CHAPTER III

OLIGOMERIZATION STUDIES OF SRL BY MALDI-TOF AND NANO-ESI MASS SPECTROMETRY
Non-covalent interactions between biomolecules play a central role in many molecular recognition processes. Examples include protein-protein, protein-ligand, enzyme-substrate and antibody-antigen interactions. Non-covalent interactions between the folded polypeptide chains or subunits are also responsible for specific aggregation of these subunits into a multisubunit structure (quaternary structure), which is essential for biological activity. Like many classes of protein systems lectins also exist as multimers under native conditions (Lis and Sharon, 1986). The oligomerization in lectins has important biological implications. The quaternary structure provides the lectin with a requisite topology that helps in multivalent binding to cells. Although the monomeric unit in any lectin is capable of sugar binding, they are generally found in nature in their oligomeric state. Nature has endowed the lectins with enormous stability by oligomerization to carry out their biological functions efficiently (Sinha and Surolia, 2005). Thus it is very important to understand the oligomeric properties of lectins in evaluating their functional roles.

Subunit interactions have traditionally been studied by techniques such as ultracentrifugation, gel permeation, size-exclusion chromatography, X-ray crystallography and nuclear magnetic resonance spectroscopy (Koltz et al., 1975). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in combination with chemical crosslinking agents has also been utilized to provide molecular mass information and subunit
stoichiometry. These methods have significant limitations and can only provide an approximate molecular mass of the complex. However, with the recent developments in soft ionization techniques, mass spectrometry (MS) has become a valuable tool for studying oligomeric state of a protein as the methods allow the accurate measurements of molecular masses.

From the initial pioneering development of electrospray ionization (ESI) by Fenn’s group (Fenn et al., 1989), the application of ESI mass spectrometry (ESI-MS) for studying non-covalent complexes has important utility in biology, biochemistry, and biomedical research (Light-Wahl et al., 1994; Przybylski and Glocke, 1996; Loo, 1997; Rostom and Robinson, 1999). The gentle ionization process in ESI allows the detection of noncovalent protein aggregates without molecular fragmentation. Further, ESI is carried out in aqueous solution (near physiological conditions). Analysis of protein complexes by ESI-MS has been improved after the introduction of nanospray ESI, since the later reduces the initial energy of the ions formed during the ionization process (Wilm and Mann, 1994). Details of applications of ESI-MS for studying non-covalently bound complexes have been discussed by Loo in several reviews (Loo, 1997; Loo, et al., 1997; Loo and Sannes-Lowery, 1998).

Several recent reports have showed that MALDI is also capable of keeping non-covalent protein interactions intact in the gas-phase vacuum of the mass spectrometer (Kiselar and Downard, 2000; Strupat et al., 2000;
Wattenberg et al., 2000; Friess et al., 2002). However, the analyses by MALDI are complicated from the fact that the matrix environment and laser irradiation dissociate most non-covalent complexes. Nevertheless, its rapidity, tolerance to impurities as well its simplicity makes it a method of choice. Recently Farmer and Caprioli (1998b) have reviewed various strategies for the determination of protein-protein interactions by MALDI-MS including specific matrix and laser combination. The merits and demerits of mass spectrometry compared to other biophysical methods, such as NMR, X-ray crystallography, and other spectroscopic techniques for investigating non-covalent interactions, are discussed in the review by Smith and Zhang (1994).

Earlier physico-chemical characterization studies in this laboratory showed that *Sclerotium rolfsii* lectin (SRL) is a dimer of molecular mass ~34 kDa with identical subunits having M_r 17 kDa as determined by SDS-PAGE and gel permeation chromatography at neutral pH. However SRL gave molecular weight of ~17 kDa by gel permeation chromatography at acidic pH. Also this subunit mass was confirmed by MALDI-MS and found to be 16,491. Thus it was concluded that SRL undergoes pH dependent oligomerization/(Swamy et al., 2001). Although our recent X-ray crystal structure of SRL determined at 1.1 Å resolution, conclusively demonstrated the dimeric nature of SRL (Leonidas et al., 2007), there were arguments in the light of reported oligomeric state of related fungal lectins.
The structural and sequence comparison of SRL with lectins from *Agaricus bisporus* (ABL) (Carrizo *et al.*, 2005) and *Xerocomus chrysenteron* (XCL) (Birck *et al.*, 2004) which belong to a newly identified family of fungal lectins revealed high degree of structural similarity. Hence SRL is considered as third member of this family, all of which share common carbohydrate specificity. Interestingly unlike ABL and XCL, which are tetramers, SRL exist as dimer at neutral pH and as monomer at acidic pH. Recently discrepancies with respect to oligomeric state were reported for a lectin from a parasitic mushroom, *Laetiporus sulphureus* (LSL), when studied by different analytical methods. LSL was shown to be tetramer with sub unit mass of 35 kDa, by SDS-PAGE and gel filtration chromatography. However the crystal structure revealed the hexameric state, which was further confirmed by analytical ultra centrifugation (Mancheno *et al.*, 2005).

Considering the importance of quaternary structure of lectins with their function it was essential to investigate further the oligomeric structure and also the pH dependent aggregation of SRL. To address this question we employed more sophisticated MALDI-TOF-MS and Nano-ESI-MS techniques. Results of these findings are presented in this chapter.

### 3.1. Materials and methods

*Sclerotium rolfsii* lectin (SRL) was purified as described earlier (II\textsuperscript{nd} chapter) and lyophilized (Swamy *et al.*, 2001). Lyophilized SRL was dissolved at a concentration of 1 mg/ml. in 20mM ammonium acetate
buffers of different pH: 3.0, 4.5, 6.8, 7.8, and 9.0 and the solutions were left at 4°C overnight before the MALDI-TOF-MS and Nano-ESI-MS analyses. Sinapic acid (98% Titration), acetonitrile (ACN >99.93% pure, HPLC grade) and formic acid (FA 96% pure) were purchased from Sigma-Aldrich. Trifluoroacetic acid (TFA, >99% pure) was purchased from Aldrich (Milwaukee, WI, USA). All other reagents used were of analytical grade and the aqueous solutions were prepared using deionized water from Milli-Q Ultrapure Water System (Bedford, MA) and filtered through 0.22μm nylon filters from Fischer Scientific (Pittsburgh, PA, USA).

3.2.1. MALDI-TOF-MS

MALDI-TOF-MS analysis was performed on Ultraflex TOF/TOF (Bruker Daltonics, Germany) mass spectrometer, equipped with a UV nitrogen laser of 337 nm. 1μl protein solution was mixed with 1μl with matrix solution (saturated solution of sinapinic acid in acetonitrile / 0.1% aqueous trifluoroacetic acid) and a total volume of 1μl of this mixture was deposited on the probe plate. The spectra were recorded in the reflectron positive ion mode after the evaporation of the solvent and spectra were acquired by Bruker Daltonics FLEX control software. The spectra were processed by Bruker Daltonics FLEX analysis software. External calibration was performed with Protein Calibration Standard–I (P.No.206355; Insulin (M+H)+ avg 5734.5600, Ubiquitin-I (M+H)+ avg 8565.8900, Cytochrom-C (M+H)+ avg 12361.0900, Myoglobin (M+H)+ avg 85
16952.5500, Cytochrome-C (M+2H) \(^{2+}\) avg 6181.0500, Myoglobin (M+2H)\(^{2+}\) avg 8476.7700) supplied by Bruker Daltonics, Germany.

3.2.2. Nano-ESI-MS

ESI mass spectra of SRL were acquired using an Esquire 3000 ion trap mass spectrometer. Samples were electrosprayed in nanoelectrospray mode; a small conical glass capillary (custom made, Nano ES spray capillaries, medium, Cat # ES391 of PROTANA, The Functional Proteomics Leader,) was used with flow rates of 30nl/min. Mild ESI interface conditions were employed for the studies of non-covalent complexes, i.e. low capillary (desolvation) temperature as low as \(~37^\circ\)C and a low capillary-skimmer-cap exit (declustering) potential (40-120V) to minimize the possible disruption of the noncovalent bonds. The tip was held at 860 V in positive ion mode. Nebulization was assisted by N\(_2\) gas (99.8\%) at a flow rate of 2 L/min at 2 psi pressure. The spray chamber was held at 37\(^\circ\)C. The Spectra were recorded in positive ion mode over an m/z range of 200-6000. Typically, 7 scans were summed to give representative spectra. The spectrophotometer was calibrated using calibration standard provided by the manufacturer (ES TUNING MIX, P.No.G2421A, Agilent, USA). Data processing was done using HP Chemstation software. The charge states were determined using the isotopic peaks.
3.3. Results and discussion

Characterization of the oligomeric structure of SRL using conventional solution phase technique such as gel filtration chromatography at acidic and basic pH has inferred that SRL exists as monomer and dimer respectively. Recently X-ray diffraction data has also revealed the dimeric state of SRL at basic pH. Although reports on the use of MALDI technique for the study of non-covalent complexes are very limited, this methodology appears to be applicable for the detection of non-covalent complexes, at least those with higher binding energies (Akashi et al., 1996; Glocker et al., 1996; Pramanik et al., 1996; Cohen et al., 1997).

MALDI mass spectra of the SRL samples incubated at different pH; 3.0, 4.5, 6.8, 7.8, and 9.0 are shown in figure 1(a) to 1(e). Figure 1(a) shows the MALDI mass spectrum of SRL at pH 3.0. The spectrum shows singly charged ion for monomer, together with different oligomer cluster ions of dimer, trimer, tetramer up to octamer form (2S+, 3S+, 4S+, 5S+, 6S+, 7S+ and 8S+). The singly charged ion of mass 16.43 kDa corresponding to subunit mass of SRL forms the base peak and constitute the major component followed by the dimer, trimer and tetramer as could be seen from their relative intensities. In addition higher aggregates were observed, however their abundance was minimum comparatively. Major constituent peak corresponding to monomer subunit could be because of the high degree
of dissociation equilibrium of native oligomer shifting towards monomer state.

MALDI spectra of SRL at pH 4.5 and at 6.8 are given in figure 1 (b) and 1 (c) respectively. The spectra revealed that the intensity of the monomer ion peak is same at pH 4.5 and 6.8. But this peak intensity is relatively much higher compared to the peak intensity observed at pH 3.0. The intensities of the dimer ion peak observed for pH 3.0 and 6.8 are similar, but are much smaller compared to intensity for the peak observed at pH 4.5. Thus at pH 4.5 abundance of dimer is higher compared to pH 3.0 and pH 6.8. On the other hand the peak intensity corresponding to tetramer is relatively higher for pH 4.5 when compared to pH 3.0 and 6.8. Figure 1 (d) and (e) shows the MALDI mass spectrum of SRL at pH 7.8 and 9.0 respectively. The spectra for the pH 7.8 and pH 9.0 show relatively lower intensities of the monomer peak compared to pH 3.0, 4.5 and 6.8, indicating relatively less population of monomer species. However it is only for pH 7.8, high intensity was observed for the tetramer ion peak indicating the abundance of tetramer only at this pH, but not in pH 3.0, 4.5, 6.8 and 9.0. The pH dependent oligomerization studies of SRL carried out using MALDI mass spectrometry appears to be very complex probably because of its anomalous behavior. Indeed these results supported our earlier findings that oligomerization of SRL is critically dependent on the pH. But the results did not provide conclusively
evidence for the monomeric state at acidic pH and dimeric state at neutral or basic pH. Nevertheless the data supported our earlier finding that dimer is also a major population in addition to predominant monomer. It is only at pH 7.8 sufficiently significant tetramer population could be seen. Also at this pH detectable population of octamer was found and this explains our earlier observation that SRL undergoes irreversible aggregation, forming an insoluble precipitate on storage (Swamy et al., 2001). Similar property was reported for Psathyrella vellutina lectin (PVL), which exists as monomer at pH 7.2 and was seen as dimer in the crystal (Cioci et al., 2006).

![MALDI-TOF mass spectra of SRL obtained at pH 3.0 with Sinapinic acid matrix. The labeling scheme for all MALDI mass spectra is as follows: $S^+$ = Monomer, $2S^+$ = Dimer, $3S^+$ = Trimer, $4S^+$ = Tetramer, $5S^+$ = Pentamer, $6S^+$ = Hexamer, $7S^+$ = Heptamer and $8S^+$ = Octamer.](a pH 3.0)
Figure 1 (b). MALDI-TOF mass spectra of SRL obtained at pH 4.5 with Sinapinic acid matrix.

Figure 1 (c). MALDI-TOF mass spectra of SRL obtained at pH 6.8 with Sinapinic acid matrix.
Figure 1 (d). MALDI-TOF mass spectra of SRL obtained at pH 7.8 with Sinapinic acid matrix.

Figure 1 (e). MALDI-TOF mass spectra of SRL obtained at pH 9.0 with Sinapinic acid matrix.
Inconclusive information obtained from the pH dependent oligomerization studies using MALDI necessitated our efforts to employ Nano-ESI mass spectrometry to determine the pH dependent oligomerization of SRL as this method is considered to be reliable for non-covalent interaction studies. SRL mass spectra were recorded as a function of the pH. ESI mass spectrum obtained for SRL in 20mM ammonium acetate, pH 4.5 is presented in figure 2 (a) and the deconvoluted masses of multiply charged ions are shown in the inset. At this pH the ESI mass spectrum showed broad distribution of multiply charged ions, m/z 1558.4, 1636.2, 1723.4, 1818.6, 1925.5, 2047.0, 2181.7, 2339.3, 2517.9, 2727.7, 2976.9, 3271.9, 3635.5 and 4091.9 with charge states ranging from +8 to +21. The deconvoluted mass spectrum yielded a protonated molecular mass of 32728.0, which is in good agreement with dimeric mass of SRL, but not in agreement with the MALDI mass spectral data where monomer population is predominant compared to dimer. With similar experimental conditions, ESI mass spectrum for SRL in 20mM ammonium acetate, pH 7.8 has been obtained and the spectrum is given in figure 2 (c). Multiply charged ions m/z 3660.6, 3873.6, 4117.3, 4390.5, 4706.2, 5066.4, 5491.2 and 5987.6 with charge states ranging from +11 to +18 corresponding to the intact tetramers, observed between m/z 3000-6000. The deconvoluted mass spectrum yielded a protonated molecular mass of 65868.1, which is in good agreement with tetrameric mass of SRL but contradictory to MALDI mass
spectral data where monomer population is predominant compared to tetramer and also indicating the presence of significant population of dimer.

ESI spectrum of SRL at pH 6.8 shows multiply charged ions, m/z 748.0, 783.5, 822.7, 865.8, 913.8, 967.5, 1027.9, 1096.2, 1174.5, 1264.6 and 1370.1 with charge states ranging from +12 to +22. The deconvoluted mass spectrum yielded a protonated molecular mass of 16431.3, which is in good agreement with monomeric mass of SRL indicating the presence of only monomer.

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**Figure 2 (a).** Positive ion nanoflow-electrospray mass spectrum of SRL in 20 mM ammonium acetate, pH 7.8. Deconvoluted masses of the multiply charged ion peaks are given in the inset.
Figure 2 (b). Positive ion nanoflow-electrospray mass spectrum of SRL in 20 mM ammonium acetate, pH 6.8. Deconvoluted masses of the multiply charged ion peaks are given in the inset.

Figure 2 (c). Positive ion nanoflow-electrospray mass spectrum of SRL in 20 mM ammonium acetate, pH 7.8. Deconvoluted masses of the multiply charged ion peaks are given in the inset.
In nano-ESI-MS, at pH 6.8 no higher order oligomers, such as dimer, trimer or tetramer, were detected. Similarly at pH 4.5 and 7.8 no peaks corresponding to monomer and other higher oligomeric aggregates except dimer and tetramers were observed. These observations indicated that the observed dimers and tetramers due to non-covalent interactions were not the result of artefactual non-specific associations. Similar results were observed with pH dependent oligomerization studies of ConA by ESI mass spectrometry (Light-Wahl et al., 1994; Van Dongen and Heck, 2000). But the pH dependence of the ESI mass spectra obtained for ConA agrees well with its known solution behavior, that is tetramer is more prevalent at pH > 7 and the dimer is the primary species at pH ~5. Below pH 6.5 the ConA tetramers dissociates into its canonical dimers. The monomer-dimer-tetramer is thought to be highly dependent on pH (Light-Wahl et al., 1994; Van Dongen and Heck, 2000). Tang et al (1994) have studied the non-covalent subunit interactions in Soybean agglutinin (SBA) as a function of acetonitrile concentration using electrospray ionization (ESI) time-of-flight mass spectrometer. The ESI mass spectral analysis of aqueous SBA solution titrated with 0 to 30% acetonitrile while keeping the ESI-MS conditions constant, including the same interface conditions. At 0 % acetonitrile concentration, SBA predominantly exist as a tetramer and the intensity of the tetramer ions decreases as the acetonitrile concentration increases, while the intensity of the monomer ions has the opposite behavior. Only monomer
ions were observed for spectra of SBA solutions containing 25% or more acetonitrile, presumably reflecting the complete dissociation of the tetramer into monomer in solution under the influence of acetonitrile. The presence of organic solvents in protein solutions is known to disrupt the weak inter- and intramolecular non-covalent bonds, which are essential for protein tertiary and quaternary structures. These results provide strong evidence that the SBA tetramer species observed by ESI-MS arise from specific non-covalent association for the subunits in solution.

Recently, the stability of monomer and tetramer of Soybean agglutinin (SBA) has been studied as a function of pH by fluorescence, CD, dynamic light scattering, and gel filtration methods. Soybean agglutinin is a tetrameric legume lectin, each of whose subunits are glycosylated. This protein shows a very high degree of stability when compared to the other proteins of the same family. The unusual stability of the agglutinin is due to a high degree of subunit interactions. A comparison of the fluorescence, CD, dynamic light scattering (DLS), and gel-filtration studies at different values of pH suggest that the protein retains its oligomeric state until pH 2.5, below which it starts dissociating. Further, the similarity of the fluorescence, CD, and DLS profiles till pH 2.5 suggests that SBA retains the tetrameric identity very strongly. Coming down to pH 2.25, it was found that the structural features were changing gradually. At this pH the onset of dissociation of the tetramer into monomers begin to occur. However, at pH
1.9 the process of dissociation is complete and SBA most populated as monomer (Sinha and Surolia, 2005).

Unlike Con A and SBA, SRL behaves differently at different pH values. At pH 4.5 SRL predominantly exist as dimer. Only monomer ions were observed for spectra of SRL at pH 6.8, presumably reflecting the complete dissociation of dimer into monomer. However, at pH 7.8 the ESI mass spectrum of SRL shows multiply charged ions corresponding to the tetramer only suggesting that the monomeric species above pH 6.8 reassociates to form tetramer.

The results of MALDI and nanospray ESI MS obtained for SRL indicate its complex oligomeric behaviour. Although these findings are intriguing and puzzling yet shed light on the complex dynamic equilibrium that exists with SRL's oligomerization warranting further investigations. Even Loo (2000) a pioneer and has authored several reviews on the application of ESI MS technique for studying non covalent complexes has expressed mixed opinion about the technique;

"There are three camps of opinion: believers, nonbelievers, and undecided. In my opinion, the believers hold their view because they had an early success in the laboratory and found ESI-MS to be useful for studying noncovalent complexes. On the other hand, nonbelievers had an early failure in the laboratory and, therefore, projected that the technique cannot possibly be used for this application; i.e., it doesn't work for my
project, so how can it be useful as a general technique?. The shrewd undecided majority has not actually attempted any (or many) experiments of this type and are waiting until more examples are reported to convince them to dive in. I am a cautious believer. Therefore, many examples will be cited to support the use of ESI-MS for studying noncovalent protein complexes".