PHYSICOCHEMICAL PROPERTIES OF A LACTOSE SPECIFIC LECTIN FROM THE SEEDS OF *ERYTHRINA SENEGALENSIS DC*

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ABSTRACT

This paper reports the purification of a lactose specific lectin from the saline extract of the seeds of *Erythrina senegalensis* and presents the data regarding its physicochemical properties. The lectin was purified by ammonium sulphate precipitation, gel filtration on Sephadex G-150 and affinity chromatography on Lactose-Sepharose 4B column. The lectin agglutinated human and rabbit erythrocytes, with a slight preference for the human O blood group. The hemagglutinating activity was inhibited by galactosyl moieties with lactose being the most potent inhibitor with MIC (minimal inhibitory concentration) of 0.195mM. *Erythrina senegalensis* lectin (ESL) is a glycoprotein with 6.5 % neutral sugars and a native molecular weight of 62kDa, composed of two identical subunits (apparent molecular weight of 30kDa). The amino acid content revealed a high proportion of acidic and hydroxyl residues and totally lacked cysteine while there are six methionine residues per mole of protein. ESL is a metalloprotein that requires both Ca^{2+} and Mn^{2+} ions for its activity. Removal of these metals by EDTA rendered the lectin inactive while their addition restored the activity. The lectin was sensitive to acidic pH as it lost all its activity when incubated with buffers at pH 3 to 6 whereas above pH 6 and up to pH 10, there was no effect on the lectin activity. The lectin was heat stable up to 65 ^{0}C but when heated at 70 ^{o}C for 20 min, it lost 60 % of its activity and was totally inactivated when heated at 80 ^{o}C for less than 10 min. Chemical modification studies of ESL with phenyl methanesulfonyl fluoride (PMSF) and 5, 5-dithiobis-2-nitrobenzoic acid (DTNB) revealed an absence of serine and cysteine in or near the ligand-binding site of the lectin. Modification of arginine with phenylglyoxal led to partial inactivation of the lectin; however total inactivation was observed upon modification of tryptophan residues with N-bromosuccinimide (NBS).

Keywords: Erythrina, seed, galactose, metalloprotein, glycoprotein, chemical modification

INTRODUCTION

Erythrina senegalensis commonly known as Senegal coral belongs to the family Fabaceae and is of the tribe Phaseolae. Most members of the genus *Erythrina* are quite similar in physical properties, mostly found as a small tree armed with stout prickles slightly recurved from a woody base usually found in the savannah and commonly planted as a hedge. Extracts from the plant, *Erythrina senegalensis* has found uses in traditional medicine in treatment of certain diseases. Recent reports have provided evidence that this plant has potentials as a new source of antibacterial agents against some antibiotic-resistant strains (Kone *et al*., 2004). Erybraedin A, a flavonoid isolated from many *Erythrina* species is an antimicrobial agent and has been shown to have a strong activity against yeast pores and also showed a high growth inhibitory potency against vanomycin resistant enterococci (VRE) and multiresistant *Staphylococcus aureus* (MRSA), these antibacterial activities were based on bacteriostatic action (Sato *et al.,* 2004) . Senegalensein, a flavanone isolated from the stem bark of *E. senegalensis* has been reported to exhibit a HIV-inhibitory activity and an antibacterial activity against *Staphylococcus aureus* and *Enterococcus faecium* (Dastidar *et al*., 2004). Erythrisenegalone and Senegalensein, two prenylated flavanones, isolated from the stem bark of this plant have also been reported to show anti-tumor activity in- vitro, while two compounds, Alpinumisoflavone and derrone isolated from the methanolic extract of *E. senegalensis* have shown moderate anti-proliferative activity against human leukemia cells(Togola *et al.,* 2008).

Lectins, proteins (or glycoproteins) that specifically and reversibly bind to carbohydrates of glycoconjugates have also been isolated from *Erythrina* plants. Among the well-studied species of the legume family lectins are *Erythrina* genus lectins which have been well characterized.Lectins have been successfully extracted and characterized from *Erythrina cristagalli* (Iglesias *et al*., 1982; Wu *et al*., 2007), *Erythrina costaricensis* (Gerardo, 1995), *Erythrina steyermarkii* and *Erythrina poeppigiana* (Quesada *et al*., 1998), *Erythrina corallodendrum* (Elgavish and Shaanan, 1998), *Erythrina speciosa* and *Erythrina indica* (Emadeldin *et al.,* 2002, 2003) and *Erythrina americana* (Ortega *et al*., 1990). Most of these lectins were found to be specific for a particular human blood group and are also inhibited by either galactose or lactose. These lectins are found in the different vegetative tissues of the plants where they play a fundamental

biological role.

This paper purified and characterized a lactose-specific lectin from the saline extract of the seeds of *Erythrina senegalensis*. The lectin's physicochemical properties are compared with other *Erythrina* lectins.

MATERIALS AND METHODS

Materials

Fresh human blood was obtained from healthy donors and animal blood from rabbits supplied by the Animal Science Department of Obafemi Awolowo University, Ile-Ife.

Red blood cells were obtained from the blood samples and fixed with glutaraldehyde according to the method of Kuku and Eretan (2004).

The seeds of the *Erythrina senegalensis* were collected from the Reafforestation Unit of Botany Department and identified in the IFE Herbarium of Obafemi Awolowo University, Ile-Ife.

Sepharose 4B, divinyl sulphone, sugars and molecular weight standards were from Sigma. All other reagents used were of analytical grades.

Methods

Preparation of Seed Extract

50 g of seeds of *Erythrina senegalensis* were ground to fine flour in a mill. The flour was defatted by stirring with petroleum ether at room temperature and air-dried. 24 g of the defatted flour was extracted overnight with 10 volume of phosphate buffered saline (PBS) pH 7.2 at 4^0C. The whole extract was filtered through cheese cloth and centrifuged for 30 min at 6000 rpm. The clear supernatant (crude extract) was collected and assayed for hemagglutinating activity.

The young leaves of the plant were also assessed for lectin activity. The leaves were deveined and homogenized in a blender with phosphate buffered saline (200 ml per 20 g of leaves). The slurry was mixed with 1-butanol (20 ml per 50 ml) and stirred at 4 °C for1 h; the butanol layer was removed by filtration and equal volume of cold acetone (50 % saturation) was added to the homogenate under stirring at 4 °C. The resulting precipitate was collected by centrifugation and dehydrated to get acetone- dried powder which was subjected to PBS extraction for 4 h with continuous stirring. The filtrate, which constitutes the saline extract, was assayed for hemagglutinating activity with human and rabbit erythrocytes.

Purification of Seed Lectin

The seed extract was brought to 70% ammonium sulphate saturation and was left overnight at room temperature. The precipitate was collected by centrifugation at 6000 rpm for 30 min. The precipitate was redissolved in PBS, pH 7.2 and dialyzed exhaustively against distilled water. The dialyzate was centrifuged at 3000 rpm for 10 min to remove debris and other undissolved materials and then lyophilized. The protein content and hemagglutinating activity were determined.

4ml of freeze dried dialyzate of ammonium sulphate precipitate (20 mg/ml) was layered on Sephadex G-150 column (2.5 x 40 cm), previously equilibrated with PBS, pH 7.2. Fractions of 4 ml were collected, elution was monitored at 280 nm and the fractions were assayed for hemagglutinating activity. The fractions in the active protein peak were pooled and dialyzed exhaustively against distilled water.

Preparation of Lactose-Sepharose 4B

10 ml of Sepharose 4B was washed with 500 ml distilled water and suspended in 0.2M carbonate buffer, pH 10.7 containing 1 ml divinyl sulphone. The suspension was shaken at room temperature for 2 h and washed with distilled water. The activated sepharose was equilibrated with 0.2 M carbonate buffer containing lactose (0.2M) and shaken at room temperature for 24 h. The sepharose was washed with distilled water and carbonate buffer and then suspended in glycine solution (100 mg in 10 ml of 0.2 M carbonate buffer, pH 10.7). The suspension was shaken for 2 h at room temperature and washed with distilled water and carbonate buffer and equilibrated with PBS, pH 7.2.

Affinity Chromatography

The lactose-sepharose 4B slurry (~7.5 ml) was packed into a column (1.5 x 10 cm) pre-equilibrated with PBS, pH 7.2. One ml of the active protein (~10 mg protein) (from gel filtration) was loaded on the column and the unadsorbed protein was washed with PBS and checked for lectin activity. The retained lectin was eluted with 0.2 M lactose in PBS. The fractions in the protein peak were pooled, exhaustively dialyzed against PBS and distilled water, lyophilized and preserved at 4^oC till further use.

Hemagglutinating Activity Assay

Hemagglutinating activity was determined in wells

of microtitre plates in a final volume of 150 µl. A serial dilution of the lectin was made by mixing 100 µl of lectin with 100 µl of PBS in the first well and transferring 100 µl of the mixture to the next well. Each well contained 100 µl of lectin solution and 50 µl of 4% (v/v) erythrocyte suspension. Agglutination was assessed after incubation for 1 h at room temperature and hemagglutinating activity was expressed as units which were taken to be the reciprocal of the highest dilution showing visible agglutination. The specific hemagglutinating activity was defined as units per mg protein.

Sugar Inhibition Test

The inhibitory effect of sugars on the hemagglutinating activity was determined by mixing serial dilutions of the sugar with the lectin before addition of erythrocytes and determining the lowest concentration giving full inhibition of agglutination (Kuku and Eretan, 2004). The following sugars were used: D-glucose, D-galactose, D-melibiose, D-arabinose, 2-deoxy D-glucose, D-glucosamine, cellobiose, fructose, maltose, lactose and trehalose

Protein Estimation

Protein concentrations were determined according to Lowry *et al.* (1951) using Bovine serum albumin as standard.

Characterization of *Erythrina senegalensis* Lectin (ESL)

Effect of Temperature and pH on ESL Activity

The effect of temperature on lectin activity was determined as described by Akev and Can (1999). Aliquots of the purified lectin were incubated in a water bath for 30 min at temperatures from 10 100 °C and then cooled to 20 °C. Hemagglutination assay was carried out and results were expressed as % hemagglutination of control kept at 20 °C for 30 min.

The pH stability study was carried out by incubating aliquots of lectin sample in buffers at different pH values (3-10) for 1 h at room temperature.The lectin samples were then assayed for hemagglutinating activity. Control was the agglutination titre of lectin in PBS, pH 7.2

Effect of EDTA and Divalent Cations on ESL Activity

The effect of EDTA and divalent cations on hemagglutinating activity was carried out according to the method of Wang *et al.* (1996). The lectin sample was dialyzed against 50 mM EDTA for 24 h, and the hemagglutinating activity of the demetallizedlectin was determined. The treated lectin was incubated with 50 µl each of the following cations: 25 mM $MnCl_2$, $MgSO_4$, $CaCl_2$, $SnCl_2$, and $CoCl_2$ for 2 h, followed by hemagglutination assay.

Polyacrylamide Gel Electrophoresis (PAGE) and SDS-PAGE

Polyacrylamide gel electrophoresis (PAGE) and SDS-PAGE for the purified lectin and molecular weight standards were carried out as described by Laemmli (1970). Gels were stained with Coomassie blue and Periodic acid-Schiff's reagent (PAS).

Carbohydrate Content of ESL

The carbohydrate content was determined by phenol-sulphuric method of Dubois *et al.* (1956) using D-glucose as standard.

Amino acid Analysis

Purified lectin was subjected to analysis of amino acid contents using phenylthiocarbamyl derivatives method of Bidlinger *et al.* (1984). The sample was hydrolyzed with 6 N HClcontaining 1 % phenol at 110 °C for 22 h, evaporated in a rotary evaporator and loaded into the Technicon sequential Multi-sample Amino Acid Analyzer (TSM) with standards.

Effect of Chemical Modification of Amino Acid Residues on Hemagglutinating Activity

Modification of tryptophan residues with N-bromosuccinimide (NBS) was carried out according to the method of Spande and Witkop (1967). 10 µl aliquot of 10 mM NBS (in water) was added to 100 µllectin sample (1 mg/ml in 50 mM sodium acetate buffer, pH 6.0) with rapid mixing, 10 µl of the reagent was then added to the lectin sample every 10 minat 20 °C for 1 h. Excess reagent (NBS) was removed by dialyzing the solution against distilled water after which 100 µl aliquot was removed from the dialyzed solution and assayed for residual hemagglutinating activity. Lectin incubated with PBS in the absence of NBS served as control.

Reduction of the thiol groups was carried out by incubating 100 µl of the lectin (1 mg/ml) in 50 mM phosphate buffer (pH 8.0) with 10 µl of 0.1

mM 5, 5'- dithiobis-(2-nitrobenzoic acid) (DTNB) at 27°C (10 µl of the reagent was added at 15 mins intervals for 1 h). Excess reagent was removed followed by determination of residual hemagglutinating activity. Lectin incubated in the absence of DTNB served as control (Sharma *et al.*, 2008).

For serine modification, the lectin (100 µg) in 100 µl of 50 mM Tris-HCl buffer (pH 7.4) was incubated with10 µl of 5 mM phenyl methyl sulfonyl fluoride (PMSF) at 27 °C (10 µl of the reagent was added at 15 min intervals for 1 h). Excess reagent was removed followed by determination of residual hemagglutinating activity. Lectin incubated in the absence of PMSF served as control (Habeeb, 1972).

Phenylglyoxal was used for modification of arginine residues (Riordan,1979). 100 µl of 1 mg/ml lectin sample in PBS, pH 7.5 was incubated with 10 µl of 10 mM phenyglyoxal (in 0.1 M sodium carbonate, pH 8.0) at room temperature for 1 h (with addition of 10 µl of reagent every 15 min). The modified lectin sample was dialyzed exhaustively to remove excess reagent and assayed for hemagglutinating activity as described above.

RESULTS AND DISCUSSION

Saline extract of *Erythrina senegalensis* seeds agglutinated human and rabbit erythrocytes with slight preference for O blood type (Table 1). This hemagglutinating activity was inhibited by both D-galactose and lactoseof all the sugars tested (Table 2), the latter being a stronger inhibitor than D-galactose with minimum inhibitory

Table 1: Blood Group Specificity of *Erythrina senegalensis* Seed Lectin

Erythrocytes		**Hemagglutination Activity (Titre)**									
		2^1	2^2	2^3	2^4	2^5	2^6	2^7	2^8	2^9	2^{10}
Human	A	+	+	+	+	-	-	-	-	-	-
"	B	+	+	+	+	-	-	-	-	-	-
"	O	+	+	+	+	+	+	+	-	-	-
Rabbit		+	+	+	+	-	-	-	-	-	-

Each experiment consisted of 100 µl of PBS with a serial dilution of 100 µl of the extract in a U-shaped microlitre plate. 50 µl of 4 % suspension of erythrocytes (from human A, B and O and rabbit blood) was added to each well.

Table 2: Sugar Inhibition of Hemagglutination activity of *Erythrina senegalensis* Seed Extract

Agglutination Titre	2^1	2^2	2^3	2^4	2^5	2^6	2^7	2^8	2^9	2^{10}
Control (saline extract)	+	+	+	+	+	+	+	-	-	-
Lactose	-	-	-	-	-	-	-	-	-	-
Sorbitol	+	+	+	+	+	+	-	-	-	-
Methy D-glucopyranoside	+	+	+	+	+	+	-	-	-	-
Arabinose	+	+	+	+	+	+	+	-	-	-
Mannitol	+	+	+	+	+	+	+	-	-	-
Glucose	+	+	+	+	+	+	-	-	-	-
Sucrose	+	+	+	+	+	+	+	-	-	-
Fructose	+	+	+	+	+	+	+	-	-	-
Glucosamine	+	+	+	+	+	+	+	-	-	-
Maltose	+	+	+	+	+	+	+	-	-	-
Mannose	+	+	+	+	+	+	+	-	-	-
Galactose	-	-	-	-	-	-	-	-	-	-

Each experiment consisted of 100 µl of serially diluted extract in a U-shaped microlitre well. 50 µl of 0.2 M sugar solution and 50 µl of 4 % suspension of erythrocytes (Human blood O) was added to each well.

concentration of 0.195 mM. This inhibitory effect suggests that the lectin can be classified as a member of galactose-specific hemagglutininThis then formed the basis of the choice of lactose-sepharose 4B as affinity matrix for purification of the lectin from the seed extract.

The saline extract of the leaves of *Erythrina senegalensis* did not show any hemagglutinating activity with both human and rabbit erythrocytes.

The dialyzate of 70 % ammonium sulphate fractionation of the seed extract when subjected to gel filtration on Sephadex G-100 (Fig.1) gave three major protein peaks, one of which showed hemagglutinating activity. The sample of the active peak from the gel filtration was further purified on lactose-sepharose 4B affinity column. The lectin was quantitatively adsorbed to the column and was eluted as a single peak with 0.2 M lactose in PBS, pH 7.2 (Fig. 2). The purified lectin after dialyzing and concentrating, when subjected to SDS-PAGE gave a single homogenous band.

The native molecular weight of the lectin as determined by gel filtration on Sephadex G-100 was 62 kDa while SDS-PAGE analysis in the presence of mercaptoethanol revealed one single band with apparent molecular weight of 30 kDa (Fig.3). This suggests the lectin is a homodimer. The lectin when stained with PAS for carbohydrate showed it is glycosylated and the neutral carbohydrate content as determined by phenol/sulphuric acid method was 6.5 % which correlates with the general range for carbohydrate content for *Erythrina* lectins of 3 12 % (Halina *et al.*, 1985; Bharracharyya *et al.*, 1986) . The lectin was sensitive to acidic pH as it lost all its activity at pH 3 to 6; above pH 6 and up to pH 10, there was no effect on the lectin activity (Fig.4). The lectin was heat stable up to 65 °C but when heated at 70 °C for 20 min, it lost 60 % of its activity and was totally inactivated when heated at 80 °C for less than 10 min (Fig.5). These results revealed the high thermal stability of *Erythrina senegalensis* lectin as compared to other *Erythrina* species lectins (Bharracharyya *et al.*, 1981: Quesada *et al.*, 1998), it however shares some resemblance to the results obtained by Emadeldin *et al.* (2003) with *Erythrina speciosa*.

The purified lectin when incubated with 50 mM EDTA for 24 h with continuous shaking and after exhaustive dialysis to remove excess EDTA, the hemagglutinating activity revealed total inactivation, however, when Ca^{2+} and Mn^{2+} (among other metal ions tested) were added to the assay medium in equimolar amounts, the activity was completely restored. Addition of Ca^{2+} or Mn^{2+} separately did not restore the activity, an indication that the lectin required both metal ions for its activity. This result correlates with what was obtained for the lectin from *Erythrina speciosa* (Emadeldin *et al.*, 2003) but however contradicts what was reported for the lectins from *Erythrina*

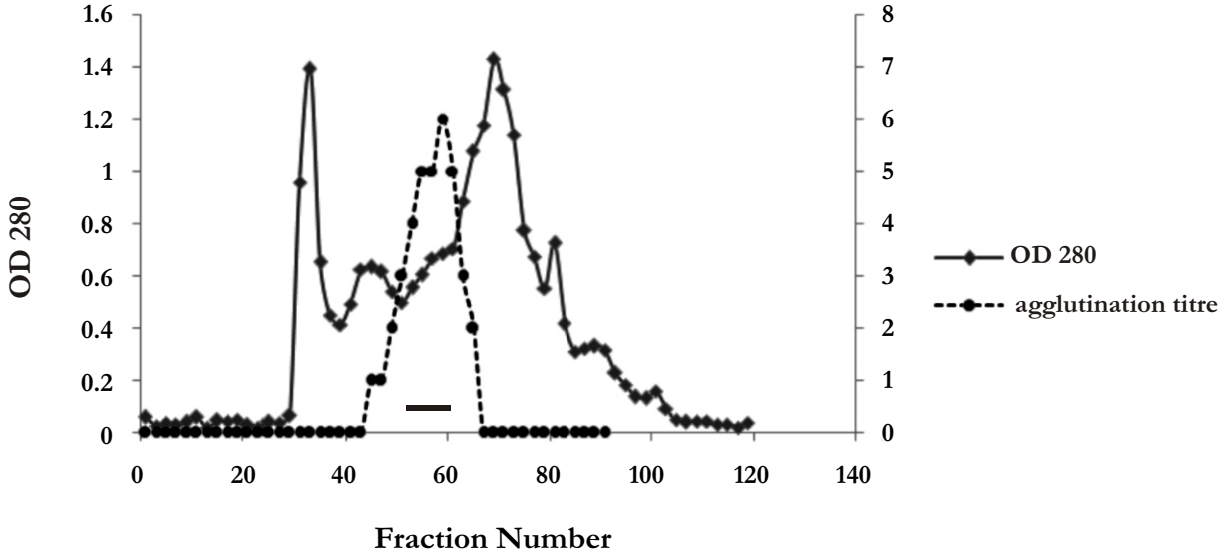

Figure 1: Gel Filtration of *Erythrina senegalensis* Seed Lectin on Sephadex G-150
4 ml of freeze dried dialyzate of ammonium sulphate precipitate (20 mg/ml) was layered on Sephadex G-150 column (2.5 x 40 cm), previously equilibrated with PBS, pH 7.2. Fractions of 4 ml were collected atthe flow rate of 20 ml/h, elution was monitored at 280 nm and the fractions were assayed for haemagglutinating activity.

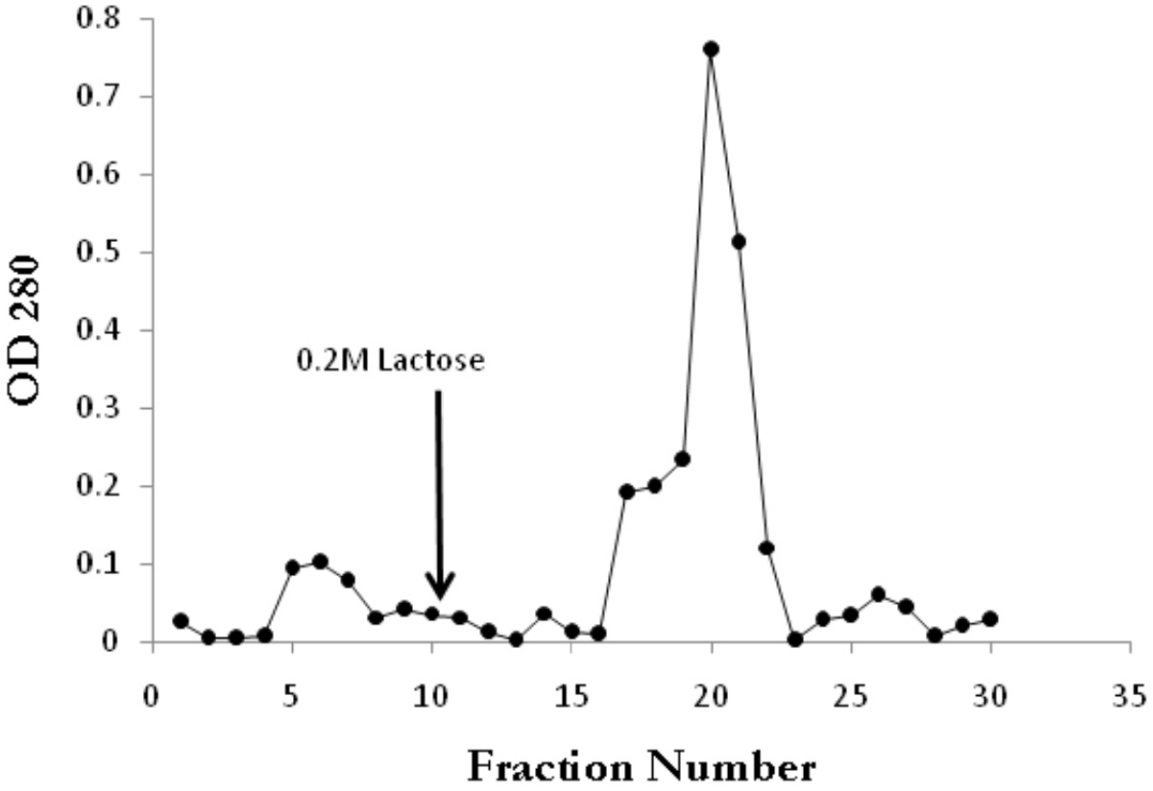

Figure 2: Affinity Chromatography of *Erythrina senegalensis* seed lectin on Lactose-Sepharose 4B Column

Lactose-sepharose 4B slurry (~7.5 ml) was packed into a column (1.5 x 10 cm) pre-equilibrated with PBS, pH 7.2. One ml of the active protein (~10 mg protein) (from gel filtration) was loaded on the column and the unadsorbed protein was washed with PBS. The adsorbed lectin was eluted with 0.2 M lactose in PBS.

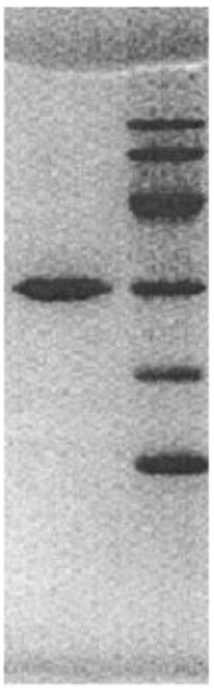

Figure 3: SDS-Polyacrylamide Gel Electrophoresis of *Erythrina senegalensis* Lectin and Molecular Weight Markers:
10 μg protein samples were loaded and gel was Coomassie blue stained.
Lane 1: *Erythrina senegalensis* Seed Lectin
Lane 2: Molecular weight standards: - lactalbumin (14 kDa), soybean trypsin inhibitor (20 kDa), carbonic anhydrase (30 kDa), egg albumin (45 kDa), bovine serum albumin (66 kDa), phosphorylase (97 kDa).

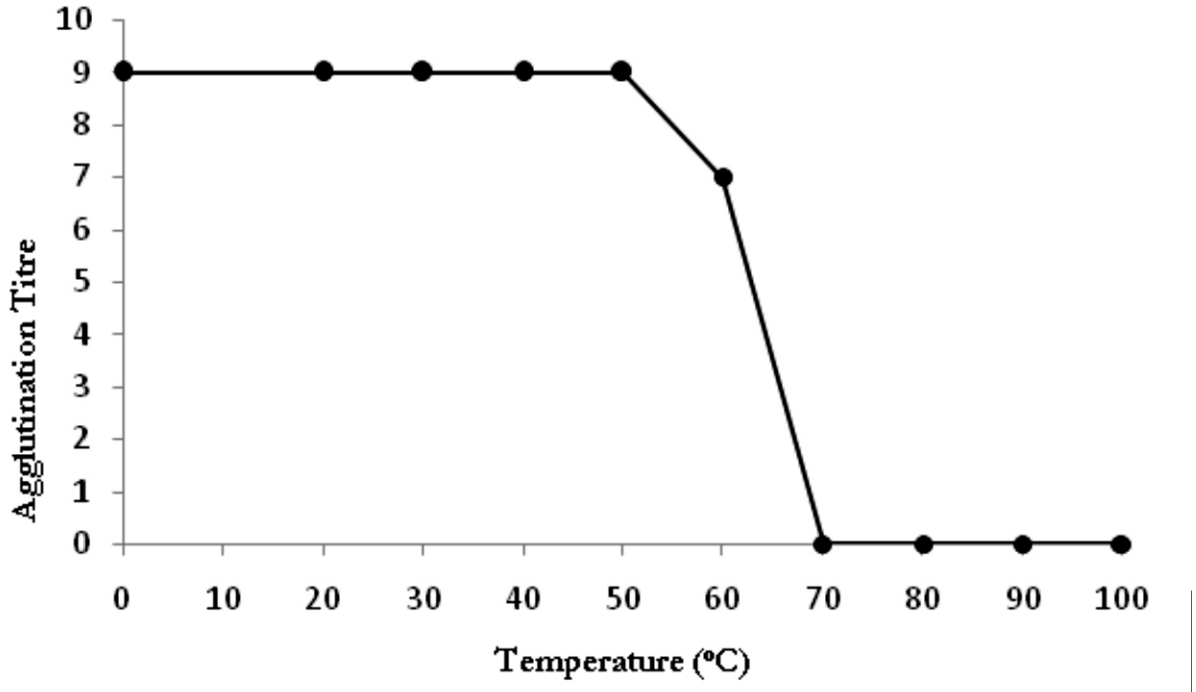

Figure 4: Effect of Temperature on the hemagglutinating activity of *Erythrina senegalenesis* Seed Lectin

Aliquots of the purified lectin were incubated in a water bath for 30 min at temperatures from 10 100 °C. and then cooled to 20 °C. The lectin samples were then assayed for hemagglutinating activity. Results were expressed as % hemagglutination of control kept at 20 °C for 30 min.

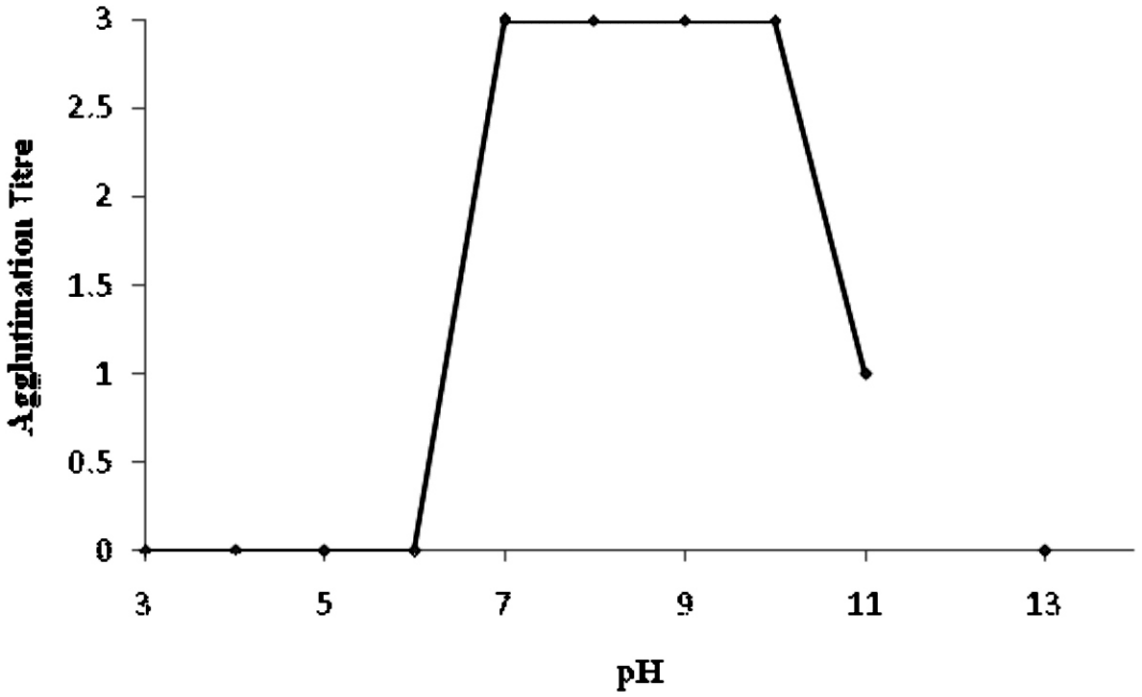

Figure 5: Effect of pH on hemagglutinating activity of *Erythrina senegalensis* Seed Lectin

Aliquots of lectin sample were incubated in buffers at different pH values (3-10) for 1 h at room temperature. The lectin samples were then assayed for hemagglutinating activity. Control was the agglutination titre of lectin in PBS, pH 7.2

Table 3: The Purification Summary of *Erythrina senegalensis* Seed Lectin

Fractions	Total protein (mg)	Total Activity (Total titre x 10^3)	Specific Activity (titre value/mg protein)	Yield (%)
Crude extract	612.0	12.84	2.11	100
Ammonium Sulphate precipitation	140.0	5.38	4.71	23.0
Gel Filtration on Sephadex G-100	27.14	0.61	5.33	4.4
Affinity Chromatography	3.11	0.03	9.84	0.5

Table 4: Amino acid composition of *Erythrina senegalensis* Seed Lectin

Amino acids	Calculated Residues/Mol	Nearest Integer	Mol %
Ala	45.2	45	7.8
Val	36.4	36	6.2
Ile	31.6	32	5.5
Leu	39.1	39	6.7
Ser	52.5	53	9.2
Thr	45.4	45	7.8
Gly	40.3	40	6.9
Cys	-	-	-
Tyr	18.8	19	3.3
Phe	28.3	28	4.8
Arg	11.1	11	1.9
Met	5.8	6	1.0
Asx	64.3	64	11.0
Glx	62.1	62	10.7
His	12.4	12	2.1
Lys	28.6	29	5.0
Pro	45.2	45	7.8
Trp	12.6	13	2.2
Total		579	100

Calculations are based on assumption of molecular weight of 62kDa

Table 5: Effect of chemical modification on hemagglutinating activity of *Erythrina senegalensis* Seed Lectin

Treatment	Modified group/amino acid	% Residual of hemagglutinating activity
Phenyl methylsulfonylfluoride (PMSF)	Serine	95
N-Bromosuccinimide (NBS)	Phenylglyoxal	0
5.5' Dithiobis-(2-nitrobenzoic acid DTNB)	**t**hiol group	100
Phenylglyoxal	arginine	50

rubrrnervia, Erythrina edulis and Erythrina indica (Gerardo, 1995, Pena *et al*., 1988; Bharracharya *et al*, 1981).

Like other *Erythrina* species lectins, ESL is distinctly rich in acidic and hydroxyl amino acids and has sufficient amount of aliphatic amino acids such as ala, val, leu and Ile; it however lacks cysteine and has a low content of methionine residues (Table 3).

A common strategy for identifying the amino acid residues essential for the biological activity of any protein is to treat the protein with specific modifying reagents; this will provide clues about amino acids involved in the biological activity. PMSF and DTNB did not produce any significant alteration in the hemagglutinating activity of ESL, suggesting that serine and cysteine are not involved in the activity. The amino acid analysis of the lectin further supported this fact as it indicated an absence of cysteine. However, the total loss of the lectin activity by NBS suggests involvement of tryptophan residues in lectin activity and stability. Partial inactivation was also observed with modification with phenylglyoxal, an indication of possible involvement of arginine residues in the lectin activity.

CONCLUSION

The lectins from *Erythrina* species share many similarities in their physicochemical properties, including sugar specificity as well as biological and molecular characteristics as can be seen from the reports of all lectins characterized from the different species of *Erythrina*. , including the one from the present study, *Erythrina senegalensis*. Nevertheless, some minor differences in characteristics still exist among this group of proteins. This may be attributed to the environmental and geographical variations in their distribution. This may also suggest that the lectins are assigned to serve specific important physiological role(s) in the various parts of the plants.

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