Chapter 6

Thermodynamics of the interaction of chitooligosaccharides with snake gourd (Trichosanthes anguina) phloem exudate lectin. Isothermal titration calorimetric and fluorescence spectroscopic studies
Summary

The interaction between snake gourd phloem exudate lectin (SGPL) and chitoooligosaccharides [(GlcNAc)₃₋₆] was studied by isothermal titration calorimetry (ITC) and fluorescence spectroscopy. Calorimetric titrations indicate that the dimeric lectin binds to two molecules of the ligand with association constants determined at 25 °C being 1.75 × 10⁵, 1.39 × 10⁵, 1.45 × 10⁵ and 3.70 × 10⁵ M⁻¹, for chitotriose, chitotetraose, chitopentaose and chitoheptaose, respectively. The binding reaction was essentially enthalpy driven with the binding enthalpy (ΔHᵇ) at 25 °C for the different chitoooligosaccharides ranging between -17.36 and -13.82 kcal.mol⁻¹, whereas the entropic contribution to the binding reaction is negative, with the value of binding entropy (ΔSᵇ) being in the range of -32.7 and -22.3 cal.mol⁻¹.K⁻¹. The enthalpically driven nature of binding reactions suggests that the main factors that stabilize the interaction of saccharides with SGPL are hydrogen bonding and van der Waals’ interactions. Fluorescence titrations indicate that the protein intrinsic fluorescence decreases upon binding of the chitoooligosaccharides, with a 3 nm blue shift in the emission maximum. Association constants determined from an analysis of the ligand-induced changes in the fluorescence intensity are in good agreement with those obtained from ITC.
Introduction

In the work reported in Chapter 5 of this thesis, purification and characterization of some of the macromolecular properties of a new chitooligosaccharide-specific lectin from the phloem exudate of snake gourd (*Trichosanthes anguina*) has been reported. The snake gourd phloem exudate lectin (SGPL) is a heterodimer with subunit masses of 50 and 55 kDa. Secondary structural analysis has shown that it consists of 9.0% α-helix, 39.6% β-sheet, 20.4% β-turns and 32.4% unordered structures (Chapter 5). Thermal inactivation studies shows that agglutination activity of SGPL is maximal in the pH range of 5.0-10.0. Thermal unfolding studies using circular dichroism spectroscopy and differential scanning calorimetry have shown that SGPL undergoes a cooperative, thermal unfolding transition at ca. 70 °C.

In the studies reported in this chapter the interaction of snake gourd phloem lectin with chitooligosaccharides was investigated by isothermal titration calorimetry (ITC) and fluorescence spectroscopy. The results suggest that the dimeric SGPL binds to two ligand molecules of chitotriose, chitotetraose, chitopentaose and chitohexaose. The binding constants were comparable for chitotriose, chitotetraose and chitopentaose but increase by a factor of 2 for chitohexaose. Both types of titrations yielded similar results with respect to binding constant and Gibb’s free energy change values. The binding reactions for chitooligosaccharides to SGPL were enthalpically driven with negative entropic contribution. The enthalpically driven nature of binding reactions suggests that the main factors that stabilize the interaction of saccharides with SGPL are hydrogen bonding and van der Waals’ interactions.
Materials and methods

Materials

Snake gourd fruits were obtained from local vendors. 2-Mercaptoethanol, chitin (from crab shells) and chitooligosaccharides (GlcNAc)₃, (GlcNAc)₄, (GlcNAc)₅ and (GlcNAc)₆, were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sodium chloride, ammonium sulfate, di-sodium hydrogen phosphate, sodium dihydrogen phosphate, sodium hydroxide, copper sulfate, potassium tartrate, sodium carbonate, ammonia solution and acetic acid were obtained from local suppliers and were of the highest purity available.

Snake gourd phloem exudate lectin (SGPL)

The lectin from the snake gourd phloem exudate has been purified by affinity chromatography on chitin as described in Chapter 5. 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 native PAGE where as in SDS-PAGE in the presence of β-mercaptoethanol it yielded two bands with comparable intensity. Lectin concentration was estimated according to the modified Lowry method of Peterson [1977].

Isothermal titration calorimetry

Calorimetric titrations were performed at 298 K on a VP-ITC isothermal titration calorimeter from MicroCal (Northampton, MA, USA). Briefly, 7 µl aliquots of the chitooligosaccharides solution were added from a 1.5 mM stock solution via a rotating stirrer syringe to a 30 µM lectin solution contained in a 1.445 ml sample cell. Samples were dialyzed against 20 mM phosphate buffer, pH 7.4, containing 150 mM sodium chloride, 2% sodium azide and 10 mM β-mercaptoethanol (PBS-
Samples were degassed prior to loading into the cell. The additions were made at 4 min intervals to allow the exothermic heat peak accompanying each addition to return to the baseline prior to next addition. Usually the first injection was found to be inaccurate; therefore, a 1 or 2 µl injection was added first and the resultant point was deleted before the remaining data were analyzed as described below.

For a system of one set of identical binding sites, the total heat evolved (or absorbed) during the binding process at the end of the $i$th injection [Wiseman et al., 1989], $Q(i)$, is given by Equation (6.1)

$$Q(i) = nP_t \Delta H_b V \left\{ 1 + X_t/nP_t + (1 + X_t/nP_t + 1/nK_b P_t)^2 - 4X_t/nP_t \right\}^{1/2}/2$$  \hspace{1cm} (6.1)

Where $n$ is the number of binding sites, $P_t$ is the total protein concentration, $X_t$ is the total ligand concentration, $V$ is the cell volume, $K_b$ is the binding constant and $\Delta H_b$ is the binding enthalpy. Obviously the heat corresponding to the $i$th injection only, $\Delta Q (i)$, is equal to the difference between $Q(i)$ and $Q(i-1)$ and is given by Equation (6.2), which involves the necessary correction factor for the displaced volume (the injection volume $dV_i$):

$$\Delta Q(i) = Q(i) + dV_i/2 V [Q(i) + Q(i-1)] - Q(i-1)$$  \hspace{1cm} (6.2)

The ITC unit measures $\Delta Q(i)$ value for every injection. These values are then fitted to Equations (6.1) and (6.2) by a nonlinear least squares method using the data analysis program Origin® [MicroCal™]. The fit process involves initial guess of $n$, $K_b$ and $\Delta H_b$ which allows calculation of $\Delta Q(i)$ values as mentioned above for all injections and comparing them with the corresponding experimentally determined values. Based on this comparison the initial guess of $n$, $K_b$ and $\Delta H_b$ is improved and the process is repeated till no further significant improvement in the
fit can be obtained. The thermodynamic parameters, $\Delta G^o_b$ and $\Delta S_b$ are calculated according to the basic thermodynamic Equations (6.3) and (6.4):

$$
\Delta G^o_b = -RT \ln K_b \quad (6.3)
$$

$$
\Delta G^o_b = \Delta H_b - T \Delta S_b \quad (6.4)
$$

**Fluorescence spectroscopy**

Binding of chitooligosaccharides to snake gourd phloem lectin was also investigated by monitoring changes in the fluorescence intensity of the protein induced by saccharide binding. Measurements were performed on a Spex Fluoromax 4 fluorescence spectrometer using slit widths of 2 and 3 nm, on the excitation and emission monochromators, respectively. Samples were excited at 280 nm and emission spectra were recorded between 290 and 400 nm. Titrations were carried out by adding small aliquots of sugar from a 500 $\mu$M stock solution in PBS to 2.0 ml of the lectin with $A_{280} \leq 0.1$ in the same buffer. Fluorescence spectra were recorded for the protein alone and after each addition of the ligand. An equilibration period of 2 minutes was given after the addition of each aliquot. All spectra were corrected for volume changes before further analysis.

**Results**

**Isothermal titration calorimetry**

Thermodynamic parameters for the binding of chitooligosaccharides to snake gourd phloem lectin was investigated by isothermal titration calorimetry. Representative calorimetric titrations for the binding of chitotriose and chitohexaose to SGPL are given in the upper panels of Fig. 6.1A and 6.1B, respectively. From this figure it is seen that the exothermic heat of binding decreases monotonically with successive injections until saturation is achieved. A plot of the incremental heat released as a
function of the ratio of chitotriose and chitohexaose to SGPL is shown in lower panels of Fig. 6.1A and 6.1B, together with a non-linear least square fit of the data to Equation (6.1). The experimental data could be fitted satisfactorily to the ‘one set of sites’ model available in the Origin software provided by the instrument manufacturer.

Fig. 6.1. (A) Calorimetric titration of SGPL with chitotriose and (B) with chitohexaose at 298.15 K. Upper panels show the ITC raw data obtained from 20 to 30 automatic injections of 7 µl aliquots of 1.5 mM chitotriose and chitohexaose into 30 µM of SGPL. Lower panels show the integrated data obtained from raw data shown in the upper panels.
The fits obtained for the data are shown as solid lines in the lower panels of Fig. 6.1A and 6.1B. The fits yielded the values of various parameters such as number of binding sites, $n = 2.10 \pm 0.012$; binding constant, $K_b = 1.75 \pm 0.1 \times 10^5 \, \text{M}^{-1}$; enthalpy of binding, $\Delta H_b = -13.82 \pm 0.13 \, \text{kcal.mol}^{-1}$; entropy of binding, $\Delta S_b = -22.30 \, \text{cal.mol}^{-1}.\text{K}^{-1}$ for the binding of chitotriose to SGPL. Corresponding values obtained with chitohexaose are: $n = 1.84 \pm 0.007$, $K_b = 3.7 \pm 0.1 \times 10^5 \, \text{M}^{-1}$, $\Delta H_b = -17.36 \pm 0.09 \, \text{kcal.mol}^{-1}$; entropy of binding, $\Delta S_b = -32.7 \, \text{cal.mol}^{-1}.\text{K}^{-1}$. These values as well as the corresponding values obtained for the calorimetric titrations performed with different oligosaccharides and at different temperatures (for chitotriose) are listed in Table 6.1. In addition, values of Gibb’s free energy $\Delta G^\circ_b$, are also listed in the above table. The stoichiometry of binding was found to be in the range between 1.84 and 2.35 ligand molecules per protein dimer, indicating that the dimeric lectin binds two chitooligosaccharide molecules.

**Table. 6.1.** Binding constants ($K_b$) and thermodynamic parameters for the binding of chitooligosaccharides to snake gourd phloem lectin SGPL.

<table>
<thead>
<tr>
<th>Sugar</th>
<th>$T$ ($^\circ\text{C}$)</th>
<th>$n$</th>
<th>$K_b \times 10^5 , \text{M}^{-1}$</th>
<th>$\Delta G^\circ_b , \text{kcal.mol}^{-1}$</th>
<th>$\Delta H^\circ_b , \text{kcal.mol}^{-1}$</th>
<th>$\Delta S^\circ_b , \text{cal.mol}^{-1}.\text{K}^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(GlcNAc)$_3$</td>
<td>15</td>
<td>2.17</td>
<td>2.15</td>
<td>7.02</td>
<td>12.30</td>
<td>18.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>1.98</td>
<td>1.77</td>
<td>7.04</td>
<td>12.60</td>
<td>19.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>2.10</td>
<td>1.75</td>
<td>7.26</td>
<td>13.82</td>
<td>22.3</td>
</tr>
<tr>
<td>(GlcNAc)$_4$</td>
<td>25</td>
<td>2.37</td>
<td>1.39</td>
<td>7.06</td>
<td>15.25</td>
<td>27.6</td>
</tr>
<tr>
<td>(GlcNAc)$_5$</td>
<td>25</td>
<td>2.35</td>
<td>1.45</td>
<td>7.08</td>
<td>16.63</td>
<td>32.1</td>
</tr>
<tr>
<td>(GlcNAc)$_6$</td>
<td>25</td>
<td>1.84</td>
<td>3.70</td>
<td>7.60</td>
<td>17.36</td>
<td>32.7</td>
</tr>
</tbody>
</table>
The association constants determined at 25 °C were nearly the same for chitotriose to chitopentaose and were found to be in the range of $1.39 \times 10^5$ to $1.77 \times 10^5$ M$^{-1}$, whereas the $K_b$ value estimated for chitohexaose was twice as high. The enthalpy of binding and entropy of binding were found to increase with increasing size of the chitooligosaccharides.

The increase in enthalpy with increasing number of GlcNAc residues in the ligand indicates that the lectin combining site contains several subsites which interact with the individual monosaccharide units of the oligosaccharide. In order to investigate this further the contributions of the different monosaccharide units of the chitooligosaccharides to the binding enthalpy, entropy and free energy have been calculated from the thermodynamic data presented in Table 6.1, by subtracting the values of the oligosaccharide containing ‘(n-1)’ monosaccharide units from the values corresponding to the oligosaccharide containing ‘n’ residues. The results obtained are presented in Table 6.2.

**Table 6.2.** Change in enthalpy, entropy and free energy per additional saccharide for the binding of chitooligosaccharides to snake gourd phloem lectin SGPL.

<table>
<thead>
<tr>
<th>Sugar</th>
<th>$T$ (°C)</th>
<th>$-\Delta\Delta H^o$ (kcal.mol$^{-1}$)</th>
<th>$-\Delta\Delta S^o$ (cal.mol$^{-1}$.K$^{-1}$)</th>
<th>$-\Delta\Delta G^o$ (kcal.mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(GlcNAc)$_3^*$</td>
<td>25</td>
<td>13.82</td>
<td>22.3</td>
<td>7.26</td>
</tr>
<tr>
<td>(GlcNAc)$_4$</td>
<td>25</td>
<td>1.43</td>
<td>5.3</td>
<td>-0.20</td>
</tr>
<tr>
<td>(GlcNAc)$_5$</td>
<td>25</td>
<td>1.38</td>
<td>4.5</td>
<td>0.02</td>
</tr>
<tr>
<td>(GlcNAc)$_6$</td>
<td>25</td>
<td>0.73</td>
<td>0.6</td>
<td>0.52</td>
</tr>
</tbody>
</table>

*Values for chitotriose are in comparison for the lectin alone. Hence the $-\Delta\Delta H^o$, $\Delta\Delta S^o$ and $-\Delta\Delta G^o$ values are for the three GlcNAc residues of the trisaccharide.
From the data presented in Table 6.2 it can be seen that the addition of the 4\textsuperscript{th}, 5\textsuperscript{th} and 6\textsuperscript{th} \textit{N}-acetylglucosamine residues to chitotriose increases the enthalpy of binding by 1.43, 1.38 and 0.73 kcal/mol. This shows that the 4\textsuperscript{th} and 5\textsuperscript{th} GlcNAc residues make roughly equal contribution to the binding enthalpy, whereas the contribution of the 6\textsuperscript{th} GlcNAc residue is about half as much. However, in the case of the 4\textsuperscript{th} and 5\textsuperscript{th} GlcNAc residues, the increase in the binding enthalpy is compensated by an increase in the binding entropy, as a result of which the \( K_b \) values for chitotriose, chitotetraose and chitopentaose are comparable. On the other hand, for the binding of chitohexaose, the entropy of binding is not altered noticeably and hence the \( K_b \) value for this sugar increases by about 2.5 fold as compared to chitopentaose.

**Fluorescence spectroscopy**

Upon excitation at 280 nm snake gourd phloem lectin gave an emission spectrum centered at 339 nm. Titration of the protein with chitooligosaccharides resulted in a decrease in the emission intensity by 32-45\% along with a 3 nm blue shift in the emission maximum. A spectrum corresponding to a representative fluorescence titration of SGPL with chitopentaose is given in Fig. 6.2. Here spectrum 1 corresponds to the protein alone and spectra 2-18 correspond to those recorded in the presence of increasing concentrations of chitopentaose. Inset of the figure gives a plot of the change in fluorescence intensity, \( \Delta F = F_o - F \) as a function of the added ligand concentration, which represents the binding curve for the titration. The fluorescence intensity at saturation binding was determined from the Y-intercept of a plot of \( F_o/\Delta F \) versus \( 1/[L]_t \), where \( F_o \) is the initial fluorescence intensity, \( F \) is the fluorescence intensity at any point during the titration, and \( [L]_t \) is the total concentration of the ligand (chitopentaose). The fluorescence titration data
was then analyzed by the method of Chipman et al. [1967] according to the equation:

\[ \log \left\{ \frac{\Delta F}{(F - F_{\infty})} \right\} = \log K_b + \log [L_f] \]  

(6.5)

where \( F_{\infty} \) is the fluorescence intensity of the protein at infinite concentration of the ligand, \( K_b \) is the association constant, and \([L_f]\) is the free ligand concentration at each point of the titration and was obtained from the following Equation:

\[ [L_f] = [L_t] - \left\{ \frac{(\Delta F/\Delta F_{\infty}) [P]}{[P]} \right\} \]  

(6.6)

Fig. 6.2. Fluorescence spectra of SGPL in the absence and after addition of defined aliquots from 0.5 mM chitopentaose stock solution. Inset shows the binding curve.

where \( \Delta F_{\infty} (= F_0 - F_{\infty}) \) is the change in fluorescence intensity at saturation binding and \([P]_t\) is the total protein concentration. The X-intercept of a double logarithmic plot of \( \log \left\{ \frac{\Delta F}{(F - F_{\infty})} \right\} \) versus \( \log [L_f] \) will yield the p\( K_b \) value for the association.
reaction. Such a plot for the binding of chitopentaose to SGPL is shown in Fig. 6.3.

![Double-logarithmic plot](image)

**Fig. 6.3.** Double-logarithmic plot for the binding of chitopentaose to snake gourd phloem lectin. The double logarithmic plot was obtained using Chipmann analysis.

From the X-intercept of this plot the association constant, $K_b$, has been determined as $1.23 \times 10^5$ M$^{-1}$. This value is in reasonably good agreement with the $K_b$ value of $1.54 \times 10^5$ M$^{-1}$ determined at 25 °C from the ITC studies. The slope of this plot is found to be $\sim 1.0$, indicating that each lectin subunit binds one saccharide molecule. The fluorescence titration data for the interaction of the other chitooligosaccharides were also analyzed in an analogous manner and the association constants obtained are presented in Table 6.3. In addition, values of change in Gibbs’ free energy, calculated according to Equation (6.3), are also listed in this Table.
Table 6.3. Binding constants, $K_b$, obtained for various chitooligosaccharides at room temperature with snake gourd phloem lectin and the corresponding Gibb’s free energy values.

<table>
<thead>
<tr>
<th>Sugar</th>
<th>$K_b \times 10^{-5} \text{ (M}^{-1})$</th>
<th>$\Delta G^\circ_b \text{ (Kcal.mol}^{-1})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(GlcNAc)$_3$</td>
<td>1.02</td>
<td>6.86</td>
</tr>
<tr>
<td>(GlcNAc)$_4$</td>
<td>1.12</td>
<td>6.92</td>
</tr>
<tr>
<td>(GlcNAc)$_5$</td>
<td>1.23</td>
<td>6.97</td>
</tr>
<tr>
<td>(GlcNAc)$_6$</td>
<td>1.34</td>
<td>7.03</td>
</tr>
</tbody>
</table>

Discussion

The affinity chromatographic purification and general characterization of snake gourd phloem exudate lectin (SGPL), which is specific for chitooligosaccharides is described in Chapter 5 of this thesis. SGPL is a heterodimer with subunit molecular weights of ca. 50 and 55 kDa. Secondary structural analysis shows that it consists of mostly β-sheet with less α-helical content. The lectin is active in the 5-10 pH range and its thermal unfolding temperature is around 70 °C. In order to investigate the binding of chitooligosaccharides to this lectin and determine the association constants and the associated thermodynamic parameters, isothermal titration calorimetric and fluorescence ligand binding experiments were carried out. The results obtained are discussed below.

Results obtained from ITC and fluorescence studies on the interaction of chitooligosaccharides with SGPL are presented in Table 6.1 and Table 6.3,
respectively. Both these methods indicate that the dimeric lectin binds two ligand molecules with comparable affinity with chitohexaose being the best ligand. The thermodynamic data are consistent with an extended binding site in this lectin. Based on thermodynamics of chitooligosaccharide binding extended binding sites were also identified in other phloem exudate lectins, viz., _Luffa acutangula_ agglutinin [Anantharam et al., 1986], _Coccinia indica_ agglutinin [Sanadi & Surolia, 1994] and _Cucurbita maxima_ (pumpkin) phloem exudate lectin, PPL [see Chapter 3]. However, PPL binds chitotriose with higher affinity as compared to chitotetraose and chitopentaose, whereas chitohexaose is recognized with a 12-15 fold higher affinity with a considerable decrease in the binding stochiometry suggesting the formation of higher order complex. Thus chitohexaose appears to bind to two different molecules of PPL simultaneously, whereas SGPL has a more extended binding site, which can accommodate up to the hexasaccharide. ITC studies on the interaction of chitooligosaccharides to _Urtica dioica_ agglutinin and wheat germ agglutinin have shown that the binding affinity increases with increase in the size of chitooligosaccharides, suggesting that these two lectins also contain extended binding sites that can accommodate a trisaccharide and a tetrasaccharide, respectively [Bains et al., 1992; Lee et al., 1998; Katiyar et al., 1999]. Calorimetric studies on the association of hevein to oligomers of _N_-acetylglucosamine also show that hevein has an extended binding site [Asensio et al., 2000]. The binding constants obtained for the association of chitooligosaccharides with snake gourd phloem exudate lectin are approximately an order of magnitude higher than the corresponding values obtained for their interaction with the _Urtica dioica_ lectin and WGA but are comparable to the values obtained with PPL and LAA. The binding reaction for [(GlcNAc)_3-6] investigated were essentially enthalpy driven with the binding enthalpy (\(\Delta H_b\)) at 298.15 K for the different chitooligosaccharides ranging between \(-13.82\) to \(-17.36\) kcal.mol\(^{-1}\), whereas the entropic contribution to the
binding reaction is negative, with the value of binding entropy ($\Delta S_b$) being -22.32 to -32.7 cal.mol$^{-1}$.K$^{-1}$.

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

**Fig. 6.4.** Enthalpy–Entropy compensation plot for SGPL-chitooligosaccharide interaction. The oligosaccharides used are: chitotriose (●), chitotetraose (○), chitopentaose (□) and chitohexaose (◆). The straight line corresponds to linear least squares fit of the data. Slope of the line is 1.04 and correlation coefficient ($R$) is 0.999.

A plot for the $-\Delta H$ versus $-T\Delta S$ shown in Fig. 6.4 yields a straight line with slope greater than unity (slope 1.04; correlation coefficient ($R$) is 0.999) which indicates that the reactions for chitooligosaccharide to SGPL are enthalpically driven as generally found in lectin carbohydrate interactions [Chapter 2; Sultan & Swamy, 2005b; Katiyar et al., 1999]. The enthalpically driven nature of binding reactions suggests that the main factors that stabilize the interaction of saccharides with SGPL are hydrogen bonding and van der Waals’ interactions.
Results from fluorescence titrations for the binding of chitooligosaccharides to SGPL are in good agreement with the results from isothermal titration calorimetry in terms of binding constant and free energy changes. The emission maximum for SGPL seen at 339 nm exhibits a 3 nm blue shift, accompanied by a decrease in the emission intensity. In contrast, the fluorescence intensity of *L. acutangula* agglutinin was found to increase upon saccharide binding and emission maximum blue shifted to 2-4 nm [Anantharam et al., 1986]. The binding constants and Gibb’s free energy values increase with increasing size of chitooligosaccharides as observed with calorimetric titrations.

In summary the titration calorimetric and fluorescence spectroscopic studies reported in this chapter clearly shows that SGPL contains an extended carbohydrate binding site that seems to accommodate up to a hexasaccharide. The binding process is governed by enthalpic forces with negative contribution from binding entropy. Enthalpy-entropy compensation was observed for the interaction between SGPL and chito-oligosaccharides attesting to the crucial role played by water structure in the overall binding process.