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Towards a better understanding of the enigmatic biological function of plant lectin: Detection and identification of endogenous receptors for *Erythrina indica* seeds lectin

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ABSTRACT

Although the studies on plant lectins have completed a century, their role in nature remained elusive. *Erythrina indica* seed lectin (EiSL) binding endogenous partners as well as those binding immobilized ConA from *Erythrina indica* seed extract (EiSE) have been studied, at different pHs values, using affinity chromatography on immobilized EiLS and ConA. At pH 7, fewer proteins bound EiLS very strongly involving sugar specific, ionic and hydrophobic interactions. However, at pH 4.6, (a pH closer to the in vivo conditions) good number of proteins interacted with EiLS solely by sugar specific interactions. Endogeneous receptors for EiSL were identified as glycosidases, acid phosphatase and or probably storage proteins. β-galactosidase which usually does not share with lectin the localization inside protein bodies, was weekly associated with EiSL, whereas hydrolytic enzymes such as α-galactosidase, α-mannosidase, N-acetyl-β-D-glucosaminidase and acid phosphatase, which are known to reside inside protein bodies, were strongly retained on immobilized EiSL. To test specificity of lectin-endogenous receptors interaction we immobilized Concanavalin A (ConA) the lectin from Canavalia ensiformis. Loading of EiSE on ConA-Speharose at pH 7.0 and subsequent elution of bound partners resulted in identification of several glycosidases that interacted with ConA by its sugar binding capacity. At pH 4.6, immobilized ConA could recognize receptors from EiSE only by ionic interactions. These results are discussed on the context of anonymous biological significance of plant lectin.

KEYWORDS

Biological role;
Protein bodies;
Interactions;
Plant lectin;
Legume;
Receptors.

INTRODUCTION

Lectins the (glyco) proteins of non-immune origin are ubiquitous in nature[1]. Though of the great progress has been made in recent years in understanding crucial roles played by lectins in many biological processes in

plant, however, mostly the function of lectins in plants remains without a conclusive statement[2,3]. Based on the presence of unique carbohydrate-binding pocket, hydrophobic binding site (some lectins), the high selective, specific and preferential interaction towards sugars and carbohydrates, a variety of functions have been postulated for this protein. These include roles as storage proteins[4], protective agents[5,6], transport vehicle[7], stress signaling[8,9], growth regulators[10], and specific attractants for Rhizobia[11]. However, at present there is no sufficient evidence to confirm any of these roles as the true physiological function of the lectins.

Majority of glycosidases along with lectin and storage proteins are long known to share a common cellular localization inside storage organelles known as protein bodies which is derived from endoplasmic reticulum[12,13]. The common localization of lectin with the storage protein, led to the postulation that lectin may be assisting in deposition of these proteins inside protein bodies[14,15]. In legumes, in particular, interactions of lectin with protein body components namely storage protein and hydrolases is reported[16]. Glycosidases and phosphatases are the lectin reactive proteins[17]

Lectin-glycoconjugate interaction has been mainly studied *in vitro*. Our previous studies with *Erythrina indica* seed crude extract indicated that if lectin was removed by affinity purification some glycosidase activities dropped down[18]. Therefore, we hypothesized that such *in vitro* lectin-glycosidase interactions were presumably occurring under *in vivo* conditions. This hypothesis was indeed proved true later, addition of the purified lectin to α-mannosidase helped in increasing the enzyme activity in a concentration dependent manner to the extent of 35%,[19]. Oliveira *et al* during their work with *Erythrina velutina forma aurantiaca* seed germination noticed the delay in lectin degradation as compared to other proteins[20]. These two results prompted us to investigate on possible endogenous receptors for EiSL which may shed some light on possible enigmatic role of plant lectins.

MATERIALS AND METHODS

Plant materials

Season fresh *Erythrina indica* and *Canavalia gladiata* seeds were collected from trees at the main campus of University of Pune. Seralose 4B is a trade name for agarose 4B, was purchased from SRL, Mumbai, India. All chemicals and reagents are of highest grade available. All experiments were carried out at 4°C unless otherwise stated.

Protein estimation

Protein quantification was done by lowry method[21] using bovine serum albumin (BSA) as the standard. A calibration curve for BSA was also prepared by monitoring absorbance at 280nm.

Glycosidase activities determination

All glycosidase activities and acid phosphatase were determined according to method of Li and Li[22] under standard assay conditions of pH and temperature. One unit of enzyme is defined as the amount of enzyme which hydrolyzes 1μmole of substrate (ρ.nitrophenyl glycoside/ phosphate) per mL per min under the assay condition.

Purification of concanavalin A

Concanavalin A (ConA) was isolated from *Canavalia gladiata* seeds essentially as shown by Agarwal and Goldstein[23].

Preparation of *Erythrina indica* seed extract

Fifty grams of good quality seeds were soaked overnight in distilled water at room temperature. The seeds coats were peeled off and softened cotyledons were homogenized in a mixer with saline I (5mL/gm of soft cotyledons). The homogenate was mixed with 1-butanol (10mL/50mL of homogenate), the mixture was stirred for 1hr at 4°C and the butaol layer was removed by centrifugation at 10000*rpm* for 30 minutes, the treatment was repeated to ensure complete removal of lipid. The homogenate was mixed with equal amount of chilled acetone under continuous stirring. The resultant precipitate was collected by centrifugation at 12000*rpm*, dehydrated by washing with chilled acetone and air dried. This precipitate is called Acetone Dried Powder (ADP). 25gm of ADP was extracted with 100mL saline I for 3hrs at 4°C. The supernatant obtained upon centrifugation at 12000*rpm* for 30minutes was called as Fraction A.

Purification of EiSL from fraction A

Erythrina indica seed lectin was isolated from

Fraction A by the affinity chromatography on lactamyl Seralose 4B matrix which was prepared in our laboratory according to[24,25]; elution of bound lectin was done with 200mM lactose prepared in 0.145M NaCl (Saline I).

Immobilization of EiSL / ConA

EiSL and ConA were immobilized by cross-linking with glutaraldehyde to aminohexyl Seralose 4B essentially according to the procedures of Brandt *et al*[26] in brief; Aminohexyl (AH) Sralose 4B was treated with 8% v/v glutaraldehyde, 5mL/mL of packed gel in 50mM bicarbonate buffer pH 8.2 (buffer I) at room temperature for 3hrs with mild stirring. The gel was washed with Buffer 1 till free from glutaraldehyde. To the activated gel EiSL / ConA (30mg/mL) containing 100mM lactose/glucose in buffer I was added and kept overnight at room temperature for cross-linking under mild stirring. The gel was washed with buffer I till washings showed no significant reading at 280nm. The gel was kept overnight in 100mM glycine in saline I to block unreacted aldehyde groups. The amount of lectin immobilized was determined by the method of Schurz and Rudiger[27].

Identification of endogenous receptors to EiSL

The detection of endogenous receptors for EiSL was done by affinity chromatography on EiSL-Sepharose 4B at two different pH values i.e. pH 7 and pH 4.6.

Preparation of fraction B

This was prepared by passing Fraction A through lactamyl seralose-4B affinity column to remove lectin. Fraction A devoiding lectin is termed Fraction B.

Identification of receptors at pH 7.0

EiSL-Seralose 4B was packed in a column (1x6cm) under the gravity, the column was equilibrated with saline I. 26mg of Fraction B was loaded onto the column under a reduced flow rate (1mL/10 minutes), recycled at least for 4 times. Column was washed with saline I with about 10 volumes of the column bed. Washings were collected till OD280nm ≤ 0.02. Trials for elution of bound receptors was done in this sequence (a) 1M NaCl (saline II) (b) 200mM lactose, and finally (c) 40% (w/v) ethylene glycol in saline II. Fractions of of 1mL/5min were collected and monitored for protein and hydrolase activities.

Identification of receptors at pH 4.6

Twenty six mg of Fraction B was exhaustively dialyzed against 100mM citrate buffer pH 4.6 (buffer II). EiSL-Sepharose 4B column was equilibrated with buffer II. Dialyzed protein was centrifuged at 10000 *rpm* for 30 minutes to remove precipitated proteins, clear supernatant was loaded onto EiSL-Sepharose 4B column under reduced flow rate 1mL/10 minutes and recycled for at least 4 times. 5mL washing fractions were collected till no more protein was recorded at 280nm. Bound proteins were initially eluted with saline II prepared in buffer II, followed by 100mM galactose in buffer II and finally 200mM lactose in buffer II. 2mL fractions were collected at flow rate 1mL/5 minutes and monitored for protein and hydrolase activities.

Receptors for ConA in EiSE

Receptors for ConA from Fraction B were studied at pH 7.0 as well as pH 4.6 values exactly as shown for EiSL endogenous receptors. Elution of bound proteins at pH7.0 was tried initially with saline II followed with different concentrations of α-methyl D-glucopyranoside in saline I, whereas elution of receptors at pH 4.6 was done only using saline I prepared in buffer II. 2mL fractions were collected at a flow rate of 1mL/5 minutes. Fractions were monitored for protein and hydrolase activities.

Native polyacrylamide electrophoresis (Native-PAGE)

Native-Polyacrylamide gel electrophoresis (PAGE) at pH 8.6 was carried out according to Williams and Reisfeld[28]. The protein bands were visualized by staining the gels with Coomassie brilliant blue R-250.

Sodium dodecylsulphate electrophoresis (SDS-PAGE)

This was performed essentially as per Laemmli's procedures[29]. The protein bands were visualized by staining the gels with Coomassie brilliant blue R-250.

RESULTS AND DISCUSSION

During our work with hydrolases and lectin from

Erythrina indica it was observed that if Fraction A was made free of lectin (Fraction B) most of hydrolases start rapidly to loss activities. The decrease in activity produced upon lectin removal was most clear in case of α-mannosidase. These observations let us to believe that lectin is likely to play an essential role in stability enhancement of these hydrolases. Furthermore, native PAGE analysis of purified EiSL always revealed a faintly stained band at the bottom edge of the gel. Testing of affinity purified EiSL for hydrolase activities showed no enzymatic activity (not shown). Therefore, we assumed that this anonymous band could either be a storage protein or hydrolase enzyme that lost activity during processing and chromatographic separation. In the search for finding a possible biological significance of plant lectin, we used immobilized EiSL and ConA to detect for probable partner(s) in the whole seed extract of *Erythrina indica*. Lectins were immobilized by cross-linking to AH-Seralose 4B beads by glutaraldehyde treatment. The amount of lectin immobilized was 10.5 and 15mg for EiSL and ConA respectively. Immobilized ConA was used to examine for EiSL-receptors interaction fine specificity. In particular, we have chosen two different pH values i.e. pH 7.0 and 4.6 to carry out this study.

EiSL endogenous receptors at pH 7.0

Fraction B was loaded onto immobilized EiSL column; column was initially washed with saline II till effluent OD280nm dropped to ≤ 0.02. Column was initially eluted with high salt to rule out any possible ionic interactions between lectin and EiSE soluble proteins. Elution of bound protein was initially tried with lectin haptenic sugar (lactose). Lactose at up to 200 mM did not elute any protein. On the other hand, trials of eluting tightly bound protein with 40% polyethylene glycol were also not successful. However, when a combination of 200 mM lactose, 40%ethylene glycol in saline II was used, protein was successfully desorbed from the column. These results indicated that at pH 7.0, EiSL interacts tightly with its endogenous receptors involving lectin sugar binding site, ionic and hydrophobic interactions. In a previous publication we have shown that *Erythrina lysistemon* seed lectin to possess a hydrophobic binding site which is independent of its sugar binding site[30]

When eluted protein was checked for hydrolase activities, three glycosidases were detected i.e.; β-galactosidase, α-galactosidase and α-mannosidase (Figure 1). On performing native-PAGE for eluent, three prominent bands were detected (Figure 2a).

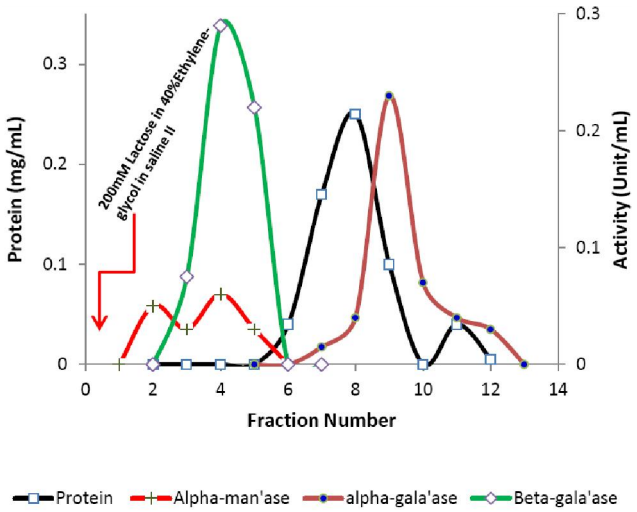

Figure 1 : Affinity chromatography of EiSL-Seralose 4B receptors at pH 7.0. Column was equilibrated with saline I pH 7.0. 26 mg protein was loaded. Elution was carried out with 200mM lactose in 40% polyethyleneglycol in saline II. Fractions of 2mL were collected at a flow rate of 1mL/ 5minutes

The surprising stability of the native structure of most lectins is thought to be caused by the hydrophobic interactions. Such hydrophobic sites, forming cavities in the lectins structure, may play an important biological role. The hydrophobic binding sites of auxins, or cytokinin and adenine, for instance, by concanavalin A may enhance the functions of lectins on the plant life cycle[31]. Another hydopthesis suggested that this hydrophobic binding site may play a role in growth regulation[32]. An interesting observation has been reported by Togun *et al* in which they have monitored *Telfairia occidentalis* seed lectin expression at different stages of germination. Authors showed the lectin to peak on day7th, decreases sharply thereafter. No lectin activity was detected after 27days of germination onset. Therefore they suggested a possible role of *Telfairia occidentalis* seed lectin as a growth regulator[4].

EiSL endogenous receptors at pH 4.6

Since the pH inside protein bodies is acidic[33], EiSL-receptors interactions was also studied at a pH that mimics the *in vivo* conditions. Since most of glycosidase and acid phosphatase possess a *pH* optima

between 4-5[18] this experiment was performed at pH 4.6. As expected, at this pH, more proteins were retained on the column (Figure 3), and that only the haptenic sugar lactose vwas able to elute them. These results indicated that at pH 4.6 EiSL interacts with endogenous partner solely by its sugar binding site. Four glycosidases i.e., β-galactosidase, α-galactosidase and α-mannosidase, N-acetyl-β-glucosaminidase besides acid phosphatase were retained on the column. Interestingly, β-galactosidase, which is known to predominantly associatewith plant cell wall[34] and rarely localized inside protein bodies[15,35], was weakly retained on immobilized EiSL at pH 4.6, on the contrary, enzymes like α-galactosidase, α-mannosidase and acid phosphatase, which are well known for their protein bodies localization[15,36] were retained to greater extend. A similar result was obtained by Freier and Rüdiger in their work with lentil lectin binding partners[15]. Native electrophoresis of eluted protein at pH 4.6 resulted into at least six protein bands (Figure 2b).

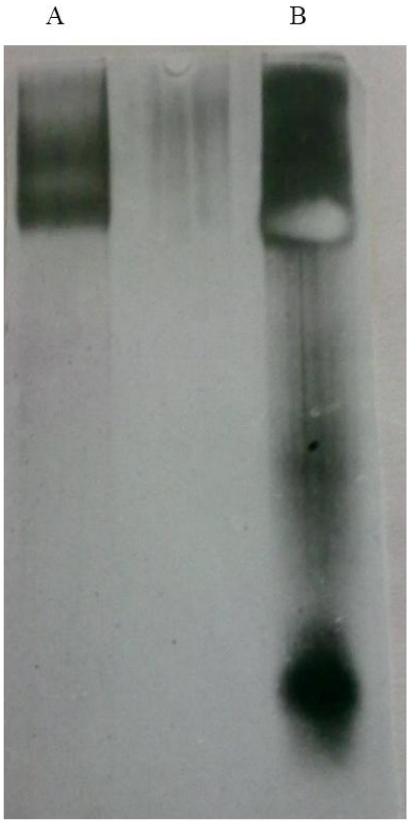

Figure 2 : Native-electrophoresis of immobilized-EiSL endogenous binders. Native-PAGE was carried out at two different pH values: A) pH 7.0, B) pH 4.6. 30µg protein was loaded on 9% gel. Gel was stained with Commassie brilliant blue.

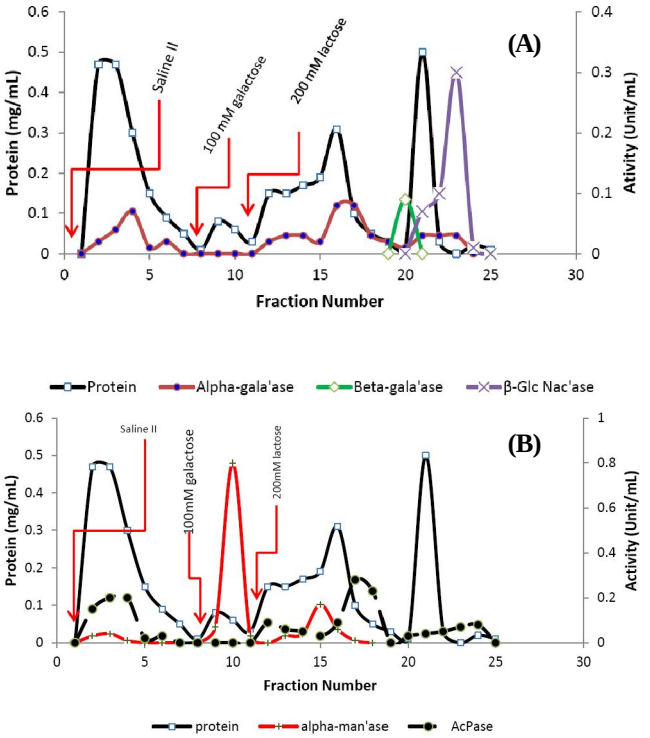

Figure 3 : Affinity chromatography of EiSL-Seralose 4B receptors at pH 4.6. *Graph was spilt into A and B for clarity and convenience.* Column was equilibrated with buffer A. 26 mg protein was loaded. Following washing of the column with saline II prepared in buffer A. Elution was carried out sequentially with the following eluents at a flow rate of 1mL/ 5min: 1) Saline, II 2) 100mM galactose and 3) 200mM lactose. All sugars were prepared in buffer A

Receptors from fraction B to ConA

In order to further study the specificity between lectin-endogenous receptor interactions, receptors to ConA from Fraction B was undertaken using ConA-Sepharose at pH 7.0. α-galactosidase, α-mannosidase, β-galactosidase, N-acetyl-β-glucosaminidase and acid phosphatase were retained on immobilized ConA (Figure 4). When Fraction A, which contains lectin, was loaded on ConA-Sepharose, even EiSL was found to be retained on the column (not shown). ConA haptenic sugar α-methyl D-glucopyranoside was alone able elute all bound proteins. If chromatography on ConA-Sepharose was carried out at pH 4.6 only acid phosphatase, N-acetyl-β-glucosaminidase and other unknown proteins were loosely retained by ionic interactions. Subsequent elution with 5 mM α-methyl D-glucopyranoside did not reveal any hydrolase activity (Figure 5). ConA is a tetramer at pH 7.0 and a dimer at acidic pH[37]. This may possibly justify the above ob-

servations. Figures 6 a & b show electrophoretic pattern of the receptors from EiSE Fraction B for ConA-Seralose at pH 7.0 and 4.6, respectively.

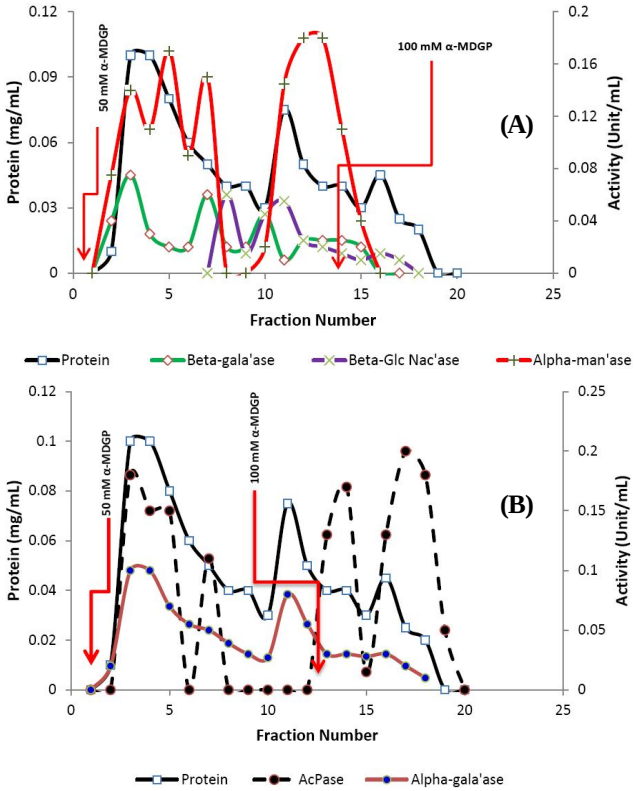

Figure 4 : Affinity chromatography of Fraction B on ConA-Seralose 4B at pH 7.0. Column was equilibrated saline I. 26 mg protein was loaded. Column was initially washed with ample quantity of Saline II. Elution of bound protein was carried out sequentially with the following eluents at a flow rate of 1mL/5min: 1) 50 α-MDGP, 2) 100 α-MDGP

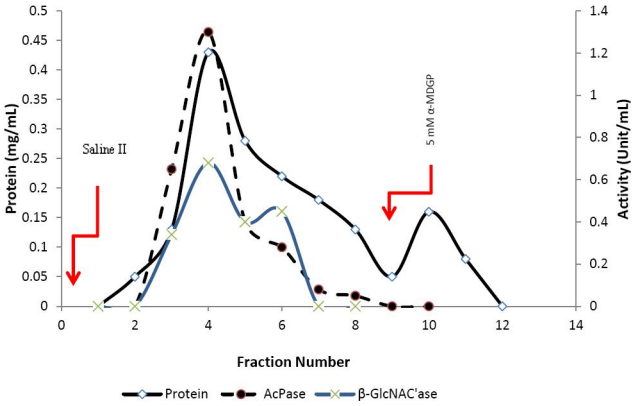

Figure 5 : Affinity chromatography of Fraction B on ConA-Seralose 4B at pH 4.6. *Graph was spilt into A and B for clarity and convenience.* Column was equilibrated with buffer A. 26 mg protein was loaded. Elution was carried out sequentially with the following eluents at a flow rate of 1mL/5min: 1) Slaine II, 2) 5 mM α-MDGP

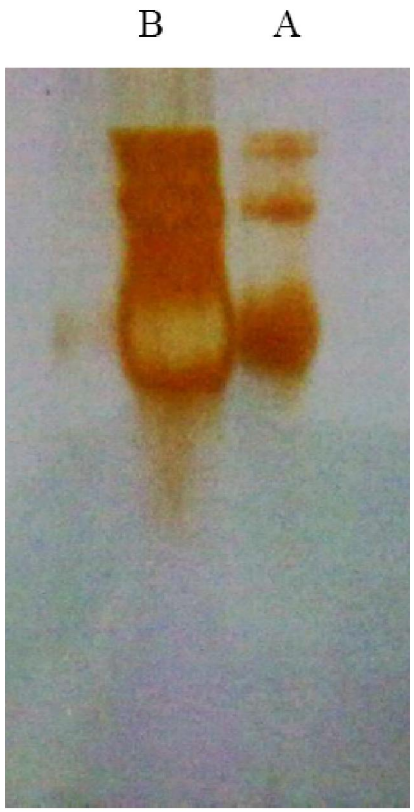

Figure 6 : Native-electrophoresis of ConA-Seralose 4B binders from Fraction B of *Eyrthrina indica* seed extract. Native-PAGE was carried out at two different pH values: A) pH 7.0, B) pH 4.6. 10 µg protein was loaded on 9% gel. Gel was stained with Commassie brilliant blue.

Rudiger and his colleagues, in several publications, had extensively studied endogenous lectin receptors. They have proposed that lectins should be involved in the packaging of storage proteins, since various lectins from leguminosae tested, were able to interact with storage proteins belonging to their own plants[38]. In another publication, they had shown that, pea lectin does interact with vicilin and legumin fraction from protein bodies of pea seeds, suggesting that the protein body membranes might be a further candidate for lectin interactions[39].

ABBREVIATIONS

ConA	:	Concanavalin A
EiSL	:	*Erythrina indica* Seed Lectin
EiSE	:	*Erythrina indica* Seed Extract
Seralose 4B	:	Trade name equivalent to Agarose 4B
Fraction A	:	*Erythrina indica* seed extract con-

taining lectin
Fraction B : Fraction A devoid of lectin
Saline I : 145mM NaCl
Saline II : 1M NaCl
ADP : Acetone dried powder
Buffer I : bicarbonate buffer pH 8.2, 50 mM
Buffer II : citrate buffer pH 4.6, 100 mM
Native-PAGE : Native-Polyacrylamide gel electrophoresis
SDS-PAGE : Sodium dodecylsulphate polyacryl amide gel electrophoresis
BSA : Bovine Serum Albumin
α-MDPG : α-methyl D-glucopyranoside
Alpha-gala'ase : alpha-galactosidase
Beta-gala'ase : Beta-galactosidase
Alpha-man'ase : Alpha-mannosidase
β-GlcNAC'ase : N-Acetyl- β -D-glucosaminidase
AcPase : Acid phosphatase

CONCLUSIONS

In the present investigation, attempt was made to detect and characterize endogenous receptors for *Erythrina indica* seed lectin. Our results showed that EiSL interacts with endogenous receptors at neutrality and acidic pH differently. Taking these results into account and recollecting our previous data on *Erythrina lysistemon* seed lectin, which describes the finding of a hydrophobic binding site that's independent of sugar binding site[30], the results of Oliveira *et al* on *Erythrina velutina forma aurantiaca* on the retarded degradation of seed lectin as compared to other storage proteins[20] and finally the increament on *Erythrina indica* seed α-mannosidase activity in the presence of endogenous lectin and the subsequent drop in the enzyme activity upon removal of lectin[19], all of these rsults taken together may allow us to conclude that studies of such interactions between lectin and components which share common *in vivo* localization, may hold good for particular species only for that case and cannot be generalized[4]. Thus, these type of studies may help in understanding, the yet to be known, biological function of plant lectins.

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