CHAPTER IV

CRYSTALLIZATION OF

SCLEROTIUM ROLFSII LECTIN

Crystal Structure of Sclerotium rolfsii lectin monomer
The structural and evolutionary relationships between fungal lectins are still poorly understood, although many fungal lectins have been studied, in some detail with respect to their biochemical properties and biological activities. And very little is known about the possible relationships between fungal and animal or plant lectins and also their in vivo functions. This is mainly because of the scarcity of information about their sequence and also complete lack of detailed structural data about fungal lectins.

At present lectins are the focuses of intense attention because of the realization that they act as recognition determinants in diverse biological processes. Mechanisms for sugar recognition have evolved independently in diverse protein structural frameworks, but share some key features. The structural variations as well as their carbohydrate binding specificities, of lectins have been paid much attention and they are the main targets of crystallographic structure analysis. The studies on the three dimensional structures of lectins and their saccharide complexes have mainly contributed for the understanding of the molecular mechanisms involved in cell-cell recognition. These lectins represent a heterogenous group of oligomeric proteins that vary widely in size, structure, molecular organization as well as in the constitution of their combining sites. Nevertheless, many of them belong to distinct families with similar sequences and structural features, which provide a novel guideline for the detection and identification of new ones (Lis and Sharon, 1986; 1998; Weis and Drickamer, 1996).
A lectin purified from the sclerotial bodies of the fungus *Sclerotium rolfsii*, exists as a monomer (17kDa) at acidic pH and as a dimer (34kDa) at neutral and basic pH. SRL is known to recognize Galβ1→3GalNAc-α-O-ser/thr residues in glycoproteins, with diminished reactivity towards sialylated glycotopes (Swamy *et al.*, 2001; Wu *et al.*, 2001). Since SRL is shown to be developmental stage specific lectin expressed at different stages of growth and involved in fungal development and morphogenesis (Swamy *et al.*, 2004) we were curious to study the structure function relationship of SRL and attempts were made to successfully crystallize SRL so as to analyse crystallographic structure of SRL.

**MATERIALS AND METHODS**

SRL was purified from the sclerotial bodies of the fungus *S. rolfsii* as described earlier (Chapter II).

Polyethylene glycol (PEG) molecular weight ranging from 1000-4000 and 2, methylpentane-2, 4-diol (MPD), were purchased from Sigma Chemical Co., St. Louis, USA. All other chemicals used were of analytical grade. All the reagents were prepared using twice glass-distilled water.

1. **Crystallization of *Sclerotium rolfsii* lectin (SRL)**: SRL purified from the fungus *S. rolfsii* (Swamy *et al.*, 2001) was used to crystallize the lectin by hanging drop vapour diffusion method, aiming to determine its preliminary x-ray crystallographic structure so as to understand the structure–function relationship of SRL.
1.1 Initial Crystallization: The freeze-dried lectin was diluted in Tris-HCl buffer (5 mM), pH 8.0 prior to crystallization trials. Initial crystallization using the hanging-drop vapour-diffusion method at a temperature of 289 K was carried out by mixing 2μl of protein solution (20 mg/ml) with an equal volume of a reservoir solution containing ammonium acetate (0.2 M), 2-propanol (30% v/v) and Tris-HCl buffer (0.1 M), pH 8.5.

1.2 Final Crystallization: As the SRL crystals grew in the presence of 2-propanol; various concentrations of 2-propanol, MPD and PEGs of various molecular weights were used to improve the crystal quality and size. Single crystals of identical morphology but of considerably better quality and size were grown within two weeks at 289 K using a reservoir solution containing ammonium acetate (0.2 M), MPD (30%), Tris-HCl buffer (0.1 M), pH 8.5 and a protein concentration in the drop of 8 mg/ml.

RESULTS AND DISCUSSION

In order to understand the structure-function relationship of SRL we crystallized the lectin by hanging drop vapour diffusion method, after several trials. The SRL crystals obtained at the initial crystallization (Fig.1.) were extremely thin, while the drops contained large amount of precipitate.

Final crystallization trials resulted in single crystals of identical morphology and considerably better quality and size (Fig. 2.) Although these conditions produced better crystals, they did not help to eliminate
Fig. 1. Photograph of SRL crystallized by hanging-drop vapour-diffusion method from the initial crystallization trials, showing small needle like crystal, on mixing 2µl of protein (20 mg/ml) with an equal volume of a reservoir solution containing ammonium acetate (0.2 M), 2-propanol (30% v/v), Tris-HCl buffer (0.1 M), pH 8.5 and were grown within two weeks at 289 K
Fig. 2. Photograph of SRL crystallized by hanging-drop vapour-diffusion method after optimization of crystallization conditions, showing crystals of better quality and size, on mixing 2µl of protein (8 mg/ml) with an equal volume of a reservoir solution containing ammonium acetate (0.2 M), MPD (30%), Tris-HCl buffer (0.1 M) pH 8.5 and were grown within two weeks at 289 K.
protein precipitation in the drop. This optimization procedure is a good example of the influence that different non-polar precipitants can have on the quality and size of protein crystals.

In the past, only a few fungal lectins, mostly from higher fungi (mushrooms), have been successfully crystallized. Notable examples are those from Flammulina velutipes, Pleurotus ostreatus and Aleuria aurentia.

Two types of Flammulina velutipes lectin crystals were obtained using 20mg/ml solution of protein in PBS. One crystal was grown from 27 to 29% (w/v) PEG 6000 in phosphate buffer (0.05 M), pH 7 containing NaCl (0.15M) and the other was obtained from ammonium sulphate (1.8 M) in phosphate buffer (0.05 M), pH 7 containing NaCl (0.15M) (Hirano et al., 1987). A lectin from Pleurotus ostreatus has been grown by hanging drop technique using ammonium sulphate (2M), sodium citrate buffer (0.1M), pH 5.6, in presence of potassium/sodium tartarate (0.2M). The hexagonal bipyramidal crystals were obtained over a period of 2-3 weeks (Chattopadhyay et al., 1999). In case of Aleuria aurentia, lectin was crystallized using 10mg/ml of lectin in saturated solution of ammonium sulphate at 4°C and the hexagonal pyramid crystals were obtained after three weeks (Nagata et al., 1991). The crystal structure of first fungal lectin from Aleuria aurentia has been reported which is composed of two
identical 312-amino acid subunits that specifically recognizes fucosylated glycans. The crystal structure of the lectin complexed with fucose reveals that each monomer consists of a six-bladed β-propeller fold and of a small antiparallel two-stranded β-sheet that plays a role in dimerization (Wimmerova et al., 2003). Recently crystallization and preliminary X-ray study of a TF antigen specific fungal lectin from Agaricus bisporus has been reported (Carrizo et al., 2004).

Partial structural characterization of the two fungal lectins from Coprinus cinereus (Cooper et al., 1997) and Rhizoctonia solani (Candy et al., 2001) has been reported based on their primary sequence. C. cinereus expresses two lectins related in sequence and carbohydrate-binding specificity to other mammalian galectins and the conservation implies that galectins evolved to perform very basic cellular functions, by interaction with glycoconjugates resembling blood group A (Cooper et al., 1997). Interestingly Rhizoctonia solani is presumed to be structurally and evolutionarily related to the superfamily of plant lectins, possessing a ricin type lectin motif. And also the sequence similarity is observed between the N-terminus of Rhizoctonia solani agglutinin (RSA) and the N-terminal subdomain of ricin-B from Ricinus communis agglutinin (RCA) (Candy et al., 2001).
Unlike fungal lectins legume lectins are most widely studied and structurally characterized (Loris et al., 1998) and much is known about their specificity, sequence and three-dimensional structure. The first lectin to be purified was Concanavalin A, from *Canavalia ensiformis* (Jack bean), which was also the first lectin that was successfully crystallized (Sumner, 1919; Sumner and Howell, 1936) and X-ray analysed (Hardman and Ainsworth, 1972; Becker et al., 1975). Subsequently lectins from cereal (Wright, 1989), viral (Wilson et al., 1981; Weis, 1997) and animal lectins (Weis et al., 1991, 1992; Liao et al., 1994; Lobsanov et al., 1993; Rini, 1995, Gabius, 1997; Weis, 1997) were also analyzed.

The wealth of information available indicates that the plant lectins represent a heterogeneous group of oligomeric proteins that vary widely in size, structure and molecular organization, and in the constitution of their combining sites. In most lectins the subunits are identical, but lectins composed of non-identical subunits are also known (Goldstein and Poretz, 1986). Lectins can be classified into two major classes based on their subunit structure. Accordingly the group I, comprise single chain lectins having either two or four identical chains of molecular weight of 25 to 30kDa. For example Con A, and *Dioclea grandiflora* (Wang et al., 1971) lectin, both glucose/mannose specific are made up of four identical subunits and belong to the phaseolae group. The second class of lectins are those
which contain two pairs of different subunits of molecular weight 15 to 20 kDa (β-chain) and 5 to 7kDa (α-chain) and these belong to viceae tribe. Other well known lectins belonging to this group include lectins from soybean and *Phaseolus vulgaris* (Baumann *et al*., 1982; Roberts *et al*., 1982).

The amino acid sequences of several hundreds of lectins from various sources and in addition the three-dimensional structures of some two hundreds of them have been elucidated (Sharon and Lis, 2004; Vijayan and Chandra, 1999) and new sequences and structures are being added every year. Comparison of the primary structures of approximately twenty lectins demonstrated that they possess a high degree of sequence homology, displaying over 20% invariant residues, most of which are involved in binding of sugar moieties and in metal ion coordination (Lis and Sharon, 1986a; Sharon and Lis, 1993). Also, the amino acid sequences of these and partial sequences of many other lectins, indicate striking homology in the α-chains and in the central part. Even, in β-chains, extensive homologies have been observed. Con A, which is a single chain polypeptide of 237 amino acids, reveals extensive homology with lentil lectin, soybean agglutinin and fava bean lectin (Sharon and Lis, 1993). Despite differences in the sugar binding and molecular properties, they have considerable homology in amino acid sequence, and are related to each other in unusual ways. This stunning realization is becoming more apparent as X-ray crystal
structures of more and more legume lectins are being solved (Rini, 1995; Vijayan and Chandra, 1999).

To the best of our knowledge, SRL is the first fungal lectin for which its preliminary x-ray crystallographic data was made available at a very high resolution of 1.1Å (Leonidas et al., 2003). Unfortunately because no other similar crystal structures were known for comparison and the N-terminal amino acid of SRL was blocked we were unable to determine the partial amino acid sequence also. Hence there was a delay in determining the complete structure of this lectin. Mean while the crystal structure of *Aleuria aurentia* was successfully studied (Wimmerova et al., 2003). Recently Leonidas et al., have determined the complete structure of both monomeric and dimeric forms of SRL. The active site determination of SRL by X-ray diffraction using different ligands is in progress (Unpublished). The crystal structure studies of SRL will help in understanding its TF antigen specificity particularly to understand the critical groups in TF antigen essential for its recognition and also its physiological role in development and morphogenesis of the fungus.