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A Chito-specific, Adenine Binding Agglutinin from *Benincasa hispida* Shows High Structural and Functional Stability

Ruby Singh[1], Sushama M. Gaikwad[1] and C. G. Suresh[1*]

[1]Division of Biochemical Sciences, CSIR-National Chemical Laboratory, Pune – 411008, India.

Authors' contributions

This work was carried out in collaboration between all authors. Author CGS has defined the problem. Author SMG has planned the experiments, analyzed and interpreted the results. Author RS has performed the experiments, analyzed the data discussing with authors SMG and CGS. Author RS also prepared the first draft of the manuscript. Author CGS has checked the analysis and edited the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To purify and characterize a novel chito-specific lectin from *Benincasa hispida* fruit.
Study Design: Protein purification and characterization using biochemical and biophysical techniques under various denaturing conditions.
Place and Duration of Study: Biochemical Sciences Division, CSIR-National Chemical Laboratory, Pune, India, from July 2013 to Jan 2015.
Methodology: *Benincasa hispida* fruit was used as lectin source and purified using chitin affinity and gel filtration chromatographic techniques. The hemagglutinating activity was detected with rabbit erythrocytes. Conformational studies were carried out using steady-state fluorescence, Circular dichroism and FTIR spectroscopic methods.
Results: The protein exists as a homodimer of 34 kDa as determined using gel filtration chromatography and MALDI-TOF/TOF and the subunit was 17 kDa as estimated from SDS-PAGE.

**Corresponding author: E-mail: cg.suresh@ncl.res.in;*

Circular dichroism spectroscopic studies showed that *Bh*L has high content of β-sheets (40%). The fluorimetric titrations with chitobiose and chitotriose sugars yielded the association constants K_a as 1.2 x10^2 M^{-1} and 1x10^4 M^{-1} at 37ºC, respectively, indicating high affinity of the lectin towards the latter sugar. Lectin also bound adenine with estimated binding constant 1 x 10^4 M^{-1} implying its physiological role in plants. The native protein has total six Trp residues out of which, two are exposed on the surface and are in electropositive environment as revealed by fluorescence quenching with KI. The lectin exhibited thermostability upto 80°C retaining 50% of hemagglutination activity and showed fairly compact and stable structure as observed in the far-UV CD spectra. Also, the lectin remained active even after incubating in 6M GDn-HCl for 24 h, or at extreme pH or in 50% organic solvents.

Conclusion: The present study revealed that the chito-specific lectin purified from *B. hispida* has novel structural and functional stability at higher temperature, in a broad pH range, and also in the presence of higher concentrations of chemical denaturants. Binding to adenine indicates its importance in plant physiology.

Keywords: Benincasa hispida; chito-oligospecific lectin; thermostability; guanidine hydrochloride; adenine binding; conformational transitions.

ABBREVIATIONS

BhL, Benincasa hispida lectin; GlcNAc, N-acetyl glucosamine; GDn-HCl, Guanidine hydrochloride; CD, Circular dichroism; DMSO, Dimethyl sulfoxide; ANS, 1-anilino-8-napthalene sulfonic acid; ACN, Acetonitrile; MRE, Mean residual ellipticity.

1. INTRODUCTION

Plant extracts possess hemagglutination activity due to the presence of lectins, which are carbohydrate-binding non-catalytic proteins of non-immune origin [1]. Lectins reversibly bind free or conjugated saccharides via multiple binding sites, varying in molecular weight, amino acid composition, structure and requirement of metal ions for sugar binding. Lectins are ubiquitous, present in all organisms including viruses but extensively studied in plants [2]. Due to easy availability and with distinct saccharide specificity, lectins have become an important tool in medical and biological research [3]. Applications of lectins in biotechnology, especially in the treatment of cancer, are extensive owing to their multivalency property [4,5], immunomodulatory function [6] and role as antimicrobials [7]. So, characterization of new lectins opens up many future implications for medical research.

Search for lectins with novel specificity and investigation of their structural characteristics has always been a topic of extensive research for protein chemists. The characterization of few lectins from Cucurbitaceae family has been reported previously. For instance, snake gourd phloem lectin [8] and Cucurbita maxima phloem lectin [9] have been purified from the phloem sap of the plant. During the course of screening of such lectins, a chito-specific lectin was detected in *Benincasa hispida* (Common name: Ashgourd), a member of Cucurbitaceae family, fruit extract. Traditionally, extracts of ashgourd is widely used for the treatment of urinary dysfunction, epilepsy, asthma, coughs, etc. It also possesses anti-inflammatory, antihelmintic and diuretic properties. Further, recent research has also shown the anti-cancer properties of ashgourd [10]. Hence, their presence in a medicinally important plant such as ashgourd makes it worthwhile to characterize their structure-function relation. There have been reports of the presence of other proteins also in ashgourd such as BPLP (*Benincasa* phloem lectin-like protein) [11], α- and β-benincasins possessing antifungal activity [12] and hispin, a ribosome inactivating protein [13].

In this report, we present the detailed analysis of conformational transition studies of chito-specific agglutinin purified from *Benincasa hispida* fruit extract. Chromatographic techniques like chitin-based affinity and gel filtration column was used to purify the lectin. The functional and conformational stability of lectin was examined by its capacity to retain hemagglutinating activity after incubating under various denaturing conditions and simultaneously monitoring fluorescence and CD spectra.

2. MATERIALS AND METHODS

2.1 Materials

Guanidine hydrochloride (GDn-HCl), acrylamide, potassium iodide (KI), 8-anilino-1-naphthalne sulfonic acid (ANS), N-bromosuccinimide (NBS), sugars like N-acetyl-glucosamine (GlcNAc) and its oligomers, chitin (from crab shells for column matrix) were purchased from Sigma, USA. All other reagents of the highest purity were procured from local suppliers. Solutions were prepared in MilliQ water for spectroscopic and CD studies. *Benincasa hispida* fruit was purchased from the local market.

2.2 Hemagglutination Assays

Hemagglutination activity (HA) was determined by a 2-fold serial dilution method [14] using 3% rabbit erythrocytes in 20 mM potassium phosphate buffer containing 150 mM NaCl (PBS) (v/v). 50 µl of lectin was serially diluted in PBS, mixed with 50 µl of erythrocytes suspension and agglutination activity was observed after 1 h incubation. The hemagglutination unit (HU) is expressed as the reciprocal of the highest dilution (titre) exhibiting visible agglutination. The specific activity of the agglutinin is expressed as the number of HU mg^{-1} of the protein. HA inhibition was tested using sugars like GlcNAc and its oligomers to determine the saccharide specificity of lectin.

2.3 Extraction and Purification

Benincasa hispida (500 g) was homogenized in deionised (DI) water at 25℃. The slurry was centrifuged at 10,000 rpm for 20 min in a refrigerated centrifuge. The clarified supernatant was kept for binding with activated chitin matrix overnight at 10℃ with continuous stirring. A chitin matrix was packed in a column and washed with DI water to remove unbound protein. The protein of interest was eluted with 50 mM glacial acetic acid and immediately dialyzed against the MilliQ water. Hemagglutination assay was performed at each stage of purification. Protein concentrations were determined according to the method of Lowry [15] using bovine serum albumin (BSA) as standard.

2.4 Molecular Mass Determination

The molecular mass of the protein was determined using Sephacryl S-200 Gel filtration chromatography and MALDI-TOF/TOF. Number of subunits in the lectin were determined by SDS-PAGE [16].

2.5 Tryptophan Estimation

Tryptophan content of the protein was estimated using NBS titration method [17]. The lectin (200 µg ml^{-1}) was titrated with freshly prepared NBS (5mM) in 25 mM citrate phosphate buffer, pH 5.0. Further, NBS aliquots were added to the protein until the OD at 280 nm started to rise. The molar absorption coefficient 5,500 M^{-1} cm^{-1} of the tryptophan modified at 280 nm was considered as standard. To estimate the total number of tryptophans modified in the denatured state, the protein was incubated with 6 M GDn-HCl for 24 h. The native lectin without any reagent was used as control for comparing hemagglutination activity on chemical modification.

2.6 Effect of Temperature, pH and Chemical Denaturants on Lectin Activity and Stability

Thermal effect on lectin activity was determined by incubating the samples at different temperatures from 30 to 100ºC for 30 min. The effect of pH on lectin activity was determined by incubating the protein samples in buffers at various pH (1-12), at room temperature for 24 h. Buffers of concentration 25 mM were used: Glycine-HCl (pH 1.0-3.0), Citrate-phosphate (pH 4.0-5.0), Sodium phosphate (pH 6.0-7.0), Tris–HCl (pH 8.0- 9.0), and Glycine/NaOH (pH 10.0–12.0). Similarly, the effect of denaturing agents such as GDn-HCl was tested by estimating lectin activity in a range of concentrations 0.5-6.0 M of the denaturant. Following the treatment with physical and chemical denaturant, an aliquot (50 µl) was taken and hemagglutination assay was performed as described above and compared that with corresponding untreated samples. Since the treated protein samples were finally assayed at room temperature and under normal assay conditions (pH 7.2, PBS) ultimately the reversibility of the denaturation was examined.

2.7 Effect of Organic Solvents on Lectin Activity

The lectin was incubated with 25-50% (v/v) ethanol, methanol, propanol, dimethyl sulphoxide (DMSO) and acetonitrile (ACN) at pH 7.4 for 24 h. The incubation mixture was sealed to

prevent evaporation of solvents. In each case the residual activity was checked by withdrawing aliquot (50 µl) at regular time intervals and performing the hemagglutination assay.

2.8 Steady-state Fluorescence Spectroscopy

The intrinsic fluorescence of *Bh*L was recorded using a Perkin Elmer Life Sciences LS50 spectrofluorimeter connected to a Julabo F20 water bath. The protein solution (0.03mg ml^{-1}) was excited at 295 nm and emission was measured in the range of wavelengths 310-400 nm setting scanning speed 100 nm min^{-1} and slit width 7 nm for both monochromators. The basic spectrum was taken at 28ºC and the background emission produced by either buffers or denaturants was subtracted for further analysis. To determine thermal unfolding profiles, the protein was incubated at desired temperatures for 5 min before recording the spectra. To see the effect of pH on fluorescence, *Bh*L was incubated in the pH buffers ranging 1-12 for 24 h. Chemical unfolding of lectin was also analyzed by incubating the protein in the presence of GDn-HCl (0-6M) for 16 h at room temperature.

2.9 Tryptophan Decomposition Analysis of Fluorescence Spectra

PFAST program (http://pfast.phys.uri.edu/pfast/) based on SIMS and PHREQ algorithm [18] was used to carry out decomposition analysis of tryptophan fluorescence.

2.10 Solute Quenching Studies

*Bh*L (0.03 mg ml^{-1}), both in native and denatured form, was titrated with stock solutions of 5 M concentration of different quenchers such as acrylamide (neutral quencher), iodide and cesium (charged quenchers) on a Perkin-Elmer LS 50B spectrofluorometer at 28℃. The protein was denatured by incubating the sample with 6 M GDn-HCl overnight at room temperature. Iodide stock solution contained sodium thiosulphate (0.2 M) to suppress tri-iodide formation (I^{-3}). Defined aliquots of quencher were added from stock till final concentration of quencher reached 0.5 M in 2 ml of lectin solution. Volume correction was done before analyzing the data taken at the wavelength corresponding to maximum emission [19]. All quenching data were analysed using Stern-Volmer equation (Eq. 1) to estimate Ksv and modified Stern-Volmer equation (Eq. 2) to determine fraction accessibility of a quencher (f_a) [20]. The following equations were used to analyse the data.

$$F_0 / F_c = 1 + K_{sv}[Q] \quad (1)$$

$$F_0 / \Delta F = f_a^{-1} + (K_q f_a)^{-1} [Q]^{-1} \quad (2)$$

Where F_0 and F_c are the respective fluorescence intensities, in the absence and presence of quencher [Q], Ksv is the Stern-Volmer quenching constant for a given quencher of a lectin and Kq is the corresponding quenching constant.

2.11 Saccharide / Ligand Binding Studies

Sugar binding studies of *Bh*L were carried out using intrinsic fluorescence titrations. To a 2 ml lectin sample (0.03 mg ml^{-1}) dialyzed in 20 mM phosphate buffer, pH 7.4, 3-100 µl aliquots of sugar solutions were added till saturation and the change in fluorescence intensity was measured after excitation at 295 nm. Similarly, ligands such as adenine and its derivatives were identified to quench the fluorescence intensity of protein's fluorophore. A stock of 5 mM adenine in 0.1M HCl was freshly prepared and titration was carried out till saturation after exciting at 280 nm. Binding constant (K_a) was calculated from the following equation [21].

$$\text{Log}[C]_f = -\log[K_a] + \log[(F_0 - F_c) / (F_c - F_\infty)] \quad (3)$$

In the above equation, F_0 is the fluorescence intensity of a free unbound protein whereas F_c is that with bound sugar of concentration [C], F_∞ is the fluorescence intensity upon saturation of all sugar/ligand binding sites. In the plot of log $[(F_0 - F_c) / (F_c - F_\infty)]$ versus log[C], the abscissa intercept gives the dissociation constant (K_d), reciprocal of which gives the association constant (K_a). The free energy parameters for binding were calculated using thermodynamic equation: $\Delta G = -RT \ln(K_a)$.

2.12 Parameter A and Phase Diagram Analysis

In steady state fluorescence, Parameter A can be defined as the ratio of fluorescence intensity at 320 nm to that at 365 nm [22]. Parameter A helps to understand the protein conformational changes under denaturation conditions [23,24]. Phase diagram analysis detects the presence of folding intermediates of the protein. It is constructed by plotting fluorescence intensity at 320 nm vs that at 365 nm at different GDn-HCl concentrations.

2.13 Structural Studies Using FTIR

Fourier Transform infrared (FTIR) spectra were recorded using Bruker Optics ALPHA-E spectrophotometer in the range of 1000-2000 cm^{-1}. The protein concentration used for the scan was 1 mg ml^{-1}.

2.14 Circular Dichroism Studies

Circular dichroism (CD) measurements of *Bh*L were recorded on a JASCO J-815-150S (Jasco, Tokyo, Japan) spectropolarimeter connected to a Peltier Type CD/FL cell circulating water bath. The cuvette for holding protein sample was sealed during high temperature studies to prevent evaporation losses. Far-UV spectra of the lectin (0.15 mg ml^{-1}) was recorded using a rectangular quartz cell of 1 mm path-length in the wavelength range 190-250 nm at a scanning speed of 100 nm min^{-1} with slit width 1 nm and response time 1s. To study the tertiary structure, the near-UV CD spectra was recorded at 1 mg ml^{-1} protein concentration in the range 250-300 nm. Each recorded spectrum was an average of 3 accumulations. Results were analysed after subtracting buffer contributions from the recorded data in terms of mean residual ellipticity (MRE) in deg cm^2 $dmol^{-1}$ defined as:

$$MRE = M\,\theta_\lambda / 10\,d\,c\,r \quad (4)$$

Where M is the molecular weight of protein, θ_λ is observed ellipticity in millidegree, d is cell pathlength in cm, c is protein concentration in mg ml^{-1} and r is the average number of amino acid residues. Secondary structure elements were calculated using CDPro software (http://lamar.colostate.edu/~sreeram/CDPro/main.html) available online. Outcome of CONTINLL showed low NRMSD values.

The thermal effect on the stability of secondary structure was studied by increasing the temperature of the protein sample at the rate of 5℃ min^{-1} within the temperature range of 25-95℃. The ellipiticity was recorded at temperature intervals of 5℃ after 5 min incubation. To study the effect of pH on the secondary structure of *Bh*L, the sample was incubated in buffers of various pH (1-12) for 4- 24 h before recording the spectra. Similarly, *Bh*L was incubated with GDn-HCl (0-4M) and organic solvents (25-50% v/v) at pH 7.4 for 4 h at room temperature. Scans of buffer solutions alone were recorded under identical conditions and subtracted from the lectin spectra for further analysis.

2.15 Hydrophobic Dye Binding

ANS emission spectra were recorded using a steady state fluorimeter in the range of wavelengths 400-550 nm with excitation at 375 nm using slit width of 7 nm. The binding of the dye was analysed by recording the fluorescence of *Bh*L incubated at extreme pH range, high temperature and in the presence of denaturants. 10 µl of 15 mM ANS was mixed with 2 ml of the protein (0.03 mg ml^{-1}). Spectrum of the blank taken with ANS alone in each condition was subtracted from the spectrum of the protein and ANS combined.

2.16 Light Scattering Studies of *Bh*L

Protein aggregation upon thermal denaturation was studied using Rayleigh light-scattering experiments with spectrofluorimeter time drive module. The same wavelength 400 nm was used for both excitation and emission. The excitation slit width was 10 nm and emission slit width was 2.5 nm. Scattering was recorded for 30 s.

3. RESULTS AND DISCUSSION

3.1 Purification of *Benincasa hispida* Lectin

Benincasa hispida lectin was purified to homogeneity using chitin affinity chromatography as a first step followed by gel filtration on Sephacryl S-200 (Fig. 1A). The progress of purification after each step is summarized in Table 1. The final yield of the pure protein was 2.5 mg from 500 g of fresh ashgourd fruit. The purified lectin showed a single band of 17 kDa on SDS-PAGE (Fig. 1B). The native molecular mass was determined to be approximately 35,481 Da (Fig. 1C) from sephacryl S-200 chromatography. When subjected to MALDI-TOF/TOF, the scan showed 2 peaks of Mr 17 kDa and 34 kDa confirming that the lectin exists as a homodimer (Fig. 1D).

3.2 Hemagglutination and Sugar Inhibition Studies

In order to determine the saccharide specificity of the agglutinin, hemagglutination inhibition by several sugars and sugar derivatives was studied. In each case the potential of sugar to inhibit the hemagglutination was measured and tabulated (Table 2). Simple sugars of monosaccharides, disaccharides and acetylated

sugars were ineffective in inhibiting hemagglutination even at very high concentrations. It is clear from the table that N, N', N"- Triacetylchitotriose (10 mM) is a potent inhibitor of the *Bh*L mediated hemagglutination but not N-acetyl glucosamine. This observation prompts us to suggest that sugar binding site for *Bh*L is comparatively larger than any of these lectins reported, multiple interactions between N-acetyl group, and amino acid residues at the binding site could be enhancing the affinity of chitobiose/triose towards the lectin. All glycoproteins showed hemagglutination inhibition. This could be due to the better orientation of the chitobiosyl conformer in these glycoproteins or due to additional interactions with the end residues present on the either side of the chitobiosyl moiety for binding to *Bh*L. This implies that with increase in the chain length and complexity of the sugar the inhibitory power of sugar increases for the lectin. In this study, no other carbohydrate showed similar inhibitory effect, implying that *Bh*L has specificity for N, N',N"- Triacetylchitotriose or sugar with higher chain length. This similarity of binding chitotriose was similar to binding property of other cucurbitaceous lectins from *Trichosanthes anguina* [8], *Cucurbita maxima* [9] and *Luffa acutangula* [25].

3.3 Influence of Organic Solvents on the Stability of Lectin Structure

The organic solvent tolerance of *Bh*L was examined as the efforts were being taken on standardization of the protein crystallization conditions where different solvents are used. *Bh*L showed good tolerance towards high concentration of organic solvents. Incubating the lectin in methanol for 24 h did not affect the hemagglutination activity, partial stability was observed in the presence of acetonitrile,

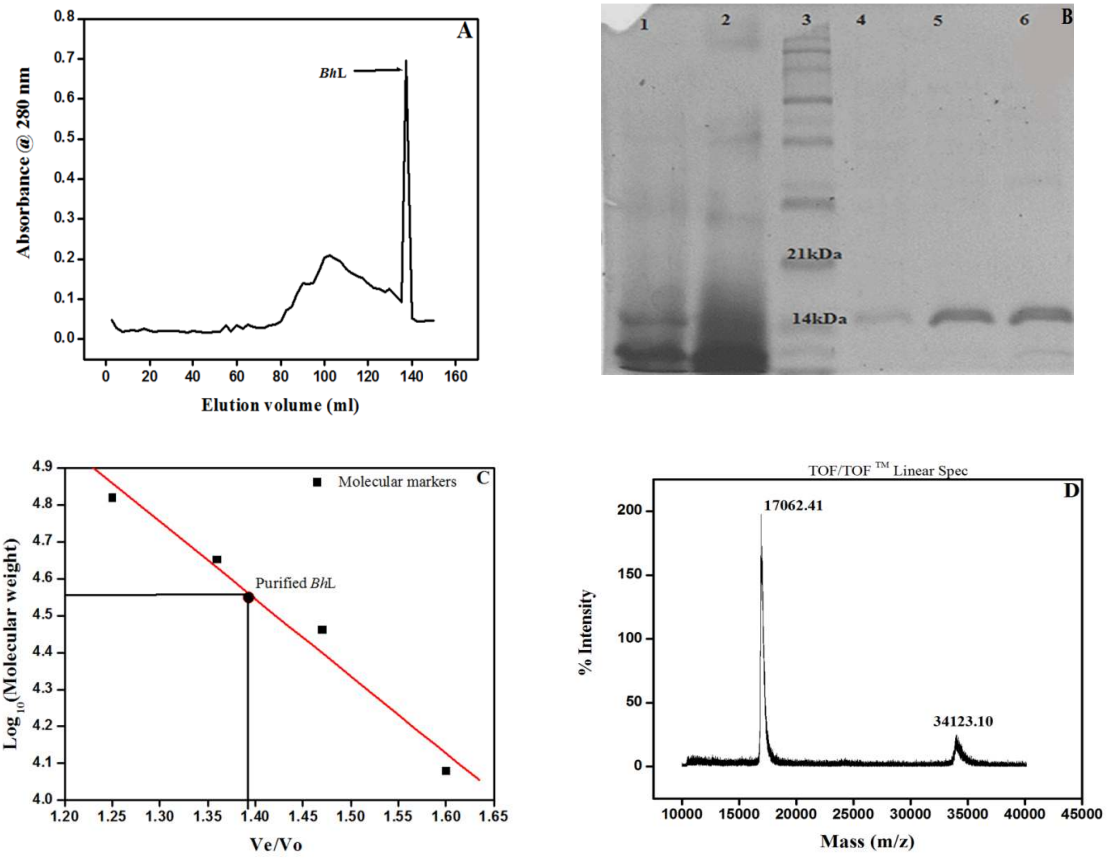

Fig. 1. Purification and molecular mass estimation of *Bh*L. A) Purification profile of lectin on gel filtration column (S-200). B) Purity check on 12 % SDS-PAGE. Lane 1: Chitin affinity column fraction; 2- loaded on sephacryl S-200 column; 3-Protein molecular marker;4-6- Pure *Bh*L. C) Plot of Molecular mass vs Ve/Vo for the estimation of native *Bh*L. D) MALDI-TOF/TOF spectra

while the protein was hardly stable in ethanol, propanol and DMSO (all 50% v/v). Far-UV CD spectra also showed no significant change in secondary structure of the lectin in presence of methanol, propanol and acetonitrile whereas complete loss of structure was seen in the presence of ethanol and DMSO (all 50% v/v) (Fig. 2). The alteration of the structure in presence of propanol was not favorable for the activity of the protein.

Table 1. Summary of purification of *Benincasa hispida* lectin

Purification step	Total protein (mg)[a]	Total activity (titre x ml)	Specific activity (HU mg^{-1})[b]	Purification (fold)[c]	Yield (%)
Aqueous crude extract (From 500 g)	728	62400	86	1.0	100
Affinity chromatography (Chitin)	10.5	8400	800	9.3	13.5
Gel filtration (Sephacryl S-200)	2.6	2800	1000	12.0	4.5

[a] Crude extract of protein from 500 g of Ashgourd fruit, [b] Specific activity: Ratio of hemagglutination unit divided by total protein concentration(mg ml^{-1}), [c] Purification fold: the ratio of minimum concentration of crude protein able to show hemagglutination and that of the protein fraction purified at each step

Table 2. Carbohydrate inhibition of *Benincasa hispida* lectin mediated hemagglutination

S. no	Sugar	Minimum concentrations needed to inhibit the hemagglutination (µg ml^{-1})
1.	Glucosamine	NA*
2.	N-acetyl glucosamine	NA
3.	N, N',N"- Triacetylchitotriose	48
4.	Fetuin	625
5.	Ovalbumin	1250
6.	Bovine submaxillary mucin	78
7.	Casein	780
8.	Thyroglobulin	40
9.	Rabbit serum	ND"

NA: No agglutination, ND": Not determined*

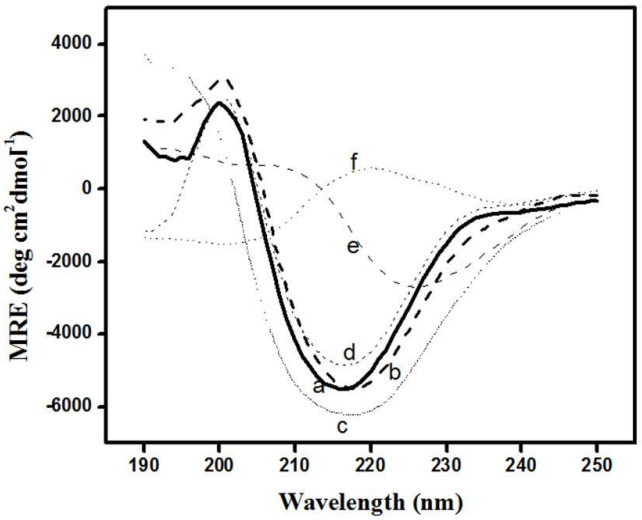

Fig. 2. Far-UV CD spectra of *Bh*L treated with 50% of organic solvent after 4 h; a-control, b- methanol, c- propanol, d-acetonitrile e-ethanol and f-DMSO

3.4 Structural Analysis

3.4.1 Steady state fluorescence and CD study

The intrinsic fluorescence of native *Bh*L gave λ_{max} of 349 nm indicating polar environment of tryptophan residues (Fig. 3A). Total of six tryptophans were estimated through NBS titration of native and denatured protein, where two tryptophans are present on the surface and four are buried. PFAST decomposition analysis of the steady state fluorescence spectrum revealed Class II (100%) tryptophan conformer. Class II represents hydrogen bond between fluorophores and structured water molecules in the protein [26].

The far-UV CD spectrum of the lectin was recorded at 28ºC (Fig. 3B). The minimum negative ellipticity wavelength of the scan was 218 nm indicating high β-sheet content of the protein. The calculation of secondary structure elements from Far-UV data, by CONTINLL program of CDPro analysis showed α-helix: 5.5%, β-sheet: 40.5%, turns: 21.6% and random coil: 32.5% (NRMSD = 0.042). Thus, *Bh*L possesses high β-sheet content and several turns as well as unordered elements found similar in structure to SGPL and PPL [8,27]. The signal in the near-UV CD spectrum showed presence of ordered structure (Fig. 3C).

3.4.2 Structure analysis using FTIR

FTIR spectrum of *Bh*L (1 mg ml^{-1}) showed prominent transmittance minima at 1640 cm^{-1} corresponding to β-sheet (amide I band) and 1698 cm^{-1} for β-turns (amide I band). Interestingly, minima was also observed at 1545 cm^{-1} corresponding to α-helix (amide II) and at 1278 cm^{-1} belonging to unordered structure (amide III) (Fig. 3D) [28]. This very well correlated with CD analysis.

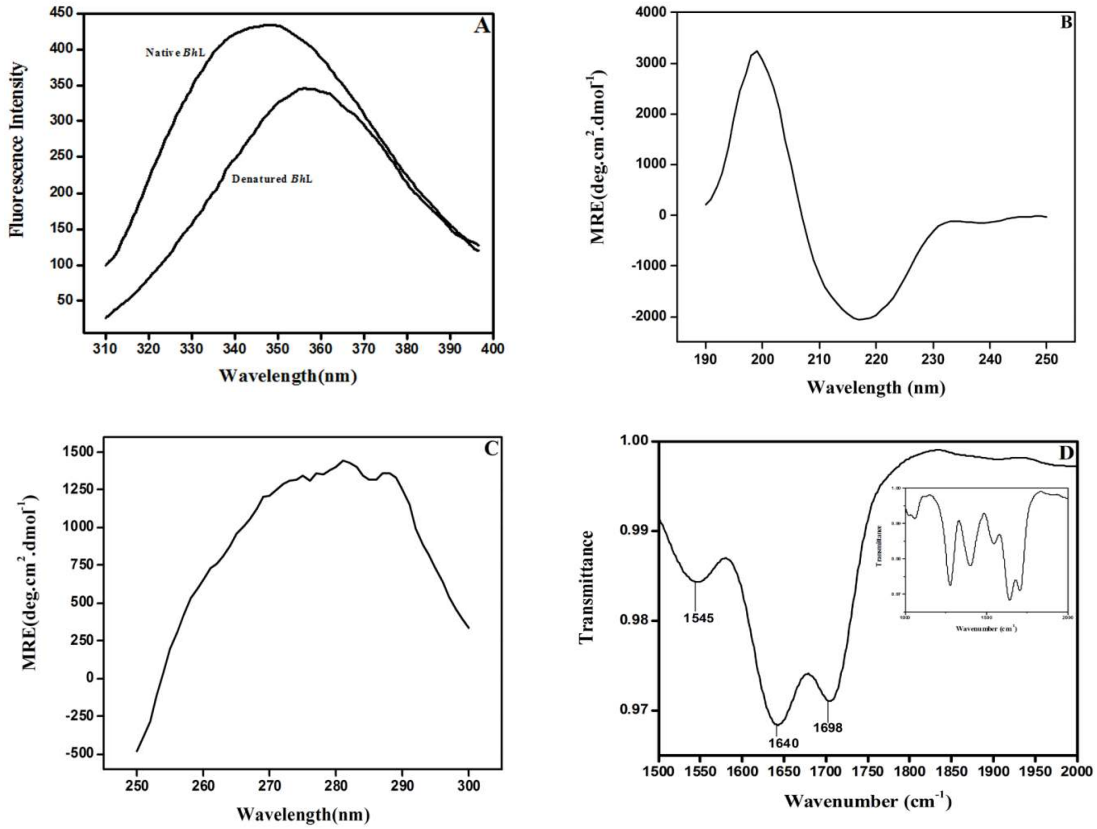

Fig. 3. Biophysical characterization of *Bh*L. (A) Intrinsic fluorescence spectra of native (λ_{max} at 349 nm) and denatured (λ_{max} at 356 nm) *Bh*L (30 µg ml^{-1} at pH7 28℃) (B) Far-UV CD spectrum (150 µg ml^{-1} at pH 7.0). (C) Near-UV CD spectra of *Bh*L (1mg ml^{-1}). (D) FTIR spectrum of *Bh*L in amide I band region (1.5 mg ml^{-1} at pH7.0). Inset shows spectrum over range of 1500-2000 cm^{-1}

3.5 Analysis of Steady State Fluorescence Quenching

Topological information about BhL structure was studied by solute quenching of intrinsic fluorescence of tryptophan present in BhL. The data obtained was analyzed by the Stern-Volmer as well as modified Stern-Volmer equation. Quenching with acrylamide gave linear Stern-Volmer plot (Supplementary Fig. S1A) in case of native BhL and showed upward curvature with denatured lectin, indicating presence of both static and collisional conformers of tryptophan. The Stern-Volmer constant Ksv for acrylamide was 8.04 M^{-1} while that for iodide is 2.74 M^{-1} for native lectin. Cesium showed no quenching, implying electropositive environment near tryptophan residues. The modified Stern-Volmer plot (Supplementary Fig. S1B) indicated significant increase in the fractional accessibility of tryptophans for KI after denaturation indicating change in the conformation of the protein. Full accessibility was not observed even after denaturation of the protein for the neutral quencher acrylamide (Table 3) indicating presence of some residual structure.

3.6 Fluorimetric Analysis of Sugar/Ligand Binding

3.6.1 Sugar binding

Specific sugar/ligand binding to lectins might vary tryptophan microenvironment by fluorescence enhancement [29]. These changes in the fluorescence intensity are exploited to estimate the binding constants of various sugars and ligands. Although *Bh*L is chito-oligosaccharide-specific, titration of the lectin with glucosamine and N-Acetyl glucosamine did not show any significant change in the fluorescence intensity. However, titration with N,N'-Diacetylchitobiose and N,N',N''-Triacetylchitoriose resulted in 23% and 37% enhancement, in the fluorescence intensity, respectively with no change in the emission maxima. From the $F_0/\Delta F$ vs 1/C and double logarithmic plots for sugars (Fig. 4A, B), the binding constants (K_a) were calculated and the one for chitotriose ($K_a = 1.0 \times 10^4\ M^{-1}$) was 83 fold more than that of chitobiose ($K_a = 1.2 \times 10^2\ M^{-1}$) indicating extended sugar binding site of *Bh*L. The negative ΔG values indicated spontaneous nature of binding (Table 4).

Table 3. Summary of parameters obtained from stern–volmer and modified stern–volmer analysis of the intrinsic fluorescence quenching of *Bh*L with different quenchers

Quencher and samples	Ksv (M^{-1})	F_a
Acrylamide		
Native	8.04	0.76
Denatured with 6M GDn-HCl	8.42	0.82
KI		
Native	2.74	0.48
Denatured with 6M GDn-HCl	3.79	0.63

Table 4. Binding constants, K_a, obtained for various chitooligosaccharides at room temperature with *Bh*L and the corresponding Gibb's free energy values

S. no	Sugar/Ligand	K_a (M^{-1})	-ΔG kJ mol^{-1}
1	Gluocosamine	NB	-
2	N-Acetyl Gluocosamine	NB	-
3	N,N'-Diacetylchitobiose	$1.2x10^2$	17.4
4	N,N',N''-Triacetylchitoriose	$1.0x10^4$	24.0
5	Adenine	$1.0x10^4$	ND

NB: No binding, ND: Not determined

3.6.2 Adenine binding

Adenine quenched the fluorescence intensity along with a red shift in the λ_{max} indicating binding as well as denaturation of the protein. The estimated binding constant for adenine to bind to *Bh*L was found to be $1 \times 10^4\ M^{-1}$. Legume lectins are known to bind adenine effectively implying its physiological importance in these plants. For example, lima bean lectin from *Phaseolus lunatus* showed maximum affinity towards adenine ($K_a = 8.3 \times 10^4\ M^{-1}$) [30] whereas winged bean lectin has K_a close to $1.5 \times 10^4\ M^{-1}$ [31]. Adenine is a hydrophobic molecule and binds to lectin at sites different from saccharide binding site and acts as plant growth regulator [32,33]. As far as we know, this is the first report of any chito-oligosaccharide-specific lectin binding to adenine.

3.7 Hydrophobic Dye Binding

Native *Bh*L showed no binding of ANS at extreme pH conditions, during thermal and chemical denaturantion (data not shown)

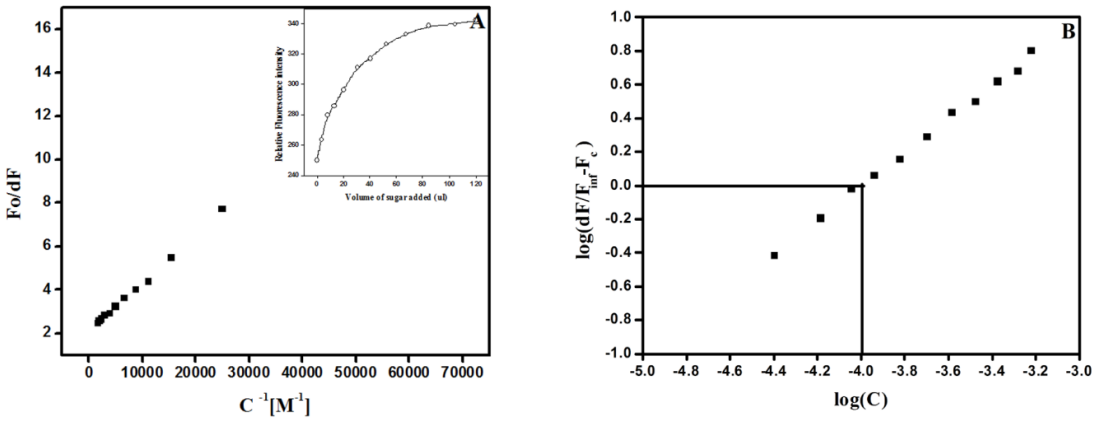

Fig. 4. Plots for the calculation of binding constants for the sugars for binding to *Bh*L (0.03 mg ml^{-1}) (A) Fo/dF vs C^{-1} (B) Log (dF/Finf-Fc) vs log (C)

indicating the absence of any hydrophobic patches coming on the surface of the lectin. May be due to this, the lectin is highly soluble with no propensity for aggregation.

3.8 Conformational Transitions

3.8.1 pH induced changes

3.8.1.1 Effect of pH on hemagglutination activity

The lectin was found to be highly stable in the broad pH range (1-12) retaining full hemagglutination activity when incubated in different pH buffers for 24 h. This indicates the high stability and compactness of the lectin at extreme pH range. Very few plant lectins possessed stability in wide range of pH, for instance, lectin from *Alocasia cucullata* [34] and *Allium sativum* [35].

3.8.1.2 Intrinsic fluorescence

A decrease in fluorescence intensity was observed under highly deprotonated and protonated conditions (Supplementary Fig. S2). No shift in emission maxima was observed at any of the pH.

3.8.1.3 CD analysis

Far-UV CD spectra also showed no change in the secondary structure throughout the pH range upon incubating for 16 h. Thus, no change in α-helix or β-sheets content was observed. Few variations were seen at MRE 200 nm implying slight decrease in ordered structure (Figs. 5A, B). Thus, *Bh*L is stable in all pH conditions with no loss of structure and function.

3.8.2 Thermostability

3.8.2.1 Effect of temperature on lectin activity

The purified *B. hispida* lectin was found to be stable till 60ºC for 1 h when it retained 100% of its hemagglutination activity. A slow decay was observed when the temperature was increased above 60ºC with gradual decrease in hemagglutinating activity at and above 70ºC. Sigmoidal fit (R^2 = 0.95) of the thermal denaturation curve indicated T_m of the protein to be 82.6℃ (Fig. 6A).

3.8.2.2 Intrinsic fluorescence

Fluorescence intensity gradually decreased with increasing temperature with no change in λ_{max}. This could be due to deactivation of the singlet excited state (data not shown). Since, there was no change in emission maximum, it can be assumed that the polarity of tryptophan environment remains unaffected. Light scattering intensity suddenly increased at 95℃ when measured in time drive module, indicating aggregation of the protein. On decreasing the temperature back to 25℃, the protein aggregated further indicating irreversible thermal aggregation (Fig. 6B).

3.8.2.3 CD analysis

No significant change in secondary structure was observed till 80ºC except an increase in content of turns by 7 % (Fig. 6C). The decrease in MRE values at 200 nm implies increase in disorderness as the temperature increases

(Fig. 6B). The sigmoidal fit analysis (R^2 = 0.99) of change in ellipticity at 218 nm gave an estimated T_m value of 82ºC which is close to estimated value using hemagglutination assay. This thermostability feature of *Bh*L is comparable to stabilities of mushroom lectin from *Stropharia rugosoannulata* [36] and seed lectin from *Trichosanthes dioica* [37].

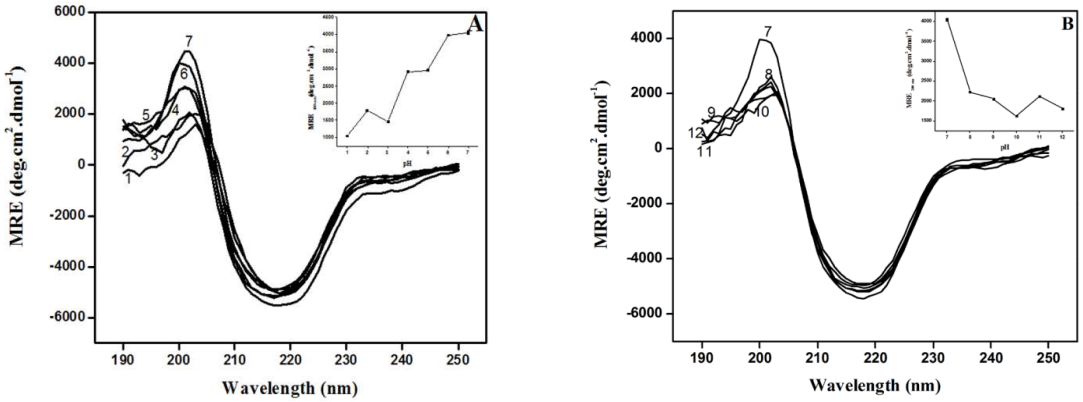

Fig. 5. Far-UV spectra of pH induced changes of *Bh*L (150 µg ml^{-1}) incubated for 24 h. The numbers indicate pH value

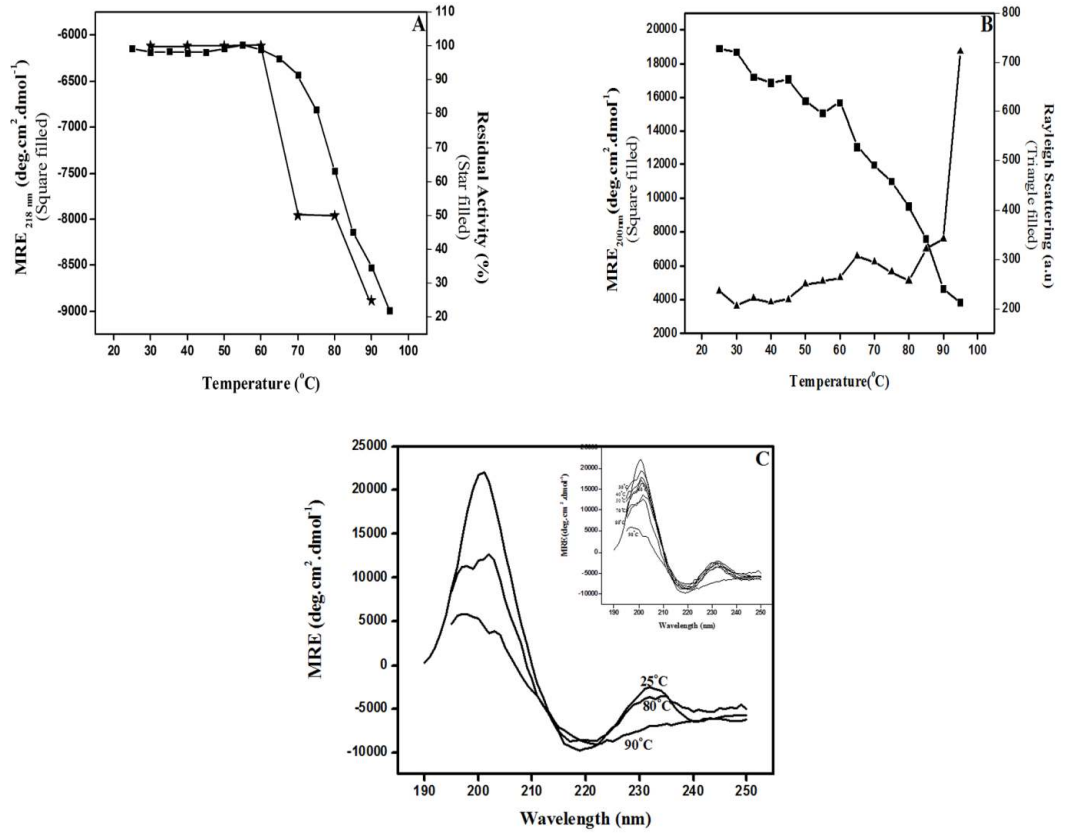

Fig. 6. Thermal denaturation of *Bh*L. (A) Effect of temperature on the hemagglutinating activity and Sigmoidal fit of the change in ellipticity at 218 nm during thermal denaturation. (B) Plot of MRE_{200nm} and Rayleigh scattering intensity versus temperature. (C) Far-UV CD spectra of *Bh*L (150 µg ml^{-1}) incubated at respective temperatures for 5 minutes. The numbers on the spectra indicate temperatures

3.8.3 Chemical denaturation of *Bh*L

3.8.3.1 Effect of denaturants on lectin activity

*Bh*L was incubated with increasing concentrations of GDn-HCl (0-6M) for 24 h and found to be stable retaining 25% hemagglutination activity in the presence of 6M GDn-HCl (Fig. 7A). The denaturant in the lectin gets diluted during serial dilution for hemagglutination; hence deaturation could be reversible to some extent.

3.8.3.2 Intrinsic fluorescence

The fluorescence of *Bh*L showed 6 nm red shift in $\lambda_{max\ in}$ presence of 5M GDn-HCl, indicating increase in polarity of tryptophan residues due to unfolding of the lectin (Fig. 7A). Parameter A (I_{320}/I_{365}) analyses the characteristics of shape and position of tryptophan spectrum, monitoring the sensitivity of lectin in different GDn-HCl concentrations. The present lectin showed multistate unfolding (Fig. 7B). Phase diagram plot (I_{320} vs. I_{365}) provides information regarding existence of intermediates during unfolding process. Two linear portions were observed and lines were plotted intersecting at 3.5 M GDn-HCl implying the presence of an intermediate (Fig. 7C). Interestingly, this intermediate exhibited pronounced secondary structure and hemagglutinating activity in the presence of 3.5 M GDn-HCl.

3.8.3.3 CD analysis

Far-UV CD spectra showed complete loss of secondary structure at 4M GDn-HCl (Fig. 7D). At this denaturant concentration, the lectin retained complete hemagglutination activity as that of the native lectin. This could be due to residual structure remaining in the protein which helps in correct refolding of the protein after diluting the reagent. Alteration in the structure of protein in presence of GDn-HCl, as observed on the basis of fluorescence and far UV CD spectra, could be reversible.

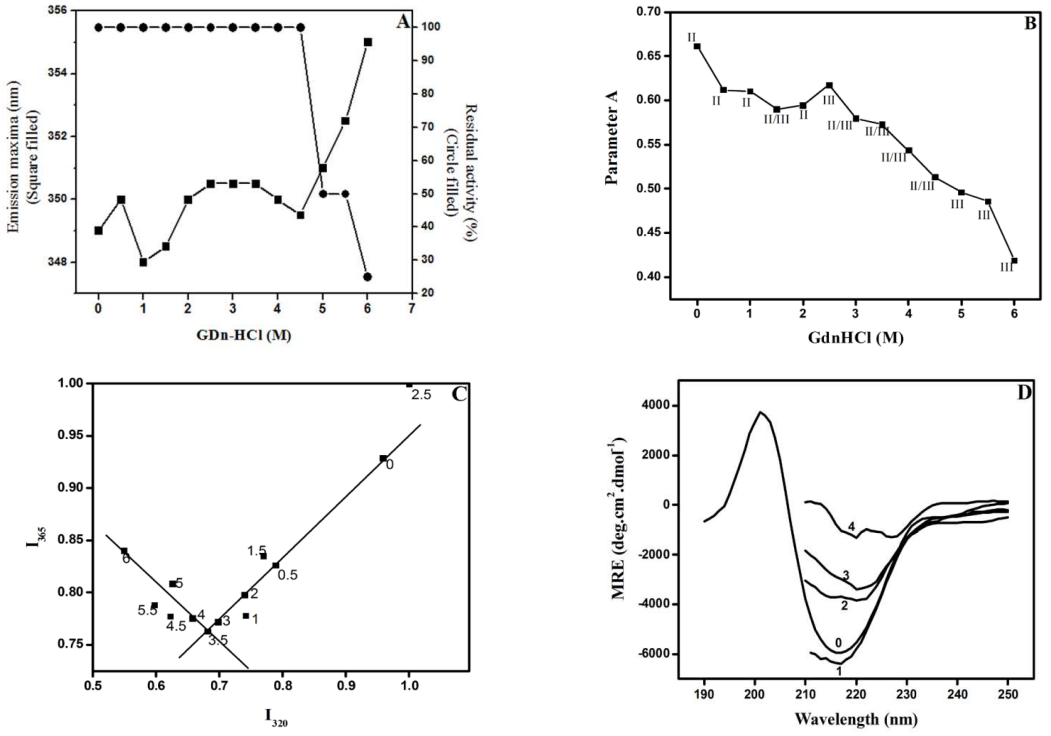

Fig. 7. Effect of GDn-HCl on *Bh*L. (A) λ_{max} shift and hemagglutination activity vs GDn-HCl concentration. Lectin was incubated with required GDn-HCl concentration, 20 mM Phosphate buffer, pH 7.4 for 16 h at room temperature (B) Parameter A analysis showing tryptophan conformer classes. (C) Phase diagram. The number indicates the GDn-HCl concentrations. (D) Effect of GDn-HCl on *Bh*L structure. The protein sample was incubated for 4 hours in respective denaturant buffers. The numbers on the spectra indicate GDn-HCl concentration

4. CONCLUSION

Our investigations on *Bh*L, a novel chito-specific fruit lectin provides better understanding of the structural and functional aspects of the lectins. The lectin is very stable in terms of structure and function 1) at high temperature, 2) under extreme pH conditions 3) in presence of GDn-HCl, and 4) in an organic solvent like methanol. The lectin seems to possess a compact structure and does not get easily unfolded at applied harsh conditions. Binding affinity of *Bh*L towards chito oligosaccharides does establish the potential applicability of the lectin. Its adenine binding property might be correlated to physiological function of *Bh*L in plant.

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COMPETING INTERESTS

The authors certify that they have no conflict of interests with or involvement in any organization or entity with any financial interest in the subject matter or materials discussed in this manuscript.

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