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# Tryptophan exposure and accessibility in the chitooligosaccharide-specific phloem exudate lectin from pumpkin (*Cucurbita maxima*). A fluorescence study

Akkaladevi Narahari, Musti J. Swamy *

*School of Chemistry, University of Hyderabad, Hyderabad 500 046, India*

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ABSTRACT

The exposure and accessibility of the tryptophan residues in the chitooligosaccharide-specific pumpkin (*Cucurbita maxima*) phloem exudate lectin (PPL) have been investigated by fluorescence spectroscopy. The emission $\lambda_{max}$ of native PPL, seen at 338 nm was red-shifted to 348 nm upon denaturation by 6 M Gdn.HCl in the presence of 10 mM β-mercaptoethanol, indicating near complete exposure of the tryptophan residues to the aqueous medium, whereas a blue-shift to 335 nm was observed in the presence of saturating concentrations of chitotriose, suggesting that ligand binding leads to a decrease in the solvent exposure of the tryptophan residues. The extent of quenching was maximum with the neutral molecule, acrylamide whereas the ionic species, iodide and $Cs^+$ led to significantly lower quenching, which could be attributed to the presence of charged amino acid residues in close proximity to some of the tryptophan residues. The Stern–Volmer plot for acrylamide was linear for native PPL and upon ligand binding, but became upward curving upon denaturation, indicating that the quenching occurs via a combination of static and dynamic mechanisms. In time-resolved fluorescence experiments, the decay curves could be best fit to biexponential patterns, for native protein, in the presence of ligand and upon denaturation. In each case both lifetimes systematically decreased with increasing acrylamide concentrations, indicating that quenching occurs predominantly via a dynamic process.



## 1. Introduction

Lectins, the ubiquitous carbohydrate binding proteins of non-immune origin are widely used in the identification, isolation and characterization of glycoconjugates [1]. Certain lectins exhibit blood group specificity while some others can distinguish between normal and tumor cells. Lectins are widely employed in the investigation of carbohydrates perched on cell surfaces and on subcellular organelles [1–3]. Although carbohydrate binding has long been considered to be the characteristic feature of lectins, more recent studies have shown that a number of plant lectins recognize a variety of noncarbohydrate ligands that are primarily hydrophobic in nature [4].

The subject of interest in the present study is the pumpkin phloem lectin (PPL), isolated from the phloem exudate of pumpkin (*Cucurbita maxima*), a widely cultivated member of the Cucurbitaceae family. PPL is a chitooligosaccharide-specific, dimeric protein with a subunit mass of 24 kDa [5]. Sequence analysis of PPL cDNA shows that it is a polypeptide of 218 amino acids with eight tryptophan and six tyrosine residues [6,7] and suggests that the protein may be hydrophobic in nature. Recently, we reported a rapid purification method for PPL using affinity chromatography on chitin as the key step [8]. Circular dichroism spectroscopic studies show that this protein contains 9.7% α-helix, 35.8% β-sheet, 22.5% β-turns and 32.3% unordered structures [8]. DSC studies indicate that PPL is a relatively stable protein and undergoes a cooperative thermal unfolding process centered at ca. 82 °C, which shifts to slightly higher temperatures upon ligand binding.

The fluorescence spectral characteristics of indole side chains of tryptophan residues in a protein are highly sensitive to their environment and can be monitored to obtain information regarding the protein structure and conformation [9–11]. In a protein molecule that has both tryptophan and tyrosine residues, the Trp residues can be excited specifically by irradiating at 295 nm, which allows the study of fluorescence from the Trp residues alone [12].

In the present study we have investigated the tryptophan exposure and environment of PPL in the absence and presence of specific carbohydrate ligand, chitotriose, and upon denaturation with 6 M Gdn.HCl, using steady-state and time-resolved fluorescence quenching measurements as well as red-edge excitation shift (REES) studies. The results obtained indicate that the Trp residues are partially buried in native PPL and are not directly involved in carbohydrate binding. The cationic quencher $Cs^+$ is unable to quench some of the Trp residues even upon denaturation due to repulsion by positive charges on adjacent residues.

* Corresponding author. Tel.: +91 40 2313 4807; fax: +91 40 2301 2460/0145.
*E-mail addresses:* mjssc@uohyd.ernet.in, mjswamy1@gmail.com (M.J. Swamy).
*URL:* http://202.41.85.161/~mjs/ (M.J. Swamy).

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## 2. Materials and methods

### 2.1. Materials

Pumpkin fruits were obtained from local vegetable vendors. Chitin (from crab shells), 2-mecrcaptoethanol, acrylamide, potassium iodide, cesium chloride, and chitooligosaccharides $(GlcNAc)_3$, $(GlcNAc)_4$ and $(GlcNAc)_5$, were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sodium chloride, ammonium sulphate, di-sodium hydrogen phosphate, sodium di-hydrogen phosphate and acetic acid were obtained from local suppliers and were of the highest purity available.

### 2.2. Pumpkin phloem exudate lectin (PPL)

The lectin from the pumpkin phloem exudate has been purified by affinity chromatography on chitin as described in [8]. The affinity eluted lectin was dialyzed thoroughly against 20 mM phosphate buffer, pH 7.4, containing 150 mM sodium chloride and 10 mM β-mercaptoethanol (PBS-βME). The lectin thus obtained gave a single band in SDS–PAGE in the presence of β-mercaptoethanol. Lectin concentration was estimated according to Peterson [13].

### 2.3. Steady-state fluorescence spectroscopy

All emission spectra were recorded on a Spex Fluoromax-3 fluorescence spectrometer. Slit widths of 3 and 5 nm were used on the excitation and emission monochromators, respectively. Lectin samples (~0.1 OD) in PBS-βME were irradiated at 295 nm to selectively excite tryptophan residues of the protein and emission spectra were collected above 305 nm. In fluorescence quenching experiments, small aliquots of the quencher from a 5 M stock solution were added to the protein sample and fluorescence spectra were recorded after each addition. The stock solution of iodide ion contained 0.2 mM sodium thiosulphate to prevent the formation of triodide ($I_3^-$). Fluorescence spectra were corrected for volume changes before further analysis of quenching data. All measurements were performed at room temperature and carried out in duplicate, which showed high reproducibility and the average results have been reported. Red-edge excitation shift (REES) experiments were performed by varying the excitation wavelength between 280 and 307 nm and emission spectra were recorded between 320 and 450 nm [14]. For REES studies the excitation and emission slits were set to 2 and 3 nm, respectively.

In order to correct the fluorescence intensity values from the inner filter effect, the absorbance at emission and excitation wavelengths of the experimental solutions were measured. Correction was made using [9]:

$$F_{corr} = F_{obs} \times \text{antilog}[OD_{ex} + OD_{em}/2] \quad (1)$$

where $F_{obs}$ and $F_{corr}$ are the observed and corrected fluorescence intensities, and $OD_{ex}$ and $OD_{em}$ are the absorption intensities of the solution at the excitation and emission wavelengths, respectively.

### 2.4. Time-resolved fluorescence studies

Fluorescence lifetime measurements were performed on an IBH-5000 single photon counting spectrofluorimeter equipped with a NanoLED excitation source and a cooled microchannel plate photomultiplier tube from Hamamatsu (Model R0839U-50). The time resolution of the spectrometer was ~50 ps. Samples of PPL in PBS-βME with $OD_{280nm} < 0.1$ were exited at 281 nm and emission was monitored at 338 nm for native PPL and at 348 nm for PPL in the presence of 6 M Gdn.HCl. All experiments were performed using excitation and emission slits with a nominal bandpass of 12 nm or less. Lamp profiles were measured at the excitation wavelength using Ludox (colloidal silica) as the scatterer. The signal/noise ratio was optimized by collecting at least 5000 photon counts in the peak channel. The fluorescence decay curves thus obtained were analyzed by a multiexponential iterative fitting program supplied by IBH.

## 3. Results

### 3.1. Quenching of the intrinsic fluorescence emission of PPL

Quenching of the intrinsic fluorescence of PPL was investigated using a neutral quencher (acrylamide), a cationic quencher (cesium ion) and an anionic quencher (iodide ion). Quenching studies were carried out with PPL under native conditions, upon binding of chitooligosaccharides and upon denaturation with 6 M Gdn.HCl. The emission spectra of the native protein recorded in the absence and presence of increasing concentrations of acrylamide, iodide and cesium ion are shown in Fig. 1A, B and C, respectively. In these figures spectrum 1 corresponds to the lectin alone, while the spectra numbered 2–20 in panels A and B and 2–16 in panel C correspond to those recorded in the presence of increasing concentrations of the quencher. The spectra in Fig. 1 also show that

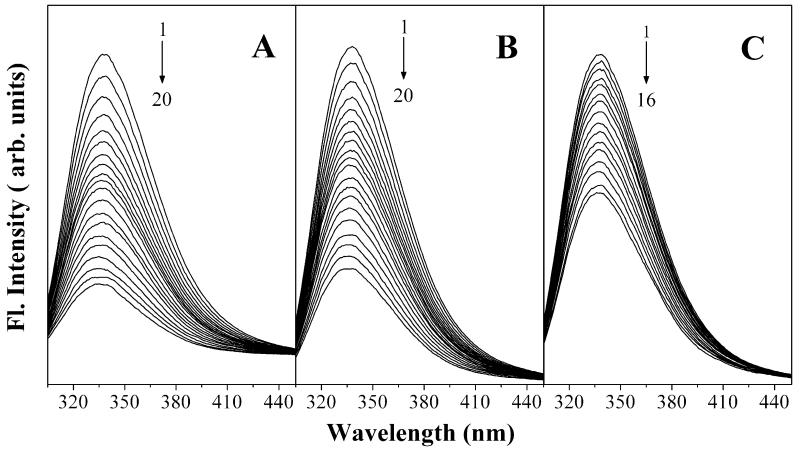


**Fig. 1.** Fluorescence spectra of PPL in absence and presence of various quenchers: (A) acrylamide, (B) iodide ion and (C) cesium ion. In each panel spectrum 1 corresponds to PPL alone and the remaining spectra correspond to those recorded in the presence of increasing concentrations of the quencher (indicated by arrow).