristagalli* lectin with 1 M acetic acid. However, no change in the lectin hemagglutinating activity was observed.

Amino acid composition and the N-terminal

The N-terminal amino acid sequence of EspecL determined by Edman's degradation methods and to position number 12 was as follows: VETISFSFSEFE. This N-terminal sequence shared 100% homology with a number of other *Erythrina* lectin sequences [7,24]. The amino acid analysis of EspecL showed the absence of cysteine and a lesser amount of methionine. The lectin was distinctly rich in acidic as well as hydroxyl amino acids (not shown).

Chemical modification studies

Modification of tryptophan. After treatment of EspecL with NBS (*N*-bromosuccinimide), a reagent that specifically modifies tryptophan, and subsequent examination of lectin hemagglutinating activity after removal of excess reagent a total loss of EspecL hemagglutinating activity was observed. A plot of the number of tryptophan residues modified by NBS versus remaining percentage residual activity of EspecL indicated that three tryptophan residues were modified under our experimental conditions, however total loss of EspecL hemagglutinating activity was evident after modification of the third tryptophan residue (Fig. 3A) which indicated the importance of this residue for lectin activity. *N*-Bromosuccinimide is also known to attack tyrosine unspecifically [18]; hence, the possibility of modification of tyrosine by NBS in the present study cannot be ruled out. Modification of other aromatic amino acids is concomitant with an increase in absorbance at 280 nm [25]; however, in the present investigation we did not observe any elevation in absorbance upon reaction of NBS with EspecL which conclusively indicated that only tryptophan was modified in this study and not tyrosine. The presence of the haptenic sugar lactose in the assay medium did not provide protection for EspecL against

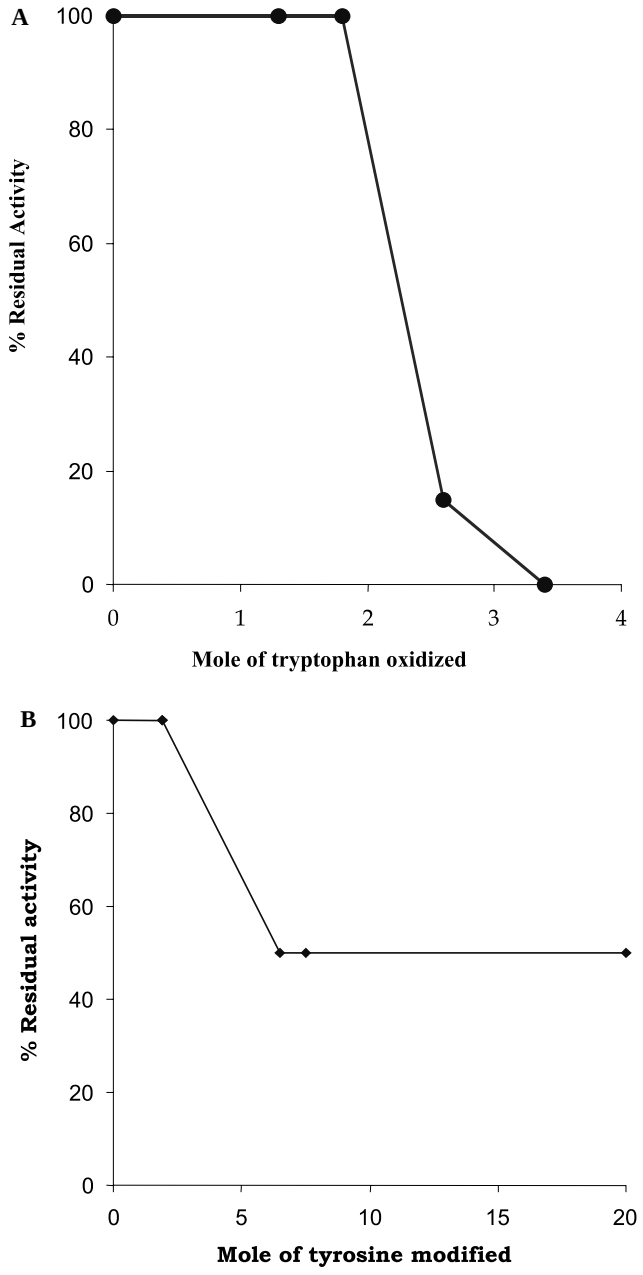

Fig. 3. Effect of modification of tryptophan and tyrosine residues on EspecL activity. (A) Inactivation of curve of NBS-modified EspecL as a function of modified tryptophan residues. (B) Inactivation curve of acetylated EspecL as a function of modified tyrosine residues.

NBS inactivation, which indicated that tryptophan might not be present in the lectin affinity site. Recently it has been shown that tryptophan 135 in the affinity site of *Erythrina corallodendron* functions basically as a binder of Ca^{2+} ion rather than direct binding of the haptenic sugar *N*-acetylgalactosamine [26].

Modification of tyrosine

Treating purified EspecL with *N*-acetylimidazole resulted in a 50% drop in the activity of EspecL, which suggested the partial necessity of tyrosine residues for the activity of EspecL. Quantitative studies for determination of the total number of modified tyrosine with NAI [19] showed that a total number of six tyrosine residues were modified in the study accompanied by a 50% drop in EspecL activity (Fig. 3B). The presence of 0.2 M lactose in the assay medium did not provide significant protection for the lectin against inactivation by NAI, which may indicate that tyrosine residues may not be present in or near the lectin affinity site.

Fluorescence studies of EspecL

The examined EspecL tryptophan intrinsic fluorescence in the presence and absence of lactose (data not shown) revealed emission spectrum of the purified lectin λ_{max} at around 330 nm, indicating the presence of tryptophan in a highly hydrophobic region. There was no significant change in the spectra upon binding of lactose to lectin, which indicated that binding of lactose to EspecL did not cause any significant change in the tryptophan environment; on the other hand, there was no significant change in λ_{max} position of native lectin and the lactose–lectin complex which may indicate that there was no change in the hydrophobic environment of tryptophan upon binding of lactose. These results taken together with the failure of lactose to protect lectin against NBS inactivation may confirm our claim that tryptophan is not present in or near the affinity site of lectin or may not be directly involved in binding the sugar receptor.

Circular dichroism studies

In the far-UV region the CD spectrum of EspecL, which monitors the secondary structure of the protein, showed a broad negative trough centered around 225 nm and a negative to positive crossover at around 203 nm (Fig. 4A). The shape of the spectra indicated that EspecL is a β-pleated-sheet-rich protein. This spectral feature also resembles that of ConA [27]. In the presence of lactose, the far-UV circular dichroism spectrum was virtually the same as that for the native EspecL, which indicated that binding of lactose to EspecL did not cause any significant change in the secondary structure of lectin. The CD spectrum of *E. corallodendron* lectin has been given by Adar and Sharon [28] which looks almost similar to our current data. For comparison, both lectins revealed λ_{min} at around 225 nm; however they differed in their ellipticity values.

The CD spectrum of EspecL in the near-UV region which reflects the contributions of aromatic amino acid residues showed a positive band at around 293 nm which could be assigned to tryptophan. The binding of lactose

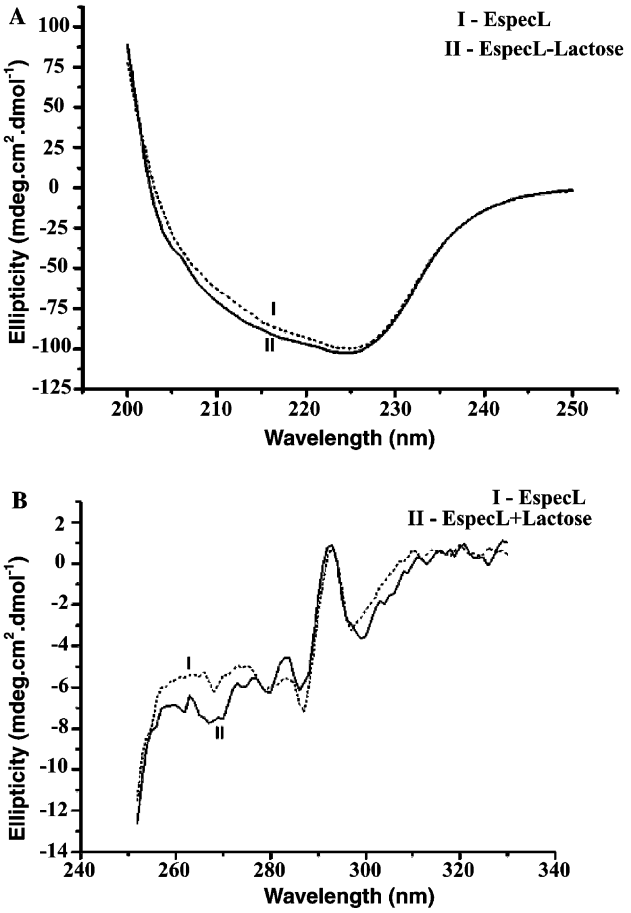

Fig. 4. (A) Far-UV, CD spectra of EspecL in the presence and absence of lactose. The protein concentration was 150 μg/mL. (B) Near-UV, CD spectra of EspecL in the presence and absence of lactose. The protein concentration was 250 μg/mL.

to EspecL did not modify the spectral pattern, which suggested that the aromatic chromophores were virtually unaltered (Fig. 4B).

Conclusion

In the present study, we have isolated and extensively characterized a D-galactose-binding lectin from seeds of *E. speciosa*. Though EspecL shares a high degree of similarity with other *Erythrina* lectins in terms of subunit structure and some of their physicochemical features including sugar specificity, it showed distinct variations from other members in its carbohydrate-binding site and biological activity, as well as importance of Ca^{2+} and Mn^{2+} ions for its activity. The differences among these members may indicate slight variations in their carbohydrate-binding pockets that have been adapted throughout evolution to serve specific, yet unrevealed, physiological significance.

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