Chapter 2

Rapid affinity-purification and physico-chemical characterization of pumpkin (Cucurbita maxima) phloem exudate lectin
Summary

The chitooligosaccharide-specific lectin from pumpkin (*Cucurbita maxima*) phloem exudate (PPL) has been purified to homogeneity by affinity chromatography on chitin. In SDS-PAGE in the presence of β-mercaptoethanol the pumpkin phloem lectin (PPL) yielded a single band corresponding to a molecular weight of 23.7 kDa, whereas MALDI-TOF mass spectrometry gave the molecular weights of the subunit and dimeric lectin as 24,812.3 and 49,721.5 Daltons, respectively. Analysis of the CD spectrum of the protein indicated that the secondary structure of PPL consists of 9.7% α-helix, 35.8% β-sheet, 22.5% β-turns and 32.3% unordered structures. Saccharide binding did not significantly affect the secondary and tertiary structures of the protein. The hemagglutinating activity of PPL was mostly unaffected in the temperature range 4-70 °C, but a sharp decrease was seen between 75 and 85 °C. Differential scanning calorimetric and CD spectroscopic studies indicate that PPL undergoes cooperative thermal unfolding process centered at ca. 81.5 °C.
Chapter 2

Introduction

Although many species from Cucurbitaceae are cultivated in large quantities across the world because fruits of many cucurbit species are edible, only a few lectins from this family have been characterized in detail. These include *Momordica charantia* lectin (MCL) and *Trichosanthes kirilowii* lectin from the seeds, and *Luffa acutangula* agglutinin (LAA) and *Coccinia indica* agglutinin (CIA) from the phloem exudate of cucurbits [Khan et al., 1981; Mazumder et al., 1981; Falasca et al., 1989; Anantharam et al., 1986; Sanadi & Surolia, 1994]. In order to characterize the cucurbit seed lectins in greater detail a long-term program was initiated by M. J. Swamy’s group in the mid 1990s and they reported on the purification, physico-chemical characterization and carbohydrate binding specificity of four new lectins from the Cucurbitaceae species, namely *Trichosanthes anguina* (snake gourd) seed lectin (SGSL), *Trichosanthes cucumerina* seed lectin (TCSL), *Trichosanthes dioica* seed lectin (TDSL) and *Trichosanthes cordata* seed lectin (TCA) characterized in greater detail, in addition carbohydrate and hydrophobic ligand binding properties for *Momordica charantia* (Bitter Gourd) seed lectin were characterized [Komath & Swamy, 1998, 1999; Kenoth & Swamy, 2003; Komath et al., 1996, 1998, 2001; Manoj et al., 2001; Padma et al., 1999; Kenoth et al., 2000, 2003; Sultan & Swamy, 2003, 2005a,b; Sultan et al., 2004a, 2009; Kavitha et al., 2009]. Additionally, the interaction of several water-soluble porphyrins with SGSL, TCSL, and MCL were investigated [Komath et al., 2000b; Kenoth et al., 2001; Sultan et al., 2004b].

In this thesis, the focus of our study is the lectins present in the phloem exudate of cucurbit species. Besides LAA and CIA which are mentioned above, strong hemagglutinating activity has been reported about three decades ago in the phloem exudate from three cucurbit species, namely *Cucurbita maxima, Cucumis sativus*
and *Cucumis melo* [Sabnis & Hart, 1978]. Among these, a lectin specific for chitooligosaccharides has been purified from the phloem exudate of *Cucurbita maxima* (pumpkin) by conventional chromatographic methods [Read & Northcote, 1983]. This protein was termed PP2 as it corresponded to the second prominent protein band in polyacrylamide gel electrophoresis. However, as this protein is a lectin, it would be more appropriate to call it a lectin and therefore hereafter we shall refer to it as pumpkin phloem lectin (PPL). Analysis of the cDNA sequence yielded the primary structure of PPL and indicated that it is a polypeptide of 218 amino acids [Read & Northcote, 1983; Bostwick et al., 1992, 1994]. To the best of our knowledge, no further work has been done on this protein. Therefore, we took up a detailed characterization of the macromolecular and carbohydrate binding properties of PPL. In this chapter a rapid and efficient purification method is reported for this protein with affinity chromatography as the key step. Physico-chemical studies show that PPL is stable up to about 75 °C, whereas CD spectroscopy indicates that its secondary structure is characterized by a large amount of β-sheet and β-turns and a very small α-helical content. DSC and CD studies indicate that the protein undergoes a cooperative thermal unfolding process around 81.5 °C.

**Materials and Methods**

**Materials**

Pumpkin fruits were obtained from local vendors. Chitin (practical grade, from crab shells), 2-meercaptoethanol, sodium dodecyl sulfate, acrylamide, methylene bis-acrylamide, molecular weight markers and chitooligosaccharides [(GlcNAc)$_2$, (GlcNAc)$_3$, (GlcNAc)$_4$, (GlcNAc)$_5$, and (GlcNAc)$_6$], were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sodium chloride, ammonium sulfate, sodium azide, di-sodium hydrogen phosphate, sodium dihydrogen phosphate,
trichloroacetic acid, sodium deoxycholate, sodium hydroxide, ammonia solution, and acetic acid were obtained from local suppliers and were of the highest purity available.

**Preparation of affinity matrix**

The chitin column was prepared essentially as described earlier [Sampietro et al., 2001]. Briefly, practical grade chitin from crab shells was suspended in 0.25 M NH₄OH and incubated for 60 minutes. The supernatant was decanted and the material was washed successively with 0.2 M NaCl, double distilled water and 20 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl and 10 mM β-mercaptoethanol (PBS-βME). The material was finally suspended in PBS-βME was packed into a glass column (16 × 4 cm) and equilibrated again with the same buffer.

**Extraction and purification of pumpkin phloem exudate lectin**

Pumpkin fruits were first washed with water and dried. Phloem exudate was then collected into ice cold PBS-βME by longitudinal, 2-5 mm deep cuts [Anantharam et al., 1986; Allen, 1979]. The buffer containing exudate was centrifuged at 9000 rpm in an Eppendorf 5810 R refrigerated centrifuge. The resulting supernatant containing soluble protein was subjected to ammonium sulfate precipitation to give 80% saturation. This solution was allowed to stand at 4 °C overnight and centrifuged at 9000 rpm and the supernatant was discarded. The precipitate obtained was dissolved in a minimal volume of water and dialysed against PBS-βME extensively at 4 °C. The solution from the dialysis bag was then centrifuged and the precipitate was discarded. The supernatant was subjected to affinity chromatography on a column of chitin (16 × 4 cm) that was pre-equilibrated with PBS-βME. The breakthrough obtained was reloaded to ensure complete binding of
the protein. The column was then washed with PBS-βME to remove unbound proteins, monitoring absorbance of the eluant at 280 nm. When the absorbance fell below 0.01 the washing was stopped and the bound protein was eluted with 0.1 M acetic acid at room temperature. Fractions of ca. ~5 ml were collected and their absorbance at 280 nm was checked for detecting the protein. Fractions showing high concentration of protein were pooled and the eluting acid was removed by extensive dialysis against PBS-βME. Purity of the dialysed protein was assessed by SDS-PAGE according to the method of Laemmli [Laemmli, 1970] and its concentration was estimated by a modified Lowry method [Peterson, 1977].

**Hemagglutination and hemagglutination-inhibition assays**

Hemagglutination assays were carried out using rabbit erythrocytes in 96-well ELISA microtiter plates. All solutions were made in PBS-βME and all dilutions were carried out using the same buffer. To each well containing 100 µl of serially diluted lectin 100 µl of 4% erythrocyte suspension was added. The plate was incubated at 4 °C for 1 hour and then the agglutination titer was visually scored. Hemagglutination-inhibition assays were carried out with purified protein. For inhibition assay 1 mM saccharide solutions were used except for chitobiose (52 mM). In the first well of microtiter plate, 50 µl of saccharide solution was placed and serially 2-fold diluted. Then 50 µl of protein solution (4 µg/ml) was added to each well. After incubating the mixture at 4 °C for 15 min, 100 µl of a 4 % erythrocyte suspension was added, the plate was incubated for one hour and the titer was scored visually.

**Mass spectrometry**

The mass spectrum of PPL was recorded using a model 4800 MALDI-TOF-TOF mass spectrometer from Applied Biosystems (Foster City, CA, USA) in the linear
mode using sinapinic acid (SA) as the matrix. The matrix was prepared by dissolving 5 mg of SA in 1 ml of 50% acetonitrile containing 0.1% trifluoroacetic acid (TFA). About 5 picomoles of the protein was spotted on the MALDI plate and the spectrum was acquired.

**Circular Dichroism Spectroscopy**

Circular dichroism (CD) spectra were recorded on a Jasco J-810 spectropolarimeter (Jasco International Co., Ltd., Tokyo, Japan, website: [http://www.jascoint.co.jp](http://www.jascoint.co.jp)) essentially as described earlier [Sultan et al., 2006]. Concentration of PPL was 1.8 µM for measurements in the far UV region (250-190 nm) and 20 µM for measurements in the near UV region (250-300 nm). Samples were placed in a 2-mm pathlength rectangular quartz cell and spectra were recorded at a scan speed of 20 nm/min with a response time of 4 s and a slit width of 2 nm. In order to investigate the effect of carbohydrate binding on the secondary and tertiary structure of PPL, spectra were recorded for the native protein as well as in the presence of 1 mM chitotriose. To investigate any structural changes that take place during thermal unfolding of the protein, CD spectra were recorded in the near UV and far UV regions at different temperatures by means of a Peltier device. Measurements were made in PBS-βME and buffer scans recorded under the same conditions were subtracted from the protein spectra before further analysis.

**Secondary Structure Prediction**

threshold = 10 and number of states = 4. For prediction using DPM the structural class was chosen as ‘all beta’. The amino acid sequence of PPL [Bostwick, et al., 1994] was imported from EMBL Nucleotide sequence database (accession number L31550).

**Differential scanning calorimetry**

Differential scanning calorimetric (DSC) studies were performed on a MicroCal VP differential scanning calorimeter (MicroCal LLC, Northampton, MA) with a scan speed of 60°/hr (Celsius scale). Thermograms were recorded with lectin samples of 12 µM concentration in PBS-βME. For experiments carried out in the presence of sugar, lectin samples contained 10 mM chitotriose. PPL was dialysed extensively against the buffer and degassed prior to loading into the cell. Buffer scans were subtracted from the thermograms corresponding to the lectin samples for analysis. Data were analysed by Origin™ software supplied by the manufacturer.

**Results and discussion**

**Affinity chromatographic purification of PPL**

Purification of two major proteins from the phloem exudate of *Cucurbita maxima* (pumpkin), named PP1 and PP2 by a combination of ammonium sulfate precipitation, ion exchange chromatography and gel filtration was reported by Read and Northcote [Read & Northcote, 1983]. Of these two proteins, PP2 (which is more appropriately referred to here as pumpkin phloem lectin or PPL) has been shown to exhibit hemagglutination activity which could be inhibited by oligomers of N-acetylglucosamine. It has been proposed that the lectin plays a role in plant’s defence mechanism by interacting with bacteria and fungi through its carbohydrate binding pocket [Read & Northcote, 1983]. In view of this it is important to investigate the macromolecular and carbohydrate binding properties of this protein.
in detail. Such studies will be facilitated by the availability of an efficient method of purification of the lectin. Although it was reported that PPL bound to ovomucoid coupled to Sepharose CL-4B in a carbohydrate-specific manner and could be eluted with chitotetraose [Read & Northcote, 1983], since ovomucoid is rather expensive, it is desirable to develop an alternative, inexpensive affinity method for its purification. In view of this, in this study we have developed a rapid purification method for PPL based on affinity chromatography on chitin, which is an inexpensive, commercially available polysaccharide. The affinity purified protein has been characterized with respect to molecular weight, secondary structure and thermal unfolding.

Since PPL was reported to be inhibited by oligosaccharides of chitin [Read & Northcote, 1983], we thought that chitin could be used as an affinity matrix for the purification of this lectin as affinity chromatography on chitin has been used as the key step in the purification of a number of lectins that specifically recognize chitooligosaccharides including wheat germ agglutinin, *Entamoeba histolytica* lectin and chitin binding lectins from *Artocarpus integrifolia, Artocarpus incisa* and *Cynchomandra betacea* [Bloch & Burger, 1974; Kobiler & Mirelman, 1980; Trindade et al., 2006; Xu et al., 1992].

PPL bound to the chitin affinity matrix eluted as a single peak when 0.1 M acetic acid was passed through the column (Fig. 2.1). In a typical purification experiment, about 80 mg of the protein was obtained from 8 ml of phloem exudate, i.e., a yield of 10 mg/ml of phloem exudate, which is comparable to the yield of 10.5 mg/ml reported earlier [Read & Northcote, 1983]. The overall yield of lectin activity recovered was about 40% (Table 2.1). Different affinity matrices such as soybean agglutinin glycopeptide coupled to Sepharose-6B and chitotriose coupled to Sepharose-4B have also been used to purify other chitooligosaccharide-specific
lectins such as potato tuber lectin and the phloem exudate lectins from ridge gourd (*Luffa acutangula*) and *Coccinia indica* [Anantharam et al., 1986; Sanadi & Surolia, 1994; Allen & Neuberger, 1973]. Sepharose-fetuin and insolubilized poly-L-leucine hog A+H blood group substance were used to purify the lectins from *Datura stramonium* and common tomato (*Lycopersicon esculentum*) [Kilpatric & Yeoman, 1978; Nachbar et al., 1980].

![Fig. 2.1. Affinity chromatographic purification of pumpkin phloem exudate lectin (PPL) on chitin. After washing the column extensively with PBS-βME, the bound lectin was eluted with 0.1 M acetic acid. Fractions (5 ml) were collected and absorbance of the column effluent at 280 nm was plotted as a function of the fraction number. The chitin affinity matrix used here is inexpensive and the method can be scaled up to process larger quantities of the phloem exudate and hence should be the method of choice for the purification of chitin-binding proteins. PPL yielded a single band in SDS-PAGE at pH 8.8 in the presence of β-mercaptoethanol (Fig. 2.2A, lane 2).](image)
Table 2.1 Purification of pumpkin phloem exudate lectin (from 8 ml of phloem fluid).

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total activity ($\times 10^4$)</th>
<th>Protein content (mg)</th>
<th>Specific activity (units/mg)</th>
<th>Percent recovery</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous extract</td>
<td>52</td>
<td>707</td>
<td>735</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>Ammonium sulfate precipitation</td>
<td>31</td>
<td>213</td>
<td>1455</td>
<td>60</td>
<td>1.97</td>
</tr>
<tr>
<td>Affinity chromatography</td>
<td>21</td>
<td>88</td>
<td>3840</td>
<td>40</td>
<td>5.2</td>
</tr>
</tbody>
</table>

Fig. 2.2. (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of PPL. Lane 1, molecular weight markers: (a) bovine serum albumin (66,000); (b) ovalbumin (45,000); (c) Glyceraldehyde-3-phosphate dehydrogenase (36,000); (d) carbonic anhydrase (29,000); (e) trypsinogen (24,000); (f) trypsin inhibitor (20,000); (g) $\alpha$-lactalbumin (14,200). Lane 2, PPL. (B) Plot of relative mobility ($R_f$) versus log $M_r$ for the estimation of molecular weight of PPL subunit, (●) marker proteins; (○) PPL. From the plot the mass of PPL subunit was estimated as 23.7 kDa.
This is consistent with the results of Read and Northcote [Read & Northcote, 1983], who reported that the lectin gave a single band in SDS-PAGE under reducing conditions at pH 8.8, but could be resolved into two closely-spaced bands at pH 9.2. By comparing the mobility of PPL with that of standard proteins (Fig. 2.2A, lane 1), the molecular weight of the subunit was estimated as ~23.7 kDa (Fig. 2.2B). In order to get the exact mass of the protein, PPL was subjected to mass spectrometric analysis. The MALDI-TOF mass spectrum of PPL is shown in Fig. 2.3. The base peak in the spectrum, seen at 24,812.3 Daltons corresponds to the subunit of PPL whereas the other prominent peak, corresponding to a mass of 49,721.5 Daltons is consistent with the dimeric protein.

![MALDI-TOF mass spectrum of PPL. The molecular ion of the subunit is seen at 24,812.3 Daltons. The peak at 49,721.5 Daltons corresponds to the dimer.](image)
Carbohydrate binding specificity

The hemagglutination activity of PPL was reported to be inhibited efficiently by chitooligosaccharides whereas a large number of other sugars investigated were ineffective [Sabnis & Hart, 1978; Read & Northcote, 1983]. To investigate the agglutination activity of the affinity purified PPL and its relative affinity towards various chitooligosaccharides, hemagglutination-inhibition assays were performed.

Table 2.2. Inhibition of the agglutination activity of PPL by chitooligosaccharides.

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Minimum concentration for 50% inhibition (µM)</th>
<th>Relative inhibitory potency</th>
</tr>
</thead>
<tbody>
<tr>
<td>chitobiose</td>
<td>700</td>
<td>1</td>
</tr>
<tr>
<td>chiototriose</td>
<td>100</td>
<td>7</td>
</tr>
<tr>
<td>chitotetraose</td>
<td>50</td>
<td>14</td>
</tr>
<tr>
<td>chitopentaose</td>
<td>12</td>
<td>60</td>
</tr>
<tr>
<td>chitohexaose</td>
<td>10</td>
<td>70</td>
</tr>
</tbody>
</table>

The results obtained are presented in Table 2.2. The lectin activity could not be inhibited by N-acetylglucosamine, but strong inhibition is seen when chitooligosaccharides are used as ligands, with the inhibitory potency of the ligands increasing with increase in the oligosaccharide size up to chitohexaose. While the concentration of chitobiose for 50% inhibition of the hemagglutination activity of PPL is about 700 µM, chitohexaose is able to achieve the same level of inhibition at only 10 µM concentration. Thus chitotriose, chitotetraose, chitopentaose and chitohexaose are 7, 14, 60 and 70 times more potent in their ability to inhibit the hemagglutination activity of PPL and suggest the presence of an extended combining site on each subunit of the lectin. These observations are qualitatively
consistent with the results of Read & Northcote [Read & Northcote, 1983], who investigated the ability of chitobiose, chitotriose and chitotetraose to inhibit the activity of the pumpkin phloem lectin. Lectins from the phloem exudate of Luffa acutangula and Coccinia indica also exhibited stronger affinity for the higher oligomers of chitin [Anantharam et al., 1986; Sanadi & Surolia, 1994]. The amino acid sequence of a chitooligosaccharide-specific, phytohormone-inducible lectin (Nictaba), isolated from the leaves of tobacco plant (Nicotiana tabacum), has been reported to be homologous to the primary structure of PPL [Chen et al., 2002]. The presence of a fraction of Nictaba in the nucleus suggested that it may be involved in a specific regulatory process in the nucleus. Glycan array screening has shown that this lectin exhibits a strong affinity for high-mannose and complex N-glycans and is most complementary to the core glycan i.e., the Manβ1-4GlcNAcβ1-4GlcNAcβ-N-Asn moiety [Lannoo et al., 2006]. Similarly, another Cucurbitaceae phloem exudate lectin, LAA was also shown to interact with the N-linked glycans of fetuin, ovalbumin and soybean agglutinin, which contain an internal chitobiosyl moiety in core glycan structure [Anantharam et al., 1986]. The binding of LAA to the above N-linked glycans has been interpreted as arising, most likely, due to the interaction of the lectin with the core chitobiosyl moiety, with some additional interactions of the protein with some of the other sugar residues of the oligosaccharide [Anantharam et al., 1986]. It is, therefore, likely that PPL also binds to N-linked glycans by interacting with the chitobiosyl moiety of the core glycan structure. Such binding may be relevant to the endogenous functions of these cucurbit phloem exudate lectins. In the light of this, it would be interesting to investigate if PPL and other Cucurbitaceae phloem exudate lectins can also interact with N-linked glycans and other sugar structures by the glycan array approach, which may shed further light on the roles played by them in their endogenous functions.
Effect of temperature on the activity of PPL

In order to investigate the effect of temperature on the activity of PPL, samples of the lectin were incubated at various temperatures for 15 minutes, followed by cooling to room temperature and then subjected to hemagglutination assay. The results obtained are presented in Fig. 2.4. It is seen from this figure that incubation at different temperatures up to 70 °C did not affect the activity of the lectin at all.

Fig. 2.4. Thermal inactivation of the agglutination activity of PPL. To investigate the effect of temperature on activity, the lectin samples were incubated at the desired temperature for 15 min. After cooling to room temperature, the sample was centrifuged, and the clear supernatant was tested for hemagglutination activity.

The activity decreased quite sharply between 75 and 85 °C; PPL incubated at 75 °C exhibits about 80% activity as compared to the native protein whereas the sample incubated at 85 °C has less than 10% activity. Incubation at 90 °C led to a complete loss of activity of the protein.
CD spectroscopy and secondary structure of PPL

Circular dichroic spectra of PPL in native state (solid line) and in the presence of chitotriose (dotted line) are shown in Fig. 2.5. The far UV spectrum of the native protein (Fig. 2.5A) is characterized by a minimum around 216 nm and a maximum around 196 nm, suggesting a predominantly β-sheet conformation for the lectin. The near UV CD spectrum of PPL (Fig. 2.5B) is characterized by three maxima at 275, 282 and 289.5 nm, which most likely arise due to the aromatic amino acids Tyr and Trp present in the protein.

![CD spectra of PPL. (A) Far UV region, (B) Near UV region. (───) lectin alone, (……..) lectin with chitotriose, (------) calculated fit obtained by using CDSSTR program.](image)

In order to derive information on the content of different structural elements in this protein, the CD spectrum was analyzed by three different methods, namely CDSSTR [Compton & Johnson, 1986; Sreerama & Woody, 2000] CONTINLL [Provencher & Glockner, 1981; Van Stokkum et al., 1990] and SELCON3 [Sreerama & Woody, 1993; Sreerama et al., 1999] employing the software routines
available at DICHROWEB (www.cryst.bbk.ac.uk/cdweb/html/) [Lobley & Wallace, 2001; Lobley et al., 2002]. A basis set containing 43 proteins was used as a reference for fitting the experimental spectrum. The results obtained from this analysis are given in Table 2.3. Among the three methods mentioned above, the best fit was obtained with CDSSTR and the resulting fitted spectrum is shown in Fig. 2.5A (dashed line) and it is seen that the fit is in good agreement with the experimental spectrum.

**Table 2.3.** Secondary structure of PPL determined from CD spectral analysis and theoretical prediction.

<table>
<thead>
<tr>
<th>Method</th>
<th>α (%)</th>
<th>β (%)</th>
<th>Turns</th>
<th>Unordered</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>From CD spectral analysis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDSSTR</td>
<td>8.0</td>
<td>42.0</td>
<td>22.0</td>
<td>31.0</td>
</tr>
<tr>
<td>CONTINLL</td>
<td>9.6</td>
<td>34.4</td>
<td>23.0</td>
<td>33.4</td>
</tr>
<tr>
<td>SELCON3</td>
<td>11.6</td>
<td>34.0</td>
<td>22.6</td>
<td>32.6</td>
</tr>
<tr>
<td>Average</td>
<td>9.7</td>
<td>36.8</td>
<td>22.5</td>
<td>32.3</td>
</tr>
<tr>
<td><strong>From theoretical prediction</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SOPMA</td>
<td>12.8</td>
<td>30.7</td>
<td>17.4</td>
<td>39.0</td>
</tr>
<tr>
<td>DPM</td>
<td>14.0</td>
<td>37.0</td>
<td>15.0</td>
<td>34.0</td>
</tr>
<tr>
<td>Average</td>
<td>13.4</td>
<td>33.9</td>
<td>16.2</td>
<td>36.5</td>
</tr>
</tbody>
</table>

The content of various secondary structures obtained from the CDSSTR analysis are: 2 % regular α-helix, 6 % distorted α-helix (giving a total α-helical content of 8 %), 26 % regular β-sheet and 13 % distorted β-sheet (adding to a total of 39 % of pleated sheet structure), 22 % β-turns and 31 % unordered structures. These values along with the values obtained from the other two methods, namely CONTINLL and SELCON3 are presented in Table 2.3; the average values obtained from the
above three methods are also given in this Table. From the above data it is clear that although CDSSTR gave the best fit to the experimental spectrum, values of secondary structures obtained from the other two methods are also in good agreement. The relatively small standard deviations obtained with the average values are quite consistent with this. Overall the results obtained from analyzing the CD spectral data indicate that PPL is a predominantly β-sheet protein with a relatively small α-helical content. LAA, another phloem exudate lectin from Cucurbitaceae was reported to contain 31% α-helix [Anantharam et al., 1986]. The far UV CD spectrum of PPL recorded in the presence of 1 mM chitotriose, is nearly identical to the spectrum of native PPL, suggesting that the secondary structure of the protein is unaltered upon ligand binding. Very minor changes are seen in the near UV CD spectrum of the protein upon binding of chitotriose, indicating that the environment of the side chains of Tyr and Trp residues is also largely unaffected by ligand binding. In contrast, significant changes were observed in the near UV CD spectrum of LAA upon binding of chitooligosaccharides [Anantharam et al., 1986]. This observation, taken together with the observation that LAA contains significant α-helical content, suggests that despite very similar sugar specificity, the two lectins differ significantly in the secondary and tertiary structures.

**Secondary structure prediction**

The results of secondary structure prediction based on the primary structure of PPL using two different methods, namely SOPMA and DPM for PPL are listed in Table 2.3. The prediction results from SOPMA indicate that 28 amino acid residues adopt α-helical structure among the 218 residues, corresponding to 12.8%. Likewise 67, 38 and 85 residues are predicted to be involved in β-sheet, β-turn and random coil, respectively. These values correspond to about 30.7% β-sheet, 17.4% β-turn and 39% random coil. Prediction by DPM gave 14, 37, 15 and 34
percentages for α-helix, β-sheet, β-turn and random coil. Secondary structure prediction values from these two methods are comparable to experimental results from circular dichroism spectroscopy.

**Thermal unfolding: DSC and CD studies**

Thermal unfolding of PPL was investigated using DSC and CD spectroscopy. Thermograms of PPL in the absence and in the presence of 10 mM chitotriose are shown in Fig. 2.6.

**Fig. 2.6.** DSC thermograms of PPL. A) PPL alone, B) PPL + 10 mM chitotriose.

DSC thermogram of PPL alone exhibits an unfolding transition centered at about 81.5 °C, which shifts to 83.8 °C in the presence of 10 mM chitotriose. It was not possible to determine the enthalpy of the transition unambiguously because the baseline at the end of the transition could not be established clearly; this could be because of aggregation of the unfolded protein as a white precipitate was observed when the sample was examined at the end of the calorimetric scan. Addition of carbohydrate ligand, chitotriose or additives such as putrescine,
hexafluoroisopropanol, spermine, and spermidine did not have any detectable effect on the aggregation of PPL during thermal denaturation (data not shown).

Far UV CD spectra of PPL recorded at various temperatures are shown in Fig. 2.7. Only minor differences are seen in the spectra recorded between 30 and 80 °C, indicating that the secondary structure of PPL is essentially unaltered in this temperature region.

On the other hand, the spectrum recorded at 85 °C has a significantly (negative) lower intensity and differs substantially from that recorded at 80 °C, clearly indicating that the protein secondary structure undergoes significant changes above 80 °C. The spectrum obtained at 90 °C exhibits further decrease in the intensity indicating a near total loss of secondary structure. These results are fully consistent with the DSC data presented in Fig. 2.6. (see above).

Fig. 2.7. Far UV CD spectra of PPL recorded at different temperatures. Spectra 1-8 corresponding to 30, 45, 60, 70, 75, 80, 85 and 90 °C respectively.
In summary, the present study reports a rapid affinity purification method for the pumpkin phloem lectin. The lectin activity was inhibited by chitooligosaccharides, with chitohexaose being the best ligand. CD spectroscopic studies indicate that the secondary structure of PPL contains mostly β-sheet and β-turns and very little α-helix, which has been supported by theoretical prediction of the secondary structure from the amino acid sequence of the protein. DSC and CD spectroscopic studies indicate that PPL undergoes thermal unfolding at ca. 81.5 °C. Carbohydrate binding has a slight stabilizing effect on the structure of PPL.