Phytochemistry 83 (2012) 7–14

Contents lists available at SciVerse ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

The enzymatic lectin of field bean (*Dolichos lablab*): Salt assisted lectin–sugar interaction

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ARTICLE INFO

Article history:
Received 1 May 2012
Received in revised form 27 July 2012
Available online 4 September 2012

Keywords:
ANS binding
Fluorescence
Lectin-ELISA
Protein sequence
Surface hydrophobicity

ABSTRACT

Field bean seed contains a Gal/GalNAc lectin (DLL-II) that exhibits associated polyphenol oxidase (PPO) activity and does not bind to its sugar specific affinity matrix. The molecular basis for this lack of binding is not known. The DLL-II gene was therefore cloned and its sequence analyzed. A conserved aromatic residue in the sugar binding site required for a stacking interaction with the apolar backbone of Gal is replaced by His in DLL-II, which explains the lack of binding. However, specific sugar binding is achieved by including $(NH_4)_2SO_4$ in the buffer. Interestingly two other salts of the Hofmeister series, K_2HPO_4 and Na_2SO_4 also assist binding to immobilized galactose. In the presence of $(NH_4)_2SO_4$ the surface hydrophobicity of DLL-II and dissociation constant for 8-anilino 1-naphthalene sulfonic acid were enhanced three fold. This increased surface hydrophobicity in the presence of salt is probably the cause for assisted sugar binding in legume lectins that lack aromatic stacking interactions. Accordingly, two other lectins which lack the conserved aromatic residue show similar salt assisted binding. The salt concentrations required for Gal/GalNAc binding are not physiologically relevant in vivo, suggesting that the role of DLL-II per se in the seed is primarily that of a PPO purportedly for plant defense.

1. Introduction

Plant lectins in particular, those isolated from the *Leguminosae* family represent the best studied class of carbohydrate binding proteins. Legume lectins found most abundantly in the mature seeds are a group of highly conserved proteins, with the ability to interact reversibly with residues of specific sugars (Ambrosi et al., 2005; Lis and Sharon, 1981; Rudiger and Gabius, 2001; Sharon, 2008; Sharon and Lis, 2004). This property has rendered them as invaluable tools for studies in biomedical research. Lectins differ in their interactions with sugars and oligosaccharides, molecular structure and agglutinating activity.

Field bean (*Dolichos lablab*) is a legume plant, the seeds of which are used in South Indian cuisine. A polyphenol oxidase (EC 1.10.3.1, PPO) purified from field bean seeds displayed associated haemagglutinating activity against human erythrocytes. The PPO and haemagglutinating activities were independent and occurred at different loci on a single protein. This bi-functional protein was therefore called "PPO-haemagglutinin" (Kanade et al., 2009). The complete primary structure of a glucose/mannose specific lectin (DLL-I) of field bean seed has been determined by conventional protein sequencing methodology (Gowda et al., 1994). PPO-haemagglutinin and DLL-I differ with respect to their sugar specificity. The haemagglutinating activity of PPO-haemagglutinin was inhibited by Gal and GalNAc. In addition it differed from DLL-1 both in mass and molecular architecture. Therefore the PPO-haemagglutinin was classified under Gal/GalNAc specific lectins. PPO-haemagglutinin displayed yet another distinctive and unusual feature: the inability to bind to its specificity sugar, Gal. This was unusual as most legume lectins reportedly bind to sugars for which they are specific. Our previous studies on the purification of PPO-haemagglutinin indicate that binding to a Gal-Sepharose affinity matrix occured in the presence of $(NH_4)_2SO_4$ (Kanade et al., 2009). A detailed study and understanding of the molecular basis for the lack of Gal binding by PPO-haemagglutinin was of considerable interest. Therefore a study of the primary structure of 'PPO-haemagglutinin' hitherto designated as DLL-II was undertaken. The gene for DLL-II was cloned and cDNA sequence obtained. Analysis of the primary sequence revealed that like all other legume lectins the triad required for general sugar binding was conserved in DLL-II. However a crucial conserved aromatic residue required for a

Abbreviations: ANS, 8-anilino 1-naphthalene sulfonic acid; cDNA, complementary; DLL-I, *Dolichos lablab* glucose/mannose specific lectin; DLL-II, *Dolichos lablab* galactose specific lectin; DBL, *Dolichos biflorus* lectin; Gal, D-galactose; GalNAc, N-Acetylgalactosamine; PBS, phosphate buffered saline; PGL, *Phaseolus glabellus* lectin; PPO, polyphenol oxidase; TBS, Tris buffered saline.

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http://dx.doi.org/10.1016/j.phytochem.2012.07.027

specific stacking interaction with Gal is replaced by a His residue in DLL-II, which explains the lack of binding. Further, the study shows that despite this substitution binding to Gal can be induced by the inclusion of $(NH_4)_2SO_4$ and other salts of the Hofmeister series in the buffer. The presence of salt increases the surface hydrophobicity of DLL-II, which favors binding. Further the Gal/GalNAc specific lectin of horse gram (*Dolichos biflorus, DBL*), with associated lipoxygenase activity (Roopashree et al., 2006), and *Phaseolus glabellus* (PGL) lectin, which also lack the conserved aromatic residue, also bind Gal-Sepharose only in the presence of $(NH_4)_2SO_4$. These results suggest that the surface hydrophobicity of Gal/GalNAc specific lectins is important for carbohydrate-protein interactions.

2. Results

2.1. Elucidation of the primary structure of DLL-II

Typically all legume lectins, which recognize a terminal Gal or GalNAc in their primary binding site comprise of a conserved Asp, which recognizes the equatorial C3–OH and axial C4–OH of D-Gal, Gly, and Asn bonded to C3–OH. In addition, a conserved aromatic residue (F/W/Y) is required at the binding site for stacking interactions with the B face of the Gal ring. The low affinity of DBL, PHA-L and PHA-E to Gal has been attributed to the substitution of this conserved aromatic residue by an aliphatic residue Leu (Hamelryck et al., 1999). Therefore to determine if such a substitution was responsible for the absence of Gal binding, the primary structure was deduced from the obtained cDNA sequence. Several degenerate primers were designed based on the determined N-terminal sequence of DLL-II and a conserved sequence among Gal specific lectins. Several gene fragments were generated and sequenced. Using these sequences, gene specific primers were designed. A 759 bp fragment obtained was cloned into pJET 1.2 and sequenced. The sequence was submitted to GenBank (Accession EF204527). 3′ Genome walking was performed to obtain the full length gene including a stop codon. A 179 bp downstream sequence was obtained, which comprised of a stop codon. A single polyA signal (AATAAA) was recognized in the 85th base position downstream of the coding region (Fig. 1).

Analysis of the cDNA shows an open reading frame encoding a protein of 252 amino acids. The predicted molecular mass of this protein is 29,095 Da. The MALDI-TOF mass spectrometric analysis showed a mass of 31,087 Da for the large subunit (Kanade et al., 2009). The difference is probably due to either glycosylation and/or a C-terminal 'ragged end' frequently observed in legume lectins (Fabre et al. 1998). The deduced amino acid sequence contains the matching sequences of five tryptic peptide obtained by Edman analyses of the purified lectin. These results strongly suggest that the isolated lectin and cloned cDNA are of the same protein. The sequence has three potential N-linked glycosylation sites commensurate with glycosylation reported (Kanade et al., 2009).

2.2. Analysis of the primary structure of DLL-II

A BLAST search of the deduced sequence showed high similarity to the Gal/GalNAc specific legume lectins. DLL-II showed maximum identity to the horse gram lectins DBL (61.3%) (Schnell and Etzler, 1987) and DB58 (59.3%). Multiple sequence alignment using MULTALIN revealed that the amino acid Asp responsible for metal binding was part of the sequence'$W^{131}D^{132}P^{133}$' present in most lectins (Lescar et al., 2002). The sugar binding region of DLL-II was compared with sequences of nine other Gal/GalNAc legume lectins (Fig. 2). The conserved sugar binding triad of DLL-II (Asp^{85}; $Gly^{103}$and Asn^{129}) is similar to the other Gal specific lectins. However, the aromatic residue (F/W/Y) that is reportedly responsible for stacking against the apolar B face of Gal is replaced by His^{127} in DLL-II (Fig. 2). DLL-II is not the only Gal specific lectin wherein the aromatic residue is substituted. The sequence alignment reveals that both *Sophora japonica* bark lectin (SJBL) and PGL of *P. glabellus,* have a His residue at this position. Moreover in DBL and PHA-L a Leu is present at this position. Interestingly both DBL and PHA-L also do not show any affinity for Gal (Hammarstrom et al., 1982; Roberts and Goldstein, 1982) suggesting that the replaced aliphatic residue could be one of the determining factors for lack of Gal binding. Our results together with these observations suggest that the His replacement probably reckons lack of the aromatic stacking thereby leading to the loss of Gal binding.

2.3. Effect of $(NH_4)_2SO_4$ on PPO and lectin binding activity

A key element of lectins is their monosaccharide binding specificity, a property that is used to produce affinity matrices for their purification. DLL-II identified to be a Gal specific lectin however, did not bind to Gal-Sepharose. Our earlier studies showed DLL-II bound to Gal-Sepharose affinity matrix only if $(NH_4)_2SO_4$ was included in the column equilibration buffer (Kanade et al., 2009). The aromatic residue required for the stacking interaction was absent in DLL-II, yet binding to Gal-Sepharose occured in the presence of $(NH_4)_2SO_4$. The key question was what changes did $(NH_4)_2SO_4$ bring about to assist binding? Therefore Gal binding in the presence of $(NH_4)_2SO_4$ was studied in detail using a pure and homogeneous preparation of DLL-II. The purity, molecular mass and glycoprotein nature of the protein used was identical to that reported earlier (Kanade et al., 2009). The pure protein used had a PPO activity of 770 U/mg and agglutinating activity of 40 HAU/mg. When DLL-II was applied to a Gal-Sepharose column equilibrated in TBS all the input protein could be consistently measured in the column flow through. As the $(NH_4)_2SO_4$ concentration increased, protein bound to the column increased (Fig. 3). At 1.2–1.5 M $(NH_4)_2SO_4$ all of the input protein bound to the matrix with no flow through in the wash. These results show that the unbound protein observed in the flow through without $(NH_4)_2SO_4$ was not reckoned by exceeding the column capacity. The results show that bound DLL-II measured as A_{280} increased in a sigmoidal manner with the increase in $(NH_4)_2SO_4$ concentration, and maximum binding was observed at 1.5 M $(NH_4)_2SO_4$ (Fig. 3). This $(NH_4)_2SO_4$ concentration is far below that required for precipitation, which is 3–4.5 M (40–60%). The bound protein eluted from the column when the eluant was changed to TBS. The binding therefore was assisted by $(NH_4)_2SO_4$. The sum of bound and unbound protein was near constant ($A_{280} = \sim 0.5$ results not shown). SDS- PAGE analyses of bound DLL-II (peak fractions) at different $(NH_4)_2SO_4$ concentration did not reveal any differences in subunit architecture.

An immuno-enzymatic method for lectin assay was studied using microtiter plates coated with asialofetuin and quantified by using a PPO-haemagglutinin specific antibody. ELISA response was dose dependent in the concentration range of 1.5 ng–2 μg/mL (results not shown). The measured lectin activity showed a sigmoidal increase with the maximum activity observed at 1.5 M $(NH_4)_2SO_4$ (Fig. 3). It is noteworthy that the trend of protein bound to Gal-Sepharose correlates with the measured lectin activity at different $(NH_4)_2SO_4$ concentrations (Fig. 3A).

The effect of varying $(NH_4)_2SO_4$ concentration on PPO activity was evaluated. PPO activity was 4460 ± 45 U/mL without $(NH_4)_2SO_4$. Increasing the concentration of $(NH_4)_2SO_4$ had no effect on PPO activity (Fig. 3). At concentrations greater than 1.5 M a marginal decrease in PPO activity was discernible. These results suggest that $(NH_4)_2SO_4$ affects only the sugar binding activity of DLL-II and not the PPO activity.

This unique and specific sugar binding only in the presence of $(NH_4)_2SO_4$ was evaluated using lactose and GalNAc affinity