CHAPTER: 3

LIGAND BINDING AND SOLUTE QUENCHING STUDIES OF THE RECOMBINANT LECTIN
SUMMARY

The recombinant chick pea lectin (rCAL) did not recognize any simple mono- or disaccharides, nor any glycoproteins other than fetuin and its asialo triantennary glycan. The binding with asialo fetuin glycan was spontaneous and enthalpically driven as revealed by thermodynamic parameters. The nucleotide sequence of rCAL showed the presence of the hemopexin fold. Binding of rCAL was checked with hemin, spermine, and thiamine using fluorescence spectroscopy. Hemin could strongly bind rCAL confirming the functional presence of the hemopexin domains. rCAL also showed binding to spermine and thiamine. Solute quenching studies showed that the microenvironment of the tryptophan in the lectin was negatively charged. Upon complete denaturation of the protein, the charge density around the tryptophan gets redistributed. The upward curvature obtained for acrylamide quenching was resolved with lifetime fluorescence measurements. The contribution of the static component ($K_s$) was more prominent than that of the collisional component ($K_{sv}$).

3.1. INTRODUCTION

Recognition is a key event in the biological functionality of the lectins. The unique and highly specific sugar-binding property of plant lectins have made these molecules useful probes for glycan detection in carbohydrates and glycoproteins for many years, providing the basis for either biochemical analysis or for development of diagnostic tools in histology, blotting and biosensor applications [1]. Lectins are used as “glycan deciphers” to interpret the carbohydrate structure in living organs and cells. The overall biological activities of the lectins are manifestation of their specificities.

It is essential to understand the mechanism of ligand binding to lectins, in order to facilitate their use as an analytical tool and for better understanding of lectin interaction with cell bound carbohydrates. Determination of association constants with a series of ligands provides considerable insight into the spatial features of the combining site of a lectin. Complementary thermodynamic data offer information on the forces involved in the binding and explain affinity differences encountered.

Since hemagglutination-inhibition studies provide semi-quantitative information, fluorescence spectroscopy has been used to obtain more quantitative information about a lectin’s binding process. The advantage of using fluorimetry in studies of carbohydrate-protein interactions is that the binding can be studied at
equilibrium without physical separation of the bound complex from the free ligand and the protein [2].

The fluorescence of the indole side chains of the tryptophan (trp) residues in a protein is extremely sensitive to its environment, and hence changes which affect the tryptophan environment can cause changes in fluorescence properties of the protein [3]. Such changes in the intrinsic fluorescence of proteins have been used to obtain information regarding structure, specificity and conformation of the proteins [4]. The accessibility of tryptophan in a protein molecule can be measured by use of small molecular weight quenchers that perturb fluorescence [5]. Fluorescence quenching by these molecules proceeds mainly via physical contacts of the quenchers with the fluorophores and hence is directly dependent on the extent to which they can approach the fluorophores in the protein. This, in turn, makes the process extremely dependent on the nature of the solvent, the degree of exposure or burial of the trp residues as well as the nature of the quenchers themselves [6]. In cases, where trp residues are at the ligand-binding site, this technique has been used to study the changes resulting from ligand binding. Lifetime studies of fluorescence decay have similarly been used to obtain information regarding the trp environment and its interaction with various quenchers [7].

The present chapter describes the functional characterization of the recombinant Cicer arietinum lectin (rCAL) with respect to its carbohydrate and ligand binding properties. The binding studies of rCAL with the asialo triantennary glycan from fetuin and with hemin, spermine and thiamine were carried out using fluorescence spectroscopy. Thermodynamic parameters for saccharide binding have also been determined. The microenvironment of the tryptophan (accessibility of trp residue) has been investigated using solute quenching studies.

3.2. MATERIALS

Cultivar BDN 9-3 of chick pea seeds was obtained from Badnapur Agricultural University, Jalna, India. Pronase-E, carboxypeptidase, aminopeptidase and neuraminidase enzymes and hemin, spermine and thiamine were obtained from Sigma-Aldrich, India. Glucose, galactose, mannose, rhamnose, xylose, fucose, raffinose, trehalose, lactose, glucosamine, mannosamine, galactosamine, Methyl-α-glucose, methyl-β-mannose, methyl-α-galactose, N-acetyl-mannosamine, N-acetyl-galactosamine, N-acetyl-D-glucosamine, D-mannitol, D-glucuronic acid, D-
polygalacturonic acid, D-glucose pentaacetate, bovine submaxillary mucin, and fetuin were obtained from Sigma-Aldrich, India. Acrylamide, cesium chloride and potassium iodide as well as all other general chemicals and buffers were procured from Sigma-Aldrich, India. Rabbit blood for the hemagglutination assay was obtained from the animal house of the National Institute of Virology, Pune, India.

3.3. METHODS

3.3.1. Protein Preparation

The recombinant *Cicer arietinum* lectin was purified to homogeneity from the induced cell lysates of *E. coli* as mentioned in Chapter 2. This purified lectin, rCAL, was the subject of the present study.

3.3.2. Preparation of asialo triantennary glycan

Triantennary-N-glycan was prepared from fetuin. One gram of the glycoprotein was dissolved in 100 ml of 20 mM Tris-HCl, pH 7.2 (containing 150 mM NaCl, 0.5% w/v sodium azide), pH 7.2 and digested by adding 50 mg of pronase-E powder at 37 °C for 72 h; 20 mg of pronase-E was added after every 24 h. The digest was lyophilized, dissolved in 5 ml of 100 mM acetic acid, centrifuged (10,000 rpm, 20 min), and the supernatant was collected. The pellet was re-extracted five times in 1 ml of 100 mM acetic acid. Two ml of clear supernatant was chromatographed on Sephadex G-25 column (1.5 x 100 cm), pre-equilibrated with 20 mM acetic acid, and eluted with the same buffer at a flow rate of 20 ml/hour. The fractions (2 ml) were collected and those showing the presence of sugar were pooled and further digested by carboxypeptidase (10 U at pH 7.0 and 25 °C for 24 h) and aminopeptidase (10 U at pH 8.5 and 25 °C for 24 h). The residual peptides were removed by chromatography on a Dowex-50 column (1.5 x 4 cm) in 20 mM acetic acid [8, 9]. The glycopeptides were desialated by incubating the sample with 5 U of neuraminidase in 20 mM Tris-HCl buffer, pH 7.2 at 37 °C for 4 h; the enzyme and sialic acid were removed by successive chromatography on Sephadex G-25 (1.5 x 10 cm) and Dowex-50 as described above. Thus, the N-linked asialo-triantennary glycan was prepared from fetuin.

Protein concentrations were determined according to the method of Lowry *et al* [10] using bovine serum albumin (BSA) as the standard.

3.3.3. Neutral sugar estimation

The sugar solutions (400 μl) were incubated with 400 μl of 5 % (w/v) phenol for 10 min at room temperature. Two ml of sulphuric acid was then added and the
mixture was allowed to cool for 20 min at room temperature. The colour developed was then measured spectrophotometrically at 490 nm. The concentration of the sugar was estimated using D-mannose as the standard [11].

3.3.4. **Erythrocyte preparation**

Rabbit erythrocytes were washed 5 to 6 times with 20 mM potassium phosphate buffer pH 7.2 containing 150 mM NaCl. A 3% (v/v) suspension of the erythrocytes in the above buffer was treated with the enzyme pronase (10 mg/ml) at 37 °C for 1 h, washed 3 times with the same buffer and used for further studies.

3.3.5. **Hemagglutination assays**

Hemagglutination assays were performed in standard microtitre plates by the two-fold serial dilution method. A 50 μl aliquot of the erythrocytes suspension was mixed with 50 μl of serially diluted lectin and agglutination was examined visually after incubation for one hour. A unit of hemagglutination activity (U) is expressed as the reciprocal of the highest dilution (titre) of the lectin that showed complete agglutination. The specific activity of the lectin is defined as the number of hemagglutination units/mg of the protein.

3.3.6. **Hemagglutination inhibition assays**

Hemagglutination inhibition assays were performed similarly, except that serially diluted sugar solutions (25 μl from 0.5 M stock) were pre-incubated for 15 min at 27 °C with 25 μl of the lectin (600 ng). Erythrocyte suspension (50 μl) was then added, mixed and the plates were examined visually after one hour.

3.3.7. **Fluorescence measurements**

Steady-state intrinsic fluorescence measurements were performed on a Perkin Elmer LS-50B luminescence spectrofluorimeter at 25 °C. Samples were excited at 295 nm (1.0 cm cell path length) and the emission spectra were recorded in the range of 310 nm to 400 nm. Slit widths of 7 nm each were set for excitation and emission monochromators and the spectra were recorded at 100 nm/min. The baseline was corrected by subtracting the contribution of the buffer. rCAL sample (150 μg/ml) in 20 mM phosphate buffer, pH 7.2 was placed in a quartz cuvette maintained at desired temperature (± 0.1 °C) by means of a Julabo circulating water bath. The sugar/ligand solution was added in 10-12 aliquots (5 to 10 μl each). A 1.8 mM stock concentration of the asialo triantennary glycan from fetuin was used. The ligands were used in the concentration of 10 μM (for hemin) and 5 mM (for spermine and thiamine). Each spectrum was an average of 5 accumulations. The fluorescence intensity at 343 nm
(λ\text{max} of the lectin) was considered for further analysis. Corrections were also made to compensate the dilution effect upon addition of sugar/ligand to the lectin. At the highest concentration of the sugar/ligand to lectin, volume change was less than 5% of the solution in the cuvette. Each data point was an average of three independent sets of experiments with SD less than 2%.

3.3.8. Ligand-binding data analysis

The association constants were calculated according to the method described by Chipman et al. [12]. The abscissa intercept of the plot of \log [C]f against \log \{(\Delta F)/(F_c–F_\infty)\}, where [C]f is the free ligand concentration, yielded pK_a value for lectin-ligand interaction according to the relationship

\[
\log \left[ F_o - F_c/F_c - F_\infty \right] = \log K_a + \log \left\{ [C]_t - [P]_t (\Delta F/\Delta F_\infty) \right\} \\
\]  

(1)

where F_c is the fluorescence intensity of the lectin at any point during the titration, [P]_t is the total protein concentration, \Delta F_\infty is the change in fluorescence intensity at saturation binding, [C]_t is the total ligand concentration, and [C]_f is the free ligand concentration, given by,

\[
[C]_f = ([C]_t - [P]_t (\Delta F/\Delta F_\infty)) \\
\]  

(2)

Free energy changes of association (\Delta G) were determined by the equation,

\[
\Delta G = -RT \ln K_a \\
\]  

(3)

Temperature dependence of the association constants was used to determine the thermodynamic parameters. Changes in enthalpy (\Delta H) were determined from the Van’t Hoff plots by using the equation,

\[
\ln K_a = (-\Delta H/RT) + \Delta S/R \\
\]  

(4)

where \Delta H is enthalpy change, R is gas constant, \Delta S is entropy change and T is the absolute temperature. The entropy change was obtained from the equation,

\[
\Delta G = \Delta H - T \Delta S \\
\]  

(5)

3.3.9. Solute quenching studies

3.3.9.1. Steady state fluorescence spectroscopy

Fluorescence quenching experiments were carried out by the addition of a small aliquot of acrylamide, potassium iodide (KI) or cesium chloride (CsCl) stock solution (5 M) to the protein solution (180 \mu g/ml) at 25 °C and the fluorescence intensities were determined. Iodide stock solution contained 0.2 M sodium thiosulfate to prevent formation of tri-iodide (I^-3). For quenching studies with denatured protein, rCAL was
incubated with 6 M GdnHCl overnight (16 hours) at 25 °C. Fluorescence intensities were corrected for volume changes before further analysis of quenching data.

The steady-state fluorescence quenching data obtained with all the quenchers used in this study were analyzed by Stern–Volmer (Eq. 6) and modified Stern–Volmer (Eq. 7) equations in order to obtain quantitative quenching parameters [5, 13].

\[
\frac{F_0}{F_c} = 1 + K_{sv} [Q] \\
\frac{F_0}{\Delta F} = f^{-1}a + 1/[K_a f_a(Q)]
\]

where \(F_o\) and \(F_c\) are the relative fluorescence intensities in the absence and presence of the quencher, respectively, \([Q]\) is the quencher concentration, \(K_{sv}\) is Stern–Volmer quenching constant, \(\Delta F = F_o - F_c\) is the change in fluorescence intensity at any point in the quenching titration, \(K_a\) is the quenching constant and \(f_a\) is the fraction of the total fluorophores accessible to the quencher. Equation (7) shows that the slope of a plot of \(F_0/\Delta F\) versus \(1/Q\) (modified Stern–Volmer plot) gives the value of \((K_a f_a)^{-1}\) and its Y-intercept gives the value of \(f^{-1}a\).

### 3.3.9.2. Time resolved fluorescence spectroscopy

Lifetime measurements were carried out on an FLS-920 single photon counting spectrofluorimeter supplied by Edinburgh Instruments. A laser pico second pulsed light emitting diode (model EPLED-295) was used as the excitation source and a Synchronization photomultiplier was used to detect the fluorescence. The diluted Ludox solution was used for measuring Instrument Response Function (IRF). The sample (200 µg/ml) was excited at 295 nm and emission was recorded at 343 nm. Slit widths of 10 nm each were used for the excitation and emission monochromators. The resultant decay curves were analyzed by a Reconvolution fitting program supplied by Edinburgh Instruments.

The static and dynamic quenching components obtained for the denatured lectin with acrylamide quenching were further resolved by fluorescence lifetime measurements using the following equations

\[
\tau_0/\tau = (1 + K_{sv} [Q])
\]

where \(\tau_0\) is the average lifetime in the absence of the quencher and \(\tau\) is the lifetime in the presence of the quencher at a concentration \([Q]\) and

\[
\frac{F_0}{F_c} = (1 + K_{sv} [Q]) (1 + K_s [Q])
\]

where \(K_{sv}\) is the Stern-Volmer (dynamic) quenching constant, \(K_s\) is the static quenching constant and \([Q]\) is the quencher concentration. The dynamic quenching
constant reflects the degree to which the quencher achieves the encounter distance of the fluorophore and can be determined by the fluorescence lifetime measurements according to the equation (9).

3.4. RESULTS AND DISCUSSION

3.4.1. Hemagglutination and hemagglutination inhibition assay

The lectin showed hemagglutination activity only against pronase-treated rabbit erythrocytes. The hemagglutination titre was obtained to be $5 \times 10^3$ hemagglutination units per mg of protein. Simple sugars like glucose, mannose, galactose, rhamnose, xylose, fucose, raffinose, trehalose, lactose, glucosamine, mannosamine, galactosamine, Methyl-α-glucose, methyl-β-mannose, methyl-α-galactose, N-acetylmannosamine, N-acetyl-galactosamine, N-acetyl-D-glucosamine, D-mannitol, D-glucuronic acid, D-polygalacturonic acid, D-glucose pentaacetate etc failed to inhibit the hemagglutinating activity of the lectin. Hemagglutination by rCAL was inhibited by glycoproteins viz. fetuin, asialofetuin, and its asialo-triantennary glycopeptides. The minimum inhibitory concentration for fetuin and its asialo glycan was found to be 32 µg. Lectins recognizing only complex glycans have been reported earlier. For example, the lectin from wild cobra lily, *Arisaema flavum* [14] shows inhibition only with asialo fetuin with an inhibitory concentration of 250 µg/ml.

3.4.2. Ligand binding with Asialo triantennary glycan from Fetuin

Fluorescence titration of rCAL with the N-linked asialo triantennary glycan prepared from fetuin (Figure 3.1(A)) resulted in quenching of the lectin fluorescence (inset, Fig 3.1(B)). The value of $F_\infty$ was derived from the plot shown in Figure 3.1(B). The binding constant $K_a$ of $1.01 \times 10^5$ M$^{-1}$ at 25 °C was determined from the plot of log $[\Delta F/(F_c - F_\infty)]$ versus log $[C]$ (Figure 3.1(C)).

Asialo-triantennary N-linked glycan of Fetuin

Galβ1→4GlcNAcβ1→2Manα1→6
Galβ1→4GlcNAcβ1→4Manα1→3
Galβ1→4GlcNAcβ1

Figure 3.1(A): Structure of the asialotriantennary glycan from fetuin
Figure 3.1(B): The fluorescence change at saturation binding ($F_\infty$) is obtained from the Y-intercept of the plot of $F_o/\Delta F$ versus $1/C$. Inset: Quenching of fluorescence of rCAL by the glycan.

Figure 3.1(C): Plot of $\log \{\Delta F/ (F_c - F_\infty)\}$ versus $\log [C]$. The X-intercept of the plot gives pK$\alpha$ value for the interaction between rCAL and the glycan.

Binding was also checked at 15 °C ($K_a, 1.88 \times 10^5$ M$^{-1}$) and 20 °C ($K_a, 1.59 \times 10^5$ M$^{-1}$). A decrease in the association constant ($K_a$) was observed with increase in
temperature. Thermodynamic parameters were calculated for the glycan binding. Van’t Hoff plots were linear (r > 0.9) in the range of temperatures studied (Figure 3.1(D)).

The change in Gibb’s free energy (ΔG) was -28.56 kJmol⁻¹ indicating binding to be spontaneous; the negative value of change in enthalpy (ΔH) of -43.65 kJmol⁻¹ demonstrated the exothermic nature of the reaction. A negative change in entropy (ΔS) (-50.65 Jmol⁻¹K⁻¹) indicated the formation of more H-bonds between the glycan and the lectin. Few reports are available on lectins showing inhibition against complex glycans, like the lectin IV from *Griffonia simplicifolia* and PHA-L from *P. vulgaris* [15]. These belong to the group termed “complex” with specificity towards N-glycans. A stronger inhibition of rCAL activity by asialo fetuin than that with fetuin was observed. The interference due to sialic acid may be abolished by the action of neuraminidase, resulting in better interaction with rCAL (due to the exposure of the Galβ1→4GlcNAc residue). A similar observation has been made for the lectin from *Fusarium solani*, by Khan *et al* [16] which interacts better with the asialo glycans of fetuin and fibrinogen than with simple galactose residue or its derivatives. The binding in this case is also enthalpically driven.

Lectin-carbohydrate interactions are generally characterized by a low affinity for monovalent ligand. In general, affinity in the milli molar range is observed for lectins binding to monosaccharides. When longer oligosaccharides act as ligands, corresponding to an extended binding site on the lectin surface, increased affinity up to
micromolar range can be observed. Branched complex glycans, due to the clustering effect, offer multiple binding sites leading to several fold increase in the affinity [17].

### 3.4.3. Ligand binding with hemin, spermine and thiamine

As a first step towards investigating its biological role, binding of rCAL with hemin, spermine and thiamine was examined using fluorescence spectroscopy.

Titration of rCAL with hemin at 25 °C resulted in gradual quenching of the fluorescence (Figure 3.2, inset). The slope of the plot of $\log \left[ \frac{\Delta F}{F_c - F} \right]$, versus $\log [C]$ was near unity, hence one binding site per monomer of rCAL was predicted. The lectin bound hemin with an association constant, $K_a = 3.55 \times 10^7 \text{ M}^{-1}$ (Figure 3.3A), indicating high affinity of binding. A similar quenching profile was observed when rCAL was titrated with 5 mM each of spermine and thiamine (Figures 3.3B and 3.3C) at 25 °C. The association constants $K_a$ obtained for spermine and thiamine binding were $1.55 \times 10^4 \text{ M}^{-1}$ and $5.37 \times 10^3 \text{ M}^{-1}$, respectively (Table 3.1), indicating significant affinity for the lectin.

![Figure 3.2](image_url)

**Figure 3.2:** The fluorescence change at saturation binding ($F_c$) is obtained from the Y-intercept of the plot of $F_o/\Delta F$ versus $1/C$. Inset: Representative quenching of fluorescence of rCAL by thiamine.
Figure 3.3: Determination of the association constant for the interaction of rCAL with (A) hemin, (B) spermine, and (C) thiamine from the plot of log $\left(\Delta F/(F_c - F_{\infty})\right)$ versus log $[C]_f$. The X-intercept of the plot gives $pK_a$ value for the interaction. The stock concentrations of the ligands A, B, C were 10µM, 5mM and 5 mM, respectively.
Table 3.1 Summary of ligand binding to rCAL with fluorescence spectroscopy

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$K_a$ (M$^{-1}$)</th>
<th>$\Delta G^*$ (kJmol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemin</td>
<td>$3.55 \times 10^7$</td>
<td>$-43.0727$</td>
</tr>
<tr>
<td>Spermine</td>
<td>$1.55 \times 10^4$</td>
<td>$-23.90509$</td>
</tr>
<tr>
<td>Thiamin</td>
<td>$5.37 \times 10^3$</td>
<td>$-21.27883$</td>
</tr>
</tbody>
</table>

$\Delta G = -RT \ln(K_a)$

3.4.4. Steady-state and Lifetime fluorescence measurements of rCAL

The gene sequence revealed the presence of a single tryptophan (trp) in rCAL. The native protein showed intrinsic fluorescence with maximum emission ($\lambda_{max}$) at 343 nm, suggesting the presence of the single trp in a partially hydrophobic environment (Figure 3.4(A)).

![Intrinsic fluorescence spectrum of rCAL (150 µg/ml)](image)

Figure 3.4(A): Intrinsic fluorescence spectrum of rCAL (150 µg/ml) (straight line: native rCAL, dashed line: rCAL denatured with 6M GdnHCl).

The lifetime of intrinsic emission decay of rCAL was studied in nanosecond domain (Figure 3.4(B)). When fitted into a bi-exponential curve ($\chi^2 < 1.26$), it could be described by two decay components $\tau_1$ and $\tau_2$ with decay times of 1.04 ns (45.40 %) and 4.14 ns (54.60 %) respectively. The average $\tau$ was found to be 2.65 ns. The single trp thus existed as two different conformers at a given time: one with the shorter lifetime lies on the surface of the protein and its fluorescence decays faster, while the longer conformer lies in the interior and decays slowly. The $\lambda_{max}$ at 343 nm is hence the cumulative intrinsic fluorescence of rCAL.
3.4.5. Solute Quenching studies

Acrylamide, being neutral by nature, was found to be the most efficient quencher for intrinsic fluorescence of rCAL in the native state, with a Stern Volmer (K_{sv}) constant of 3.17 M^{-1} (Figure 3.5(A)). 73% of the tryptophan (trp) fluorescence was accessible to it. Among the ionic quenchers, fluorescence could be quenched only by CsCl and not by KI, indicating a negative charge density around the trp. The biphasic curvature obtained for CsCl quenching (Figure 3.5(A)) indicated a heterogeneous ionic environment around the trp with the presence of two conformers – one getting quenched earlier than the other (K_{sv1} = 3.3 M^{-1} and K_{sv2} = 1.02 M^{-1}).

The ionic solute quenching profile was reversed for the denatured protein. The lectin denatured with 6 M GdnHCl could be quenched only by KI and not by CsCl, indicating a reorientation of charge density around the trp from negative to positive. K_{sv} of 5.59 M^{-1} and 70 % accessibility was obtained for KI (Figure 3.5(B)). The trp environment was examined in the partially unfolded state. In the vicinity of 1.75 M
GdnHCl, rCAL was partially unfolded with a $\lambda_{\text{max}}$ of 348 nm (Chapter 4, Figure 4.6 (A)). Even at this stage, the fluorescence quenching with CsCl was very low ($K_{sv} = 0.35 \text{ M}^{-1}$), while that with KI was significant ($K_{sv} = 2.97 \text{ M}^{-1}$) (Figure 3.5(C)).

![Figure 3.5: Stern-Volmer plots for quenching of rCAL.](image)

Accessibility of acrylamide to fluorescence increased to 100% upon denaturation of the lectin with 6 M GdnHCl. An upward curvature was obtained in the Stern Volmer’s Plot, indicating the involvement of both collisional and static components (Figure 3.5(B)). The static mechanism is a consequence of complex formation, while the dynamic mechanism involves collisions with acrylamide during the lifetime of the excited trp [18].

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The average lifetimes were calculated using the following equations [19, 20]:

\[
\tau = \Sigma_i \alpha_i \tau_i / \Sigma_i \alpha_i \quad \text{Eq. 10}
\]

\[
<\tau> = \Sigma_i \alpha_i \tau_i^2 / \Sigma_i \alpha_i \tau_i \quad \text{Eq. 11}
\]

For \( i = 1, 2, 3 \ldots \), and where \( \tau \) and \( <\tau> \) are the average lifetimes obtained by two different approaches and \( \alpha \) is the weighting factor (see Table 3.2).

Table 3.2: The lifetimes of fluorescence decay of denatured rCAL and the corresponding pre-exponential factors along with calculated average lifetimes for acrylamide quenching.

<table>
<thead>
<tr>
<th>Q[M]</th>
<th>( \tau_1 ) (ns)</th>
<th>( \alpha_1 )</th>
<th>( \tau_2 ) (ns)</th>
<th>( \alpha_2 )</th>
<th>( \tau_3 ) (ns)</th>
<th>( \alpha_3 )</th>
<th>( \tau ) (ns)</th>
<th>( &lt;\tau&gt; ) (ns)</th>
<th>( \chi^2 )</th>
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<td>0.000</td>
<td>1.67</td>
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<td>1.21</td>
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<td>0.076</td>
<td>2.87</td>
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<td>0.002</td>
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<td>1.43</td>
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<td>0.58</td>
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<td>0.049</td>
<td>5.01</td>
<td>0.004</td>
<td>1.08</td>
<td>1.66</td>
<td>1.12</td>
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<td>0.165</td>
<td>0.64</td>
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<td>1.72</td>
<td>0.043</td>
<td>4.83</td>
<td>0.004</td>
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<td>1.07</td>
</tr>
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<td>0.018</td>
<td>13.10</td>
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<td>0.002</td>
<td>0.90</td>
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<td>0.72</td>
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<td>2.87</td>
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<td>0.000</td>
<td>0.91</td>
<td>1.31</td>
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</tr>
<tr>
<td>0.305</td>
<td>0.49</td>
<td>0.156</td>
<td>1.72</td>
<td>0.029</td>
<td>5.80</td>
<td>0.002</td>
<td>0.74</td>
<td>1.38</td>
<td>1.07</td>
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<tr>
<td>0.355</td>
<td>0.44</td>
<td>0.176</td>
<td>1.48</td>
<td>0.032</td>
<td>4.96</td>
<td>0.003</td>
<td>0.66</td>
<td>1.27</td>
<td>1.04</td>
</tr>
<tr>
<td>0.405</td>
<td>0.44</td>
<td>0.160</td>
<td>1.23</td>
<td>0.039</td>
<td>4.26</td>
<td>0.004</td>
<td>0.67</td>
<td>1.2</td>
<td>1.01</td>
</tr>
<tr>
<td>0.455</td>
<td>0.47</td>
<td>0.194</td>
<td>1.91</td>
<td>0.020</td>
<td>6.66</td>
<td>0.001</td>
<td>0.63</td>
<td>1.18</td>
<td>1.03</td>
</tr>
<tr>
<td>0.505</td>
<td>0.36</td>
<td>0.188</td>
<td>1.03</td>
<td>0.047</td>
<td>4.23</td>
<td>0.005</td>
<td>0.57</td>
<td>1.19</td>
<td>1.06</td>
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</tbody>
</table>

Using the average lifetimes obtained from the analysis of time resolved fluorescence data (Table 3.2), the value of \( K_{sv} \) for the lectin was obtained as 3.67 M\(^{-1}\) (Figure 3.6) (from earlier equations 8 and 9).

\[
\frac{\tau_0}{\tau} = (1+K_{sv} [Q])
\]

\[
\frac{F_o}{F_c} = (1+K_{sv} [Q]) (1+K_s [Q])
\]

Putting this value in Eq. (9) and plotting a graph of \( (F_o/F_c) / (1+K_{sv}[Q]) \) against [Q], the value of the static quenching constant (\( K_s \)) was found to be 7.52 M\(^{-1}\) (Figure 3.6). The static component hence contributes more to the quenching by acrylamide than the dynamic component. The bimolecular quenching constant, \( k_q \) (calculated as \( k_q = K_{sv} / \tau \)), was found to be 1.39 x 10\(^9\) M\(^{-1}\)s\(^{-1}\). According to Lehrer et al [13], this value of \( k_q \) corresponds to a negatively charged indole-3-acetate moiety; it correlates with our trp microenvironment which is negatively charged in the native state. Lectins showing lack of positive charge density around the tryptophan residues in their native state not been reported yet.
Incorporating the values of $K_{sv}$ and $K_s$ in the expression $(1+K_{sv}[Q])(1+K_s[Q])$, the values obtained were plotted against [Q]. It was observed that the values of $F_0/F_C$ and $(1+K_{sv}[Q])(1+K_s[Q])$ match very well (Figure 3.7).

**Figure 3.6: Acrylamide quenching of denatured rCAL.** Calculation of the collisional ($K_{sv}$) (filled squares) and static ($K_s$) (open squares) quenching constants for the upward curvature obtained with acrylamide for rCAL denatured with 6M GdnHCl.

**Figure 3.7: Coincidence plot for rCAL.** The plot of $F_0/F_C$ and $(1+K_{sv}[Q])(1+K_s[Q])$ against [Q] corresponding to quenching of denatured rCAL with acrylamide (open squares: $F_0/F_C$; closed squares $(1+K_{sv}[Q])(1+K_s[Q])$).
The summary of quenching parameters obtained for rCAL is given in Table 3.3.

<table>
<thead>
<tr>
<th>Quencher</th>
<th>$K_{sv}$ (M$^{-1}$)/ $K_s$ (M$^{-1}$)</th>
<th>$f_a$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acrylamide</strong></td>
<td></td>
<td></td>
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<tr>
<td>Native</td>
<td>3.17</td>
<td>0.73</td>
</tr>
<tr>
<td>6 M GdnHCl- treated</td>
<td>3.67* / 7.52*</td>
<td>1.05</td>
</tr>
<tr>
<td><strong>Cesium chloride</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Native</td>
<td>3.3, 1.02</td>
<td>0.5</td>
</tr>
<tr>
<td>1.75 M GdnHCl-treated</td>
<td>No fluorescence quenching observed</td>
<td></td>
</tr>
<tr>
<td>6 M GdnHCl- treated</td>
<td>No fluorescence quenching observed</td>
<td></td>
</tr>
<tr>
<td><strong>Potassium iodide</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Native</td>
<td>No fluorescence quenching observed</td>
<td></td>
</tr>
<tr>
<td>1.75 M GdnHCl-treated</td>
<td>3.02</td>
<td>0.73</td>
</tr>
<tr>
<td>6 M GdnHCl- treated</td>
<td>5.59</td>
<td>0.70</td>
</tr>
</tbody>
</table>

* Derived from time resolved fluorescence.

Several lectins expressing complex sugar specificity have been reported. Examples include lectins from *Moringa oleifera* [21], *Arisaema curvatum* [22], *Sauromatum venosum* [23], *Acacia constricta* [24], *Scilla campanulata* [25], *Ficus cunia* [26], *Salvia sclerea* [27], and *Glechoma hederocea* [28] etc.

rCAL is an albumin by nature, hence binding properties of homologous plant seed albumins for other ligands were checked for rCAL. It was found that rCAL showed similar binding properties. Similar studies of binding with hemin, spermine or thiamine have not been reported for other lectins, though there are reports of lectins known to bind hydrophobic ligands like adenine [29, 30].
REFERENCES


