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A hemagglutinin from *Pongamia pinnata* (L.) Pierre seed

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Lectins are diverse proteins that bind to carbohydrates and are involved in many important physiological processes. They are highly specific to the sugar molecules they bind and therefore, have many therapeutic and diagnostic applications. There are several lectins that display antiviral, antibacterial antifungal and antitumour activities. Characterization of new lectins paves way for comprehension of their diverse biological roles and mechanism of action, thus aiding in further exploration of lectins in various domains of biology. Here, we endeavoured to purify and characterize lectin from the seed of the legume,Pongamia. *Pongamia pinnata* (L.) Pierre (syn. *Millettia pinnata*), seed lectin (PPSL) was purified conventionally by ammonium-sulfate precipitation followed by size exclusion chromatography. The further lectin was physicochemically characterized by CD, fluorescence spectroscopy, mass spectroscopy and isothermal calorimetry. Hemagglutination studies with various mono and disaccharides showed specificity towards galactose. This specificity was reaffirmed by isothermal studies with appreciable thermodynamic parameters. Lectins have tremendous diagnostic applications. They are used as second-generation drug delivery systems.

Keywords: Drug delivery, Indian Beech tree, Karanja, Lectin, Lectin-carbohydrate interactions, Hemagglutination, Isothermal calorimetry, *Millettia pinnata*, Nitrogen fixing tree, Pongame oil tree, Pongamia seed lectin

Pongamia pinnata (L.) Pierre (syn. *Milletti pinnata*) is a deciduous rustic legume of subtropical regions. As a legume, it fixes nitrogen into the soil. It is frequently planted as a windbreak between fields on farms. Biodiesel is extracted from Pongamia seed oil by a binary step acid and alkali-catalyzed trans-esterification of free fatty acids[1]. With its anti-inflammatory, antiplasmodial, antinociceptive, anti-hyperglycemic, anti-lipid peroxidative, antidiarrheal, anticancer, anti-hyperammonemic, antioxidant, anti-convulsant, cytotoxic and immunomodulatory properties, Pongamia is extensively used in the traditional Indian medicine system as a curative for several ailments. Most of these functions are due to chemical compounds, such as karanjin, pongamol and cycloartane (triterpene); however, cytotoxic, immune-modulatory and apoptotic effects are chiefly attributed to lectins[2].

Lectins are ubiquitous in nature and are found in bacteria, fungi, invertebrates, plants and animals. They are carbohydrate-binding proteins that are highly specific for sugar moieties of other macro-molecules[3]. They are also known as hemagglutinins. The sugar-protein interactions are non-covalent, reversible and highly specific[4]. Lectin interactions are very weak with dissociation constants in the millimolar range[5]. Lectins are used as cancer diagnostic markers. Almost all oncogenic biomarkers presently used in the clinical setup are glycoproteins, which have either structurally transformed or eccentric sugar moieties. Amongst various other post-translational modifications, glycosylation is crucial in oncogenic changes, as they are vehemently involved in cancer progression and metastasis. Glycosylation changes associated with cancer include sialylation, fucosylation, increased GlcNAc-branching of N-glycans. Hence, lectin-based approaches are in vogue to screen novel cancer biomarkers[6]. Further, lectins possess antiviral potential and are known to avert HIV infection and also have shown inhibitory activities against coronaviruses[7]. The legume lectins are one of the most extensively characterized families of plant proteins. Typically, legume lectins contain two or four identical or nonidentical subunits of 25-30 kDa each of about 250-300 amino acids with one or two N-linked oligosaccharides. Each subunit ideally contains a carbohydrate-binding site, a firmly bound Ca^{2+} and a transition metal ion, usually Mn^{2+}. About 20% of amino acid residues in legume lectins

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are constant and another 20% being similar renders their subunit structures nearly superimposable, irrespective of sugar specificity[8].

Advances in X-ray crystallography have shed light on lectin-ligand interactions, providing the basis for protein interactions with natural ligands. Typically, lectins exhibit fastidious specificities for di, tri and tetra- saccharides with higher association constants than monosaccharides. Although amino acid residues are conserved, the heterogeneity of sugar-binding stems from other amino acid residues in the combining pocket. In spite of having highly conserved primary and tertiary protein structures, the lectins display an array of heterogeneity in their carbohydrate specificity, post-translational modifications and quaternary structure. Although more than 20 legume lectins have been cloned and the three-dimensional structures of 8 have been precisely ascertained, there is no substantial framework to enumerate the basis of the occurrence of these diverse properties and linkages amongst them. Assembled genomic and proteomic data of the Pongamia plant is currently unavailable in the literature[9]. Therefore, in the present study, we explored purifying a Pongamia seed lectin (PPSL) using simple conventional methods for high yield. Detailed studies based on the biophysical and functional aspects of PPSL were undertaken using the purified protein.

Materials and Methods

Purification of lectin

Seeds of *Pongamia pinnata* (2 kg) were obtained from a local market and the sample was validated by the department of studies in Botany from the University of Mysore. The seeds were ground and defatted by extraction with n-hexane. The defatted seed meal was extracted in 0.5 M NaCl for 2 h at 25°C. The extract was centrifuged at 15346×g at 25°C for 30 min and the supernatant was precipitated with salt at 100% saturation of ammonium sulfate. The supernatant was extensively dialyzed against 30 mM Tris-HCl pH 8.0 and 300 mM NaCl for 48 h. The extract was concentrated and further fractionated by gel filtration chromatography AKTA (GE Healthcare, Little Chalfont, UK Life Sciences). The purified protein was analyzed on SDS-PAGE as described earlier[10].

Gel filtration

Size exclusion chromatography was performed using the AKTA protein purification system (GE Healthcare Life Science)[11]. Gel filtration chromatography was carried out using a Superdex S-200 column with a bed volume of 24 mL. The column was equilibrated with 30 mM Tris-HCl pH 8.0, 200 mM NaCl, at a flow rate of 0.30 mL min^{-1}. The column was calibrated using thyroglobulin (670 kDa), gamma globulin (158 kDa), Ovalbumin (44 kDa), myoglobin (17 kDa) and vitamin B12 (BioRad 151-1901).

Circular dichroism

CD experiments were performed on the JASCO J-810 spectrophotometer using a quartz cuvette with a sample volume of 300 µL and a path length of 1 mm. Far UV (190-250 nm) spectrum was collected. All spectra were collected at a 3-nm bandwidth and a 4s response time. Three replicates were performed for each spectrum collected. These recorded replicates were averaged and corrected for buffer spectrum.CD spectra of protein at concentration of 0.2 mg mL^{-1} was recorded in 200 mM NaCl, 30 mM Tris-HCl buffer pH 8.0. The spectra were averaged, smoothened with a Savitzky-Golay filter and the CD readings obtained in mdeg were converted into molar ellipticity ([θ]), deg cm^2dmol^{-1} using the R tool software. The spectrum revealed more propensity for beta strands and agrees with other well characterized lectins.

Fluorescence spectroscopy

The intrinsic fluorescence of the protein was measured using a Perkin-Elmer LS-55 luminescence spectrofluorimeter and emission spectra were recorded from 250 to 400 nm on excitation at 280 nm. The excitation and emission slits were set at 2.5 and 3.5 nm, respectively with a scan speed of 120 nm min^{-1}. Data from scans were averaged and corrected for buffer background.

Mass spectrometry

Peptide samples in acetonitrile/water without TFA were subjected to LC MS analysis on a Q-TOF Maxis Impact mass spectrometer (Bruker Daltonics, Germany), utilizing standard electrospray ionization (ESI) source annexed to an Agilent (Santa Clara, CA, United States of America) HPLC system accommodated with a reverse-phase C8 column. Acetonitrile (ACN) and water gradients (5% ACN to 95% ACN) in 60 min with a flow rate of 0.2 ml min^{-1} were used. Mass was detected in the m/z range of 100-3000 Da. Calibration was accomplished with an Agilent tune mix (m/z 332-2721). Data were analyzed using Bruker (Newark, DE, United States of America)

data analysis software 4.1. Some spectra were also acquired on an HCT ultra ion-trap spectrometer (Bruker Daltonics) fitted with an Agilent HPLC system. Both Q-TOF and ion-trap mass spectrometers were used for MS/MS experiments.

Dynamic light scattering

Dynamic light scattering measures the translational diffusion coefficient of macromolecules in solution. The homogeneity of a protein in the solution can be assessed by DLS and hence it is extensively used for examining the suitability of the protein sample for crystallization. DLS measurements were performed on Spectrosize 300, Xtal instrument using a 20 µL cuvette, with a data acquisition duration of 10 s for each of the 100 readings. Protein concentration of 6 mg mL^{-1} in 30 mM Tris-HCl pH 8.0 and 200 mM NaCl was used.

Hemagglutination and hemagglutination inhibition assay

Hemagglutination assay and inhibition assay were performed as described in Surolia *et al*.[12] Lectin and blood was stored at $4C^0$ before the commencement of the experiment. Lectin in PBS was subjected to 2-fold serial dilutions in a round-bottomed ELISA plate and an equal amount of 4% rabbit erythrocyte suspension in PBS was added. The mixture was incubated at room temperature for 2 h and agglutination was visualized by eyeball examination. The hemagglutination inhibition assay was carried out with different sugars in a similar manner as the hemagglutination assay[12].

Isothermal titration calorimetry

Titrations were carried out using a Malvern Microcal PEAQ-ITC calorimeter at 298 K. 0.28 mL of the degassed protein solution was carefully added to the sample cell, ensuring that no air bubbles were trapped, and was then equilibrated to the appropriate preset temperature. The ligand solution loaded in the injection syringe was added to the sample cell as a series of 20 injections (aliquots of 2 µL) separated by 2 min intervals with constant stirring at 750 revs min^{-1}.

Results

Protein purification

A fairly pure, thick band corresponding to 21 kDa was visualized on the SDS-PAGE gel (Fig 1A), same band was visible in the 100% ammonium sulfate supernatant fraction (Fig. 1B). This result encouraged us to carry out purification by the method cited in the materials and methods section. The yield of the protein was appreciable and was approximately 2 g kg^{-1} of pulverized seed meal.

Physicochemical characterization of PPSL

SDS PAGE of the purified protein (Fig. 1B) showed that it has a subunit mass of 21.64 kDa. The subunit mass was confirmed by ESI-MS (Fig. 1C). It depicts a subunit mass of 21.64 kDa. Purified protein was resolved on Superdex 200 column and its oligomeric state was ascertained by the methods described in the methods section Along with a major peak corresponding to 21.64 kDa, another peak corresponding to the molecular mass of 129 kDa was observed suggesting that PPSL could be a homo hexamer (Fig. 2A). he fluorescence emission spectrum revealed the exposed tryptophan and tyrosine residues at the surface (Fig. 2B). The Mean residual ellipticity of the lectin was found to be 2.5×10^3 deg cm^2dmol^{-1} (Fig. 2C) The mono-dispersed phase of the protein was confirmed by dynamic light scattering studies (Fig. 3C).

A dynamic light scattering experiment was carried out to ascertain the aggregation and mono or polydispersed phase of the protein. As per the

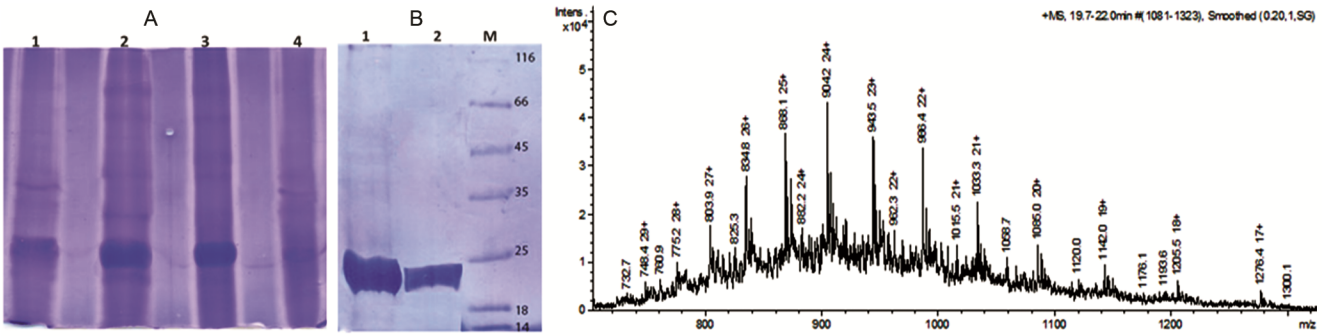

Fig. 1 — Purification of *Pongamia* seed lectin. (A)10% SDS-PAGE crude extract in water and 0.5M NaCl. Lane 1 and 4 100-µg protein extracted in water. Lane 2 and 3 100-µg protein extracted in 0.5M NaCl;(B) Lane 1-ammonium sulphate precipitated dialysed protein, Lane 2-purified protein, Lane3-Marker; and (C) ESI-MS of intact PPSL protein of subunit mass 21.64 kDa

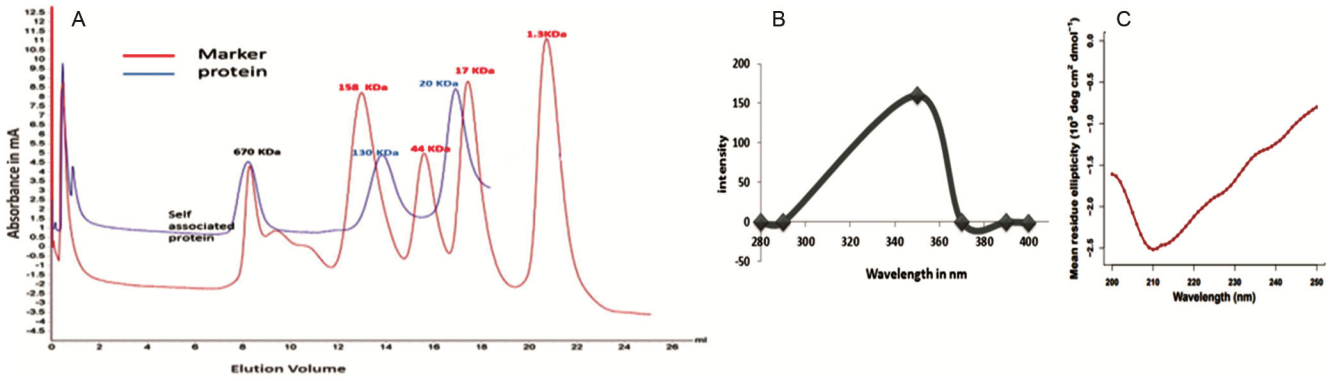

Fig. 2 — (A) Analytical gel filtration profile of PPSL with a marker;(B) Fluorescence Spectrum, Tryptophan and Tyrosine emission spectrum; and (C) Far UV CD Spectrum of PPSL

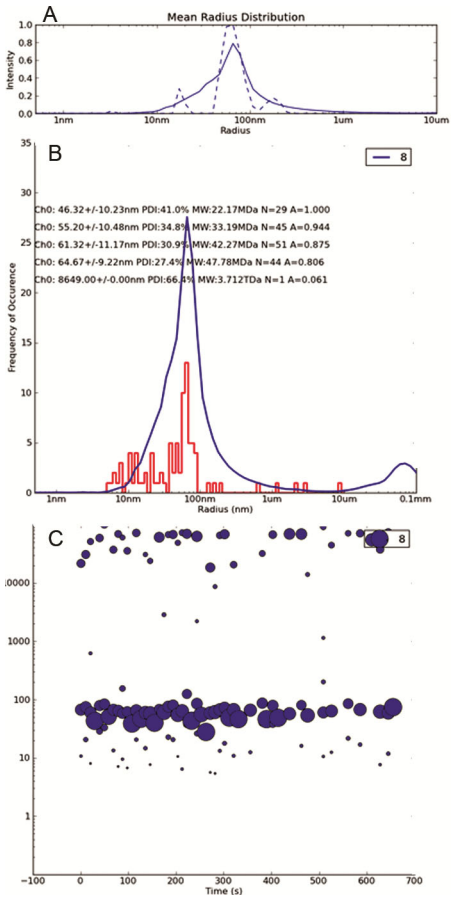

Fig. 3 — (A) DLS mean radius distribution of *Pongamia*; (B) DLS Histogram of PPSL; and (C) DLS radius plot of PPSL

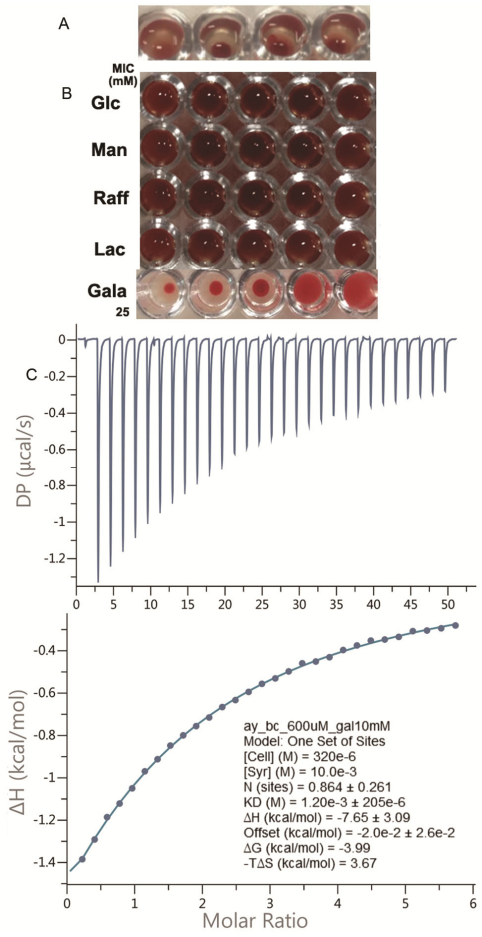

Fig. 4 — Functional characterization of PPSL from *Pongamia* (A) Hemagglutination assay; (B) Hemagglutination inhibition assay of PPSL; and (C) Upper panel shows ITC results upon the interaction of PPSL with galactose (raw data). The lower panel shows plots of heat released as a function of ligand concentration for the titration shown in raw data. Solid lines represent the best least square fits for the data obtained.

dynamic light scattering data, particle size is 100 nm (Fig. 3). The mean radius of the aggregated protein was found to be 100nm (Fig 3A). This data is depicted in the form of a histogram in (Fig 3B). Functional characterization of PPSL: it agglutinated RBCs (Fig. 4A). Hemagglutination studies with glucose, mannose, lactose, rhamnose, raffinose and galactose confirmed the specificity of sugar. It is a galactose binding lectin with a minimum inhibitory concentration of 25 mM (Fig. 4B). ITC reassured the specificity of sugar with thermodynamic parameters

with KD of $1.20e^{-3}$M, enthalpy change (ΔH)-7.65 kcal mol^{-1} and free energy change (ΔG) −3.99 kcal mol^{-1} and -TΔS 3.67 kcal mol^{-1} (Fig. 4C).

Discussion

Purification of PPSL

Purification of the lectin was carried out by the protocol described in the materials and methods section. Protein was extracted in water and 0.5 M NaCl. In Figs 1A lanes 1 and 4 correspond to protein extraction in water whereas lanes 2 and 3 correspond to protein extraction in 0.5 M NaCl on 10% SDS-PAGE. The band intensities on SDS-PAGE encouraged us to solubilize protein in salt. A homogenous band corresponding to 21 kDa was visualized on the SDS- PAGE gel (Fig. 1B) with appreciable yield[13,14].

Physicochemical characterization of PPSL

The purified protein has a subunit mass of 21 kDa on SDS-PAGE. The subunit mass was corroborated by ESI-MS (Fig. 1C). A major peak corresponding to a subunit mass of 21.64 kDa was recorded. Purified protein was loaded on the Superdex 200 column and its oligomeric status was confirmed by the methods described in the methods section. The major peak of protein at 21.6 kDa was recorded. Besides this major peak, another peak corresponding to the molecular mass of 129 kDa was observed indicating that PPSL could be a homo hexamer (Fig. 2A). Typically, legume lectins display oligomerization and this physical feature capacitate functional adroitness in terms of saccharide binding[15]. The monomers of lectin oligomers are bestowed with vital characteristics which are in congruence with carbohydrate-binding.

Oligomerization has a direct influence on carbohydrate-binding efficacy. Oligomerization of sugar-binding domains is of vital significance to produce multivalency for lectins in order to establish multiple sugar-protein interactions[16]. Few carbohydrate-binding domains are di or trivalent (ricin B domain)[17]. Generally, legume lectin domains are monovalent. Oligomerization of protomers is a salient feature of all plant lectin families. However, radical differences are seen with respect to the mode of association of monomers as far as structure, type and numbers are concerned. Indeed, oligomerization stabilizes lectins and mandates essential hallmarks of binding sites manifesting their pivotal biological functions. Self–association is a reiterating feature of a huge number of plant lectins[18]. Each monomer has a single carbohydrate recognizing niche. Hence, oligomerization is obligatory in some of the lectins to create a multi arena platform to have multitudinal valencies potentiating aggressive cell agglutination. However, the size exclusion profile indicated that the lectin tends to undergo self-association and aggregation. At low concentrations of protein (2 mg mL^{-1}) monomeric, hexameric and aggregated forms were noticed in the profile (Fig. 2A). There is a likelihood of equilibrium between monomeric and hexameric forms but the biologically active form could not be ascertained precisely. This was also confirmed by dynamic light scattering experiments.

Such huge size is attributed to protein aggregates or self-associated polymers which agrees with the results reported for some of the legume lectins. Peanut agglutinin is a 110 kDa tetramer of subunit mass of 27 kDa. Dynamic light scattering data confirmed the mono-dispersed phase of protein (Fig. 3C). The secondary structure of purified PPSL was estimated by CD spectral analysis. PPSL showed a CD spectrum typical of globular well-folded proteins with a minimum at 218 nm (Fig. 2C). K2 D2 software analysis of the spectrum revealed that the PPSL lectin has 37% β-sheets and 11% α-helix. These values are comparable to those of other legume lectins which have high β-sheet content[9]. The CD spectrum revealed more propensities for beta-sheets with mean residual ellipticity of 2.5×10^3 deg cm^2dmol^{-1}. Lectins are known to contain approximately 2% tryptophan by weight. The intrinsic fluorescence emission spectrum of purified PPSL obtained upon excitation at 280 nm is shown in (Fig. 2B). As evident, the emission maximum was at 343 nm suggesting some of the tryptophan and tyrosine residues could be external which accounts for the total fluorescence of the protein. The genomic data of the plant is unavailable currently in the database which bottlenecks the protein sequencing.

Functional characterization of PPSL

Lectins exhibit multifarious valencies and interact either with aqueous phase or membrane-anchored sugars glycoproteins or glycolipids of cells. These reactions are highly reversible[19]. This feature earmarks lectins for simple hemagglutination tests. Hemagglutination assays are highly sensitive. It is a semi-quantitative assessment method of establishing the sugar-binding specificity of lectin. Lectins agglutinate red blood cells by recognizing sugars on the cell surface. Hemagglutination can be visualized at even nanogram concentrations of proteins. Hence,

the hemagglutination assay is a golden standard for the confirmation of lectins. The lectin was serially diluted on 96-well microtitre plates by adding 4% RBCs in 1:24 dilution in PBS. Rabbit blood was used for agglutination studies. Agglutination was monitored by eyeball examination. Like other lectins, PPSL could hemagglutinate the RBCs (Fig. 4 A and B). The large particle size of PPSL could be responsible for the agglutination of RBCs.

Hemagglutination inhibition assays were performed with different monosaccharides and disaccharides. Hemagglutination is inhibited by the sugars that specifically bind to lectin, validating the identity of protein as lectin. The hemagglutination inhibition studies carried out with monosaccharides like glucose, mannose and galactose for PPSL was comparable (Fig. 4B) and the agglutination was inhibited by galactose with a minimum inhibition concentration (MIC) of 25 mM (Fig. 4B). Hemagglutination studies with a panel of other sugars like GlcNAc and GalNAc were not promising, and hence, the data is not reported.

Isothermal calorimetry (ITC) studies on PPSL

Hemagglutination indicates the binding of lectin to specific sugars but the affinity of binding and binding dynamics cannot be assessed. Binding dynamics give insights into the thermodynamic parameters like binding constants, enthalpy change, entropy change, free energy change, and a number of binding sites. These parameters are very crucial in understanding the mechanism of lectin-sugar interactions[19]. Thermodynamic parameters can be precisely established through ITC studies. Hemagglutination studies clearly established the galactose binding specificity of the lectin. Hence, ITC studies were carried out using galactose sugar to understand the energetics of lectin-carbohydrate interactions. Lectin-sugar interactions *per se* are very weak with low binding constants. Results of a conventional ITC experiment carried out at 298 K, involving the addition of 10 mM of galactose to 600 µM of PPSL solution are shown in Fig. 4C. Monophonic decrease in the heat liberated with the addition of ligand indicates that PPSL probably has one binding site. It is obvious that detectable binding at normal concentrations occurred unambiguously with K_D of $1.20e^{-3}$M, enthalpy change (ΔH)-7.65 kcal $mol^{-1}$and free energy change (ΔG) −3.99 kcal mol^{-1} and -TΔS 3.67 kcal mol^{-1}. The reaction is exothermic. ITC studies with disaccharides and trisaccharides were not promising and unyielding. Upon examination of thermodynamic parameters, it is apparent that the binding of PPSL to galactose is enthalpy driven[20-24]. Substantially plant lectin-sugar interactions are enthalpy driven. This inference is in corroboration with former findings on a number of plant lectin-sugar interactions[25-27]. However, interaction studies with other disaccharides were not fruitful.

Conclusion

The above study describes a simple and easy method of *Pongamia pinnata* seed lectin (PPSL) purification and its elementary biophysical and functional characterization. Lectins play a pivotal role in recognition at the cellular and molecular level. Further in-depth studies on lectin-sugar interactions would be required for exploring the use of PPSL for biomedical applications.

Conflicts of interest

The authors declare no competing interests.

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