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Fluorescence and circular dichroism studies on the accessibility of tryptophan residues and unfolding of a jacalin-related α-D-galactose-specific lectin from mulberry (*Morus indica*)

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ABSTRACT

MLGL (Mulberry Latex Galactose-specific Lectin) is an α-D-galactose binding lectin isolated from the latex of mulberry (*Morus indica*) tree and contains two tryptophan residues in each of its subunits. The fluorescence emission maximum of native MLGL seen at 326 nm shifts to 350 nm upon incubation with 6 M guanidinium thiocyanate (Gdn.SCN), suggesting that the tryptophans are located inside the hydrophobic core of the protein and become fully exposed upon denaturation. Fluorescence quenching studies revealed that the neutral acrylamide exhibits the highest quenching, with ~33% of total fluorescence in the native protein being quenched at a quencher concentration of 0.5 M, whereas iodide (~24%) and cesium (~4%) ions showed significantly lower quenching. With the denatured protein, acrylamide quenching involves both dynamic and static processes as evident from an upward curving Stern-Volmer plot. Time-resolved fluorescence studies showed two lifetime components of 3.7 ns and 1.3 ns for the native protein, while three lifetime components were observed for the denatured protein. MLGL showed high resistance to urea (up to 8 M) and guanidine hydrochloride (up to 6 M), whereas treatment with 6 M Gdn.SCN completely denatured the protein, via a broad sigmoidal transition with a transition midpoint at ~3.75 M. Circular dichroism studies and hemagglutination assays showed that the secondary and tertiary structures as well as lectin activity of MLGL were unaffected up to 70 °C. Additionally, pH dependent studies showed that the secondary structure of MLGL is unaltered in the pH range 6.2 to 8.5, but a decrease in lectin activity is observed (~50%) at pH 6.2.

1. Introduction

Lectins are a diverse group of proteins which can recognize specific carbohydrate structures in a non-catalytic and reversible manner [1]. The binding to specific carbohydrate molecules is mediated by various non-covalent interactions, e.g., H-bonding, hydrophobic interactions, van der Waals' forces, metal coordination bonds as well as by water molecules [2]. Lectin-carbohydrate interactions are highly specific but the strength of these interactions is relatively weak with a binding constant in the 10^3–10^4 M^{-1} range for monosaccharides. Nevertheless, these proteins show high affinity and specificity for oligosaccharide structures of cell-surface glycoconjugates thereby agglutinating various types of cells [3,4]. Besides their binding to carbohydrates on cell surfaces, their interaction with different non-carbohydrate, predominantly hydrophobic molecules such as phytohormones (e.g. indole acetic acid, gibberellic acid and abscisic acid) and porphyrins may also be relevant from a physiological standpoint [5]. Lectins are ubiquitously present in all kinds of organisms and have various applications in biomedical and biological research owing to their ability to recognize specific sugars. These are, for instance, in targeting tumor markers, in affinity chromatography for the purification of glycoconjugates or oligosaccharides, in new techniques such as lectin blotting and microarray technology as well as in developing lectin-based biosensors [6].

In recent work, we reported the purification of an α-D-galactose specific lectin (MLGL) from the latex of mulberry (*Morus indica*) and characterized it in terms of its amino acid sequence, secondary structure and thermal stability [7]. MLGL belongs to the family of jacalin-related lectins (JRLs), a unique family of plant lectins which shows high affinity towards the tumor-associated T-antigen, Galβ1-3GalNAc [8,9]. MLGL is a glycoprotein with a subunit mass of 18 kDa and each of its four subunits has two polypeptide chains, a longer chain with 133 amino acids and a shorter chain with 20–24 amino acids

Abbreviations: CD, circular dichroism; DSC, differential scanning calorimetry; Gdn.HCl, guanidine hydrochloride; Gdn.SCN, guanidinium thiocyanate; JRL, jacalin-related lectins; MLGL, mulberry latex galactose-specific lectin; MDCK cells, Madine-Darby canine kidney cells; MCF-7, Michigan cancer foundation-7; MeαGal, methyl-α-D-galactopyranoside

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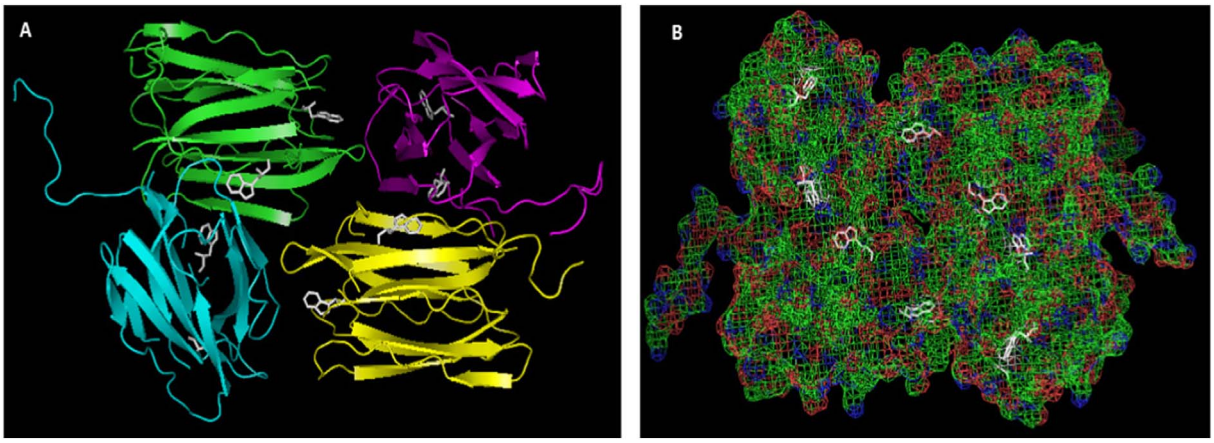

Fig. 1. A 3-dimensional model of MLGL (Uniprot accession number – C0HK14) generated using homology-modeling server MODELLER [10]. Jacalin (PDB code - 1JAC) has been used as the template. (A) A cartoon representation and (B) a mesh representation of four subunits of MLGL. Tryptophans are shown as white sticks.

which are non-covalently linked with each other. The longer chain contains a pauci-mannose [$GlcNAc_2(Fuc)Man_3(Xyl)$] or complex-type [$GlcNAc_2(Fuc)Man_3(Xyl)GlcNAc(Fuc)Gal$] glycan covalently attached to Asn_{21}. A 3-dimensional model of MLGL was built using the homology-modeling server MODELLER as shown in Fig. 1. This primarily β-sheet-rich lectin is thermally quite stable and shows a denaturation temperature of 77.6 °C. Moreover, the high cytotoxicity exhibited by MLGL towards MDCK and MCF-7 cell lines led us to speculate that this lectin might have a role to play in the protection of the plant against phytophagous insects or higher animals [7].

MLGL has two tryptophan residues in each of its subunits, Trp_{123} in the heavier chain and Trp_{18} in the lighter chain. To investigate the exposure, accessibility and microenvironment of these tryptophans and their possible involvement in ligand binding, we have performed steady-state fluorescence quenching studies employing neutral, anionic and cationic quenchers as well as fluorescence life time measurements. Tryptophans located on the protein surface and those buried inside the hydrophobic core are not accessible to the quenchers to the same extent. Furthermore, a neutral quencher can penetrate much better into the structure of a protein than an ionic quencher due to the hydrophobic effect. Therefore, quenching of tryptophan fluorescence by neutral and ionic quenchers can yield valuable information about the structure of the protein [11,12]. Additionally, the unfolding behavior of MLGL was investigated by monitoring changes in the tryptophan emission characteristics in the presence of Gdn.HCl, Gdn.SCN and urea in order to assess the stability of the protein in the presence of these chaotropic agents.

In previous work, the thermal stability of MLGL was investigated by differential scanning calorimetry [7]. In this study, circular dichroism (CD) spectroscopy was employed to observe temperature-induced changes in its tertiary and secondary structures as well as the effect of pH on the stability of MLGL. Furthermore, hemagglutinations assays were performed at different temperatures and pH to assess its lectin activity under various conditions.

2. Materials and Methods

2.1. Materials

Fresh latex was obtained from the mulberry plantation located in the campus of University of Hyderabad. Guanidine hydrochloride, urea, guanidine thiocyanate, acrylamide and potassium iodide were obtained from Sigma (St. Louis, MO, USA). Cesium chloride was obtained from Cisco Research Laboratories (Mumbai, India).

2.2. Purification of MLGL

Collection of mulberry latex and subsequent purification of MLGL were performed as described earlier [7]. In brief, latex collected from mulberry leaf stems into TBS (10 mM Tris-HCl buffer containing 150 mM NaCl, pH 8.0) was stored at − 20 °C for 24 h and then centrifuged at 10,000 rpm after thawing at room temperature. The crude extract was then passed through a cross-linked guar-gum column [13,14] and the bound protein was eluted with 0.2 M galactose [7]. The protein was dialyzed extensively against PBS (20 mM phosphate, 150 mM NaCl, pH 7.4) buffer.

2.3. Steady-state Fluorescence Spectroscopy

All emission spectra were recorded on a Fluoromax-4 fluorescence spectrometer from Horiba Jobin-Yvon. Slit widths of 3 and 5 nm were used on the excitation and emission monochromators, respectively. Absorption measurements were made using a Shimadzu UV3101PC double beam spectrometer. MLGL samples ($OD_{280} \leq 0.1$) were irradiated with 295 nm light, to selectively excite tryptophan residues of the protein. Emission spectra were recorded above 305 nm. Quenching titrations were performed as described earlier [15]. Briefly, small aliquots were added from a 5 M stock solution of the quencher to the protein sample and fluorescence spectra were recorded after each addition. Fluorescence intensities were corrected for volume changes before further analysis of the quenching data. To correct the fluorescence intensity values for inner filter effect, the absorbance at excitation and emission wavelength of the sample were measured. Correction was made by using the following equation [11]:

$$F_{corr} = F_{obs} \times \text{antilog}\,[(OD_{ex} + OD_{em})/2]$$

Here, F_{corr} and F_{obs} are the corrected and observed fluorescence intensities, respectively and OD_{ex} and OD_{em} are the absorbances measured at excitation and emission wavelengths, respectively. For chemical denaturation studies, MLGL samples were incubated overnight with various concentrations of denaturants (urea, Gdn.HCl and Gdn.SCN) and the fluorescence spectra were recorded (λ_{ex} = 295 nm) thereafter.

2.4. Fluorescence Lifetime Measurements

Time-resolved fluorescence measurements were performed using a time-correlated single-photon counting (TCSPC) spectrometer from Horiba Jobin Yvon IBH (Glasgow, UK). A Delta diode laser source (λ_{exc} = 285 nm) was used for excitation and a Hamamatasu photomultiplier (R3809U-50) was used to detect the fluorescence. The instrument response function, which was limited by the fwhm (full