Chapter III

Binding Studies of SRL
With
Salivary Glycoproteins
Increasing interest has developed in using saliva to diagnose systemic
diseases because of the simplicity in its collection. Testimony to this
growing interest is reflected from the reviews published on diagnostic uses
of saliva (Mandel, 1990; Malmud, 1993; Wu et al., 1994; Amerongen and
Veerman, 2002; Streckfus and Bigler, 2002; Kaufman and Lamster, 2002).
However, there are only a few studies in the literature concerning the use of
saliva to detect malignancies remote from the oral cavity. These reports
deal with the identification and quantification of cancer related proteins in
saliva, which were previously described to be present in/on cancer tissues
or elevated in the serum of cancer patients. For example the elevated levels
of salivary α amylase activity in oral cancer patients (Bassalyk, et al., 1992)
or the identification of serum cancer antigens such as CA125 (Chen et al.,
1988; 1990). Thus the importance of such studies envisages the basis for
continued cancer biomarker research.

Majority of constituent salivary proteins are complex mucoproteins
or mucins, which contain a large portion of carbohydrates. Major class of
mucins is secreted by submandibular gland and minor salivary glands. These
glycoproteins contain carbohydrate chains of varying size, predominantly
rich in galactose, mannose, hexosamines (glucosamine and galactosamine),
fucose and sialic acid (Tenovuo, 1989). Human mucins constitute a family
of at least two classes; multisubunit higher molecular weight species,
designated MG 1, and a lower molecular weight single subunit species MG
Salivary proline-rich proteins and amylases also occur in multiple forms as glycosylated or non-glycosylated species (Kauffman et al., 1987; Bennick, 1987; Oppenheim et al., 1987).

Salivary mucins are the major constituents of saliva and are characterized by high sugar to protein ratio and occur as complex diverse group with wide ranging molecular masses falling in the range $10^2 \text{kDa}$ to $10^4 \text{kDa}$. Most contain about 30% N-acetylhexosamines, a variable amount of sialic acids (8% - 33%), trace to 15% galactose and fucose, little or no mannose, and sometimes sulphate esters. In many mucin molecules, the oligosaccharide sequence is the same as that which determines the specificity of blood group substances and that populate the extra cellular surface of the plasma membrane. Mucins bearing a large proportion of relatively short carbohydrate side chains are valuable tools for the purification and characterization of lectins (Wu et al., 1994) which in turn are useful in the insitu localization of glycoconjugates in tissue sections, in defining host-parasite reactions and as cancer markers.

The high molecular-weight mucin MG1 and the low molecular-weight mucin MG2 have been previously isolated and characterized biochemically as glycoproteins (Levine et al., 1987). Cloning studies have shown that MG1 is actually a mixture of mucin gene products consisting predominantly of MUC5B and, to a lesser extent, MUC4 (Nielsen et al.,
1997). MG 2 is the product of the MUC7 gene (Troxler et al., 2000). Two isoforms of MUC 7 have been reported from saliva till date, which differ in the degree of sialylation. MUC 7 can bind to wide variety of microorganisms, which supports its protective role (Bolscher et al., 1999). This function is not limited to the oral cavity but also concerns the respiratory and alimentary tracts. The carbohydrate moiety of MUC 7 is mainly composed of mono-, di-, and tri-saccharides (Bolscher et al., 1999). Little is known about the existence of heterogeneity in the carbohydrate moiety of MUC 7 and how individual, tissue specific, and pathologically induced differences in glycosylation may influence its protective activity.

A seemingly subtle change in sugar sequence in these mucous glycoproteins may indicate the onset of disease, such as is observed in many types of cancer. The loss of peripheral monosaccharide residue may unmask epitopes that in healthy individuals are safely protected (Croce et al., 2001). The immunotherapeutic and / or diagnostic, metastatic potential of some of these mucin structures such as Galβ1-3 GalNAc (TF antigen), sialosyl-Tn and Tn have been demonstrated in human saliva (Wu et al., 1994).

Synthesis and secretion of mucin are common features of glandular epithelial tissues and the expression of mucin antigens have been investigated mainly in adenocarcinomas (Akyurek et al., 2002; Carvalho et al., 1999; Byrd and Bresalier, 2004). Normal squamous cells do not secrete mucins, but they were observed in the serum of esophageal squamous cell
carcinoma (ESCC) (Kannan et al., 2003) and cervical SCC patients (Reddi et al., 2000). Altered expression of mucin-type glycoproteins has been reported also from oral SCC (Croce, 2001). There were no reported studies, which utilized lectins to recognize, and to quantify cancer associated mucin-type glycoproteins in saliva. This prompted us to use SRL, a novel TF antigen binding lectin, which was identified and purified in our laboratory.

Lectins are a class of carbohydrate binding proteins, commonly detected by their ability to precipitate glycoconjugates in solution or to agglutinate cells. Lectins form cross-links between polysaccharides or glycoproteins in solution and induce their precipitation, which can be inhibited by the sugar ligands for which the lectins are specific. Analogy of lectin-saccharide interactions with those of antibody-antigen reactions prompts the application of lectins in the detection, quantification and purification of glycoproteins and glycopeptides in solution (Lis and Sharon, 1998). Most antibodies to mucins are directed against carbohydrate determinants. Mucus glycoproteins may be difficult to assay using conventional antibody techniques, because antibodies generally have narrow specificity. Therefore the broader specificity of lectins makes them particularly useful for quantification of mucins (Rhodes et al., 1995). Since altered glycosylation is a universal feature exhibited by cancer cells and certain types of glycan structures are well known markers for tumor progression (Dennis, 1992; Fukuda 1996; Kim and Varki, 1997), lectins
binding to such marker antigens are widely employed for detection of cancer-associated glycosylation changes (Sharon and Lis, 2003; Gabius et al., 2004). Cancer associated antigens occurring in mucins and mucin type glycoproteins have been of special interest (Bhavanandan, 1991), in cancer biology for studying novel cancer cell surface molecules (Gabius et al., 2004).

Lectin from the phytopathogenic fungus Sclerotium rolfsii (SRL), purified and characterized in this laboratory has a complex sugar specificity that recognizes DGalβ1→3GalNAcα-O-ser/thr. This disaccharide is called Mucin Core 1 structure and is considered as oncofetal antigen, TF antigen, expressed on cells during metastasis. Many such TF antigen binding lectins are known, but SRL differs from these lectins by having additional requirement for intact peptide back bone attached to the carbohydrate moiety (Swamy et al., 2001; Wu et al., 2001). It does not bind to free Gal or GalNAc or disaccharide DGalβ1→3GalNAc. Thus SRL with its unique TF antigen binding property would be promising tool for monitoring aberrant glycosylation and the changes associated with cancer. Present chapter discusses the studies carried out to investigate the expression of cancer associated TF antigen in the saliva of oral cancer patients. Investigation included lectin precipitation assays, SDS PAGE of salivary glycoproteins and lectin blot studies by western blotting to localize mucin-like cancer-associated antigen in the saliva of cancer patients.
3.1. Materials and General Methods

3.1.1. Saliva samples

Saliva samples collected for the earlier studies described in the first chapter were used for these studies. However the glycoproteins were prepared from these samples by dialysis followed by freeze-drying. Freeze dried samples after estimating protein and carbohydrate contents were used for all the studies described in this chapter.

3.1.2. Reagents and chemicals:

Bovine serum albumin (BSA), CM Cellulose, Neuraminidase (*Clostridium perfringens*), N-hydroxysuccinimidobiotin, standard protein molecular weight markers were obtained from Sigma Chemical Co., St. Louis, USA. Bio Gel P-60 was purchased from Bio-Rad Laboratories, Richmond, California. Sephadex G-75 was obtained from Pharmacia Fine chemicals, Upasala, Sweden.

Acrylamide and N-N’-Methylene bisacrylamide were purchased from Koch-Light Lab Pvt. Ltd., Colabrook Berks, England. N N N N’-tetramethylethylene diamine (TEMED) was procured from BDH Chemicals Ltd., Poole, England and nitrocellulose membrane (0.45 μm) was purchased from Sartorius AG, Germany. Silver nitrate was purchased from Qualigens Fine Chemicals, Mumbai, India. Streptavidin peroxidase conjugate (6-8
units/mg) and tetramethyl benzidine / hydrogen peroxide (TMB / H₂O₂), were procured from Bangalore Genei Pvt. Ltd. India.

All other chemicals used were of analytical reagent grade. All the reagents were prepared in twice glass-distilled water. Samples of *Sclerotium rolfsii* lectin (SRL) and Peanut agglutinin (PNA) were purified as per the methods described.

3.1.3. *Sclerotium rolfsii* Lectin (SRL)

3.1.3.1. **Purification of SRL:** *Sclerotium rolfsii* lectin (SRL) was purified from the sclerotial bodies formed from the fresh cultures grown on corn-sand culture medium by adapting the protocol developed in this laboratory (Swamy *et al.*, 2001).

For the purification of SRL, *Sclerotium rolfsii* was grown on corn-sand semi solid medium as it produces larger and higher yields of sclerotial bodies. Thoroughly washed clean coarse sand, sterilized by autoclaving, was mixed with crushed corn in the ratio of 9:1 (w/w) and moistened with distilled water. This semi solid medium was distributed in 500ml Erlenmeyer flasks and autoclaved. The medium was inoculated with sclerotial bodies. The flasks were incubated at room temperature for several days and were harvested for matured sclerotial bodies after their formation (approx. 30 days).
Matured sclerotial bodies of *S. rolfsii* were harvested from the cultures grown on sand-corn medium and were ground finely using pestle and mortar, the fine powder stored under desiccation at 4°C till further use. This dried powder of sclerotial bodies was used as starting material for the purification of lectin.

3.1.3.2. Preparation of crude lectin extract: Dried powder of sclerotial bodies (10 gm) was suspended in 500 ml of 50mM acetate buffer containing 150mM NaCl, pH 4.3 and kept for extraction by stirring overnight at 4°C. The homogenate was centrifuged at 10,000 x g, for 30 min, at 4°C, and the supernatant obtained was dialyzed extensively against 50mM acetate buffer, pH 4.3, containing 50mM NaCl. Light brown coloured dialyzed extract (crude extract) was subjected to (0 – 30 %) methanol precipitation.

3.1.3.3. Methanol precipitation: The crude lectin extract (500ml) maintained at 0°C, was subjected to methanol precipitation by adding chilled methanol dropwise using peristaltic pump, with constant stirring to reach 30% methanol concentration. The resulting precipitate was clarified by centrifugation at 10,000 x g at 0°C for 30 minutes. The precipitate formed at 0 - 30% was dried over a stream of cold air and dissolved in 5.0 ml of 50mM acetate buffer (pH 4.3) containing 75mM NaCl and dialyzed against the same buffer. After dialysis the precipitate formed was removed by centrifugation and the lectin activity, protein and total sugar content were estimated in the supernatant fraction.
3.1.3.4. **Cation exchange chromatography on CM Cellulose:** Methanol precipitate (0-30%) obtained from the fractionation step was subjected to Cation exchange chromatography on CM Cellulose. Methanol precipitate (obtained from 10 gm powdered sclerotial bodies) dialyzed against 50mM acetate buffer containing 75mM NaCl, pH 4.3 was applied on to a CM Cellulose column (20 x 2.0cm), which was previously equilibrated in 50mM sodium acetate buffer, containing 75mM NaCl, pH 4.3, at a flow rate of 20ml/hr. Fractions of 3.0ml were collected on FRAC-100 automatic fraction collector (Pharmacia). Washing the column with equilibration buffer, eluted unbound proteins and the washing continued till the absorbance of the eluting fractions read zero at 280nm (double beam spectrophotometer, Hitachi DU 2800). Strongly adsorbed proteins were eluted using salt gradient (75mM-500mM NaCl) in the equilibration buffer. Lectin fractions eluted were monitored by measuring the absorbance at 280nm and determining the lectin activity in each fraction after suitable dilutions. Peak fractions containing lectin activity were pooled, dialyzed against 50mM pyridine acetate buffer pH 4.3 and lyophilized.

3.1.3.5 **Gel filtration Chromatography on Bio Gel P-60:** Since efforts to further purify SRL either by hydroxyapatite or phenyl Sepharose column were unsuccessful, gel filtration chromatography on Bio Gel P-60 column was used.
Bio Gel P-60 (particle size 100-200 mesh) was suspended in 50mM acetate buffer, pH 4.3 containing 100mM NaCl, allowed to swell and fine particles were removed by decantation. The gel slurry was de-gassed by keeping in a vacuum desiccator for 48 hours. The swollen gel was packed into a column (45 x 2.5cm) and equilibrated with the same buffer. The flow rate of the column was maintained at 12ml/hr using a peristaltic pump. The lyophilized lectin sample eluted from CM Cellulose column was dissolved in equilibration buffer (1.5ml) containing 3% sucrose was layered carefully on the column. The column was eluted with the same buffer at a constant flow rate of 12ml/hr. Fractions of 3.0ml were collected and the elution of the lectin peak was monitored by determining the absorbance at 280nm and by determining the hemagglutination activity in the eluted fractions.

3.1.3.6. Gel filtration Chromatography on Sephadex G-75: The major lectin peak from Bio Gel P-60 column was dialyzed against 50mM pyridine acetate buffer, pH 4.3 and lyophilized. The lyophilized protein was dissolved in 1.0ml of 50mM sodium acetate buffer, pH 4.3 containing 100mM NaCl. This sample was loaded on to the Sephadex G-75 column (100 x 1.5cm), equilibrated in 50mM sodium acetate buffer pH 4.3 containing 100mM NaCl. The gel filtration chromatography on Sephadex G-75 was carried out under similar conditions as mentioned for gel filtration chromatography on Bio Gel P 60. The eluted lectin sample
was dialyzed against 50mM pyridine acetate buffer, pH 4.3 and lyophilized and stored at -20 °C till further use.

3.1.4. Purification of peanut agglutinin (PNA)

PNA required for the lectin blotting studies was prepared according to the method of Lotan et al., (1975), using lactose sepharose affinity chromatography. Lactose - sepharose 4B affinity matrix was prepared by using divinyl sulfone coupling as coupling reagent as described by Porath and Errson (1973).

Defatted peanut powder (10 gm), kept for extraction with 250 ml of PBS, pH 7.2, overnight at 4 °C by stirring magnetically. Resulting extract was clarified by centrifugation at 3000 rpm and the supernatant was subjected to 0-60 % ammonium sulfate precipitation at room temperature, by adding solid ammonium sulfate. Precipitate (0 – 60%) formed was collected by centrifugation at 5000 rpm at 22 °C, suspended in 10 ml of PBS and dialyzed extensively against water to remove ammonium sulfate.

Dialyzed extract was passed through lactose – sepharose 4B affinity column (1.5 x 15 cm) equilibrated with PBS at a flow rate of 25 ml / hr. After loading, the column was washed with PBS till the absorbance of the eluting fractions read zero absorbance at 280 nm (Hitachi DU 2800) and fractions of 3.0 ml were collected. Affinity bound lectin was eluted by using 150 mM lactose in PBS, and the protein fractions were pooled and extensively dialyzed against water and the lectin activity was determined by
hemagglutination assay. Homogeneity of the purified lectin sample was determined by SDS PAGE. Purified lectin was freeze-dried and stored at -20 °C till further use.

3.1.5. Preparation of Biotinylated SRL and PNA

Biotinylated lectins SRL and PNA required for lectin blot studies were conjugated with N-hydroxysuccinimidobiotin as described by Duk et al., (1994). Lectin (SRL / PNA), dissolved in 100 mM sodium phosphate, 0.15 M NaCl, pH 7.2, at a concentration of 4mg/ml to which D N-hydroxysuccinimidobiotin dissolved in DMSO to a final concentration of 20mg/ml was added and mixed well. The concentration of biotin added to the lectin solution was to get 20-fold molar excess of biotin reagent over the lectin. The reaction was allowed to proceed at room temperature for an hour. Excess of biotin was removed by extensive dialysis against PBS and biotinylated lectins were stored at 4°C till further use.

3.1.5.1. Confirmation of biotinylation: Prior confirmation of biotinylation of the lectins was done as described by Zanetta et al., (1993) by serially diluting the biotinylated lectins (20μl) with saline in ELISA microtitre plate which was dried in an oven at 37°C. The wells were then saturated with 100μl of 3% p-BSA in PBS and incubated for one hour at room temperature. After removal of the liquid and three washes with PBS, Streptavidin-HRP (1→1000 dilution) in PBS was added to all the wells and
incubated for one hour at room temperature. The plates were repeatedly washed with PBS (one hour) and water (last wash) and finally incubated with TMB/H$_2$O$_2$ (1→10 dilution) in water at 37°C and the color intensity was observed.

3.1.6. Preparation of periodate-BSA (p-BSA)

Periodate treated bovine serum albumin (p-BSA) required for blocking the nonspecific binding of lectin on lectin blots was prepared according to the procedure described by Glass et al., (1981). BSA was subjected to periodate oxidation to release the associated glycans and was converted to p-BSA. BSA (fraction V) in 0.1M sodium acetate buffer, pH 4.5 (4g/100ml) was treated with 10mM periodic acid for 6 hours at room temperature. Then excess of periodate was eliminated by adding glycerol to a final concentration of 10mM and later the solution was dialyzed extensively against 10mM PBS and lastly against water and freeze-dried.

3.1.7. Analytical methods

3.1.7.1. Determination of haemagglutination activity: The lectin activity in the extract of sclerotial bodies was determined by haemagglutination assay using trypsinized rabbit erythrocytes. Trypsinized erythrocytes for haemagglutination were prepared by the method described by Lis and Sharon (1972).
Haemagglutination activity in the extract of sclerotial bodies was assayed by the serial two-fold dilution technique of Liener and Hill (1953) in U-bottomed microtitre assay plates, with some modifications. 50 µl of saline was added to all the wells of a row. 50 µl of lectin sample was added only to the first well of the assay plate. The contents of the first well (100 µl) were mixed well and 50 µl was transferred to second well. The process was repeated serially for the remaining wells. Thus, the lectin extract was serially two fold diluted to which 50 µl of trypsinized erythrocyte suspension was added and gently mixed on a rotary shaker. After incubation for one hour at 37°C, the plates were visually examined for haemagglutination. The highest dilution of the extract, causing visible haemagglutination was regarded as the 'titre'. The protein content in the highest dilution causing visible agglutination is referred to as 'one unit' of haemagglutinin activity. It is otherwise expressed as MCA (minimum concentration of protein required for agglutination). The specific haemagglutination activity is expressed as units of activity per mg of protein.

3.1.7.2. Estimation of total protein and total sugar: Protein content in the extracts was estimated by the method of Lowry et al., (1951) using bovine serum albumin (BSA) as standard. The total sugar present in different samples was estimated by the phenol-sulphuric acid method of Dubois et al., (1956) with some modifications (Saha and Brewer, 1994).
3.1.7.3. Lectin - precipitation assays: Quantitative precipitation assays were carried out to determine the SRL reacting salivary glycoproteins from the saliva of normal individuals and cancer patients. These assays were performed using saliva samples collected from 10 healthy subjects and 10 patients of oral SCC. Precipitation assay was performed in duplicate in 1.5ml centrifuge tubes as described by So and Goldstein (1967).

Purified SRL (80 µg) in PBS was incubated with 0 – 140µg , salivary glycoproteins (as estimated by phenol-sulfuric acid method) in different tubes in a total volume of 300µl for 1 hour at room temperature. Tubes were later incubated at 4°C for 4 days and the precipitate formed was collected by centrifugation at 3000rpm for 15 minutes at room temperature. Supernatant was carefully decanted and the precipitates were allowed to drain with the tubes in an inverted position on blotting paper. Precipitates were washed twice by suspending in 500 µl of 50mM phosphate buffer containing 1.0M NaCl, pH 7.2, agitating with a vortex mixer, centrifuging and draining. The tubes were centrifuged and the clear supernatant was used for determining protein by the method of Lowry et al., (1951). PBS containing only saliva (80µg sugar) and PBS containing only SRL were used as controls. Saliva samples from control group and test group were used for the assay. Protein precipitated in micrograms was plotted against the salivary glycoprotein concentrations added.
3.1.7.4. SDS-PAGE of salivary glycoproteins: SDS-PAGE of salivary glycoproteins was performed according to the method of Laemmli (1970), to study the electrophoretic profile of salivary proteins in healthy individuals and oral SCC patients. Lyophilized saliva samples were prepared in sample buffer (500 mM tris-HCl, pH 6.8) containing 2% SDS, 10% glycerol and 0.05% Bromophenol blue (tracking dye) and incubated at 37°C overnight to ensure efficient reduction.

Electrophoresis was performed in 10% vertical slab gel of 1 mm thickness using Mighty Small II SE 250 (Hoefer Scientific instruments, Sanfransisco, USA) electrophoresis unit. Stacking gel of 4.5% and resolving gel of 10% were used. 5μl of each preparation (1μg protein / μl) was loaded in to the sample wells of stacking gel. A mixture of standard protein molecular weight markers (5μl) containing phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20.1 kDa) and lysozyme (14.3 kDa), prepared in the same sample buffer was also electrophoresed simultaneously along with saliva samples. Electrophoresis was initially carried out for 20 minutes with voltage set to 90V in stacking gel and then for 45 minutes with 150V in resolving gel.

3.1.7.5. Identification of salivary glycoproteins by Silver staining:

After the electrophoresis, the gel was separated from the unit carefully, and transferred to staining box. All steps were performed in glass
containers on a shaker at room temperature. Rapid silver staining procedure (30 minutes) was used (Nesterenko et al., 1994). Stock solutions used in silver staining were 50% acetone, 50% TCA, 10% sodium thiosulphate and 20% silver nitrate. After the electrophoresis, the gel was fixed using fixative containing 60 ml acetone stock with 1.5 ml TCA stock and 25 μl formalin for 5 minutes. The gel was repeatedly rinsed and washed with double distilled water for 5 minutes to ensure removal of salts. The gel was pretreated with 60 ml acetone stock for 5 minutes followed by sodium thiosulphate solution (100 μl stock in 60 ml double distilled water. After rinsing the gel with double distilled water, it was treated with silver nitrate solution containing 0.8 ml of silver nitrate stock, 0.6 ml formalin in 60 ml double distilled water for 8 minutes. The gel was rinsed using double distilled water and treated with developer solution containing 1.2g sodium carbonate, 25μl formalin and 25μl sodium thiosulphate in 60ml double distilled water till the protein bands acquired desired intensity (usually less than 15 seconds). The gel was immediately transferred to stop solution containing 1% glacial acetic acid in double distilled water followed by gentle rinsing with double distilled water and the gel was preserved in 1.5% glycerol in double distilled water.

3.1.7.6. Lectin blotting studies of Salivary Proteins: After the electrophoresis, the gel was carefully taken out of the unit and stacking gel and dye front were excised. The gel size was measured and the trimmed gel
was transferred to a large petri dish containing transfer buffer (2 mM Tris, 250 mM glycine (pH 8.3), 0.1% SDS). Six Whatman filter paper (No. 3) strips and one nitrocellulose membrane were cut exactly to the size of the trimmed gel. Immediately, the nitrocellulose membrane was transferred to a glass petri dish with transfer buffer. Semidry blotting method was used to transfer the salivary proteins. The graphite plates were gently moistened with transfer buffer using cotton swab. Three Whatman filter paper strips were dipped in transfer buffer and placed onto the center of the lower graphite electrode surface. Pre wetted nitrocellulose membrane was placed on these filter paper strips followed by trimmed gel. Remaining filter paper strips were wetted with transfer buffer and placed carefully on the gel. A clean glass rod was gently rolled over this sandwich to remove any trapped air bubble. The upper graphite electrode plate was closed gently on the sandwich. 75 mA of current was used for the electrophoretic transfer for 3 hours (1 hour at room temperature and 2 hours at 4°C).

3.1.7.7. Localization of SRL-binding salivary glycoproteins: After the electrophoretic transfer, the nitrocellulose membrane was removed using forceps and the separated salivary glycoproteins binding to SRL / PNA were localized on the membrane by using biotinylated lectins.

Salivary glycoprotein blots were washed thrice with PBS, rinsed and incubated in PBS for 15 minutes. Since PNA binds only to desialylated TF
antigen, the blots were pre incubated with neuraminidase (0.1 unit/ml) at room temperature for 30 minutes. Blots were rinsed with PBS for 15 minutes. Nonspecific binding sites for the lectins on the blots were blocked by suspending the blots in 0.1% carbohydrate free BSA (pBSA) in 0.1M PBS solution at room temperature for one hour followed by washing with PBS pH 7.2, several times. The blots were then incubated in biotinylated SRL/ biotinylated PNA (20 µg/ml) in PBS, overnight at 4°C. Lectin blots were then rinsed and washed extensively with PBS to remove unbound biotinylated lectin and transferred to clean glass trays containing sufficient volume of streptavidin-HRP (1 → 1000 dilution) in PBS to immerse the blots. After incubation for one hour at 37°C, blots were rinsed with PBS three times, every time suspending and with gentle agitation for 15 minutes with a last rinse in double distilled water. Finally the blots were incubated with chromogen substrate TMB. H₂O₂ for 5 minutes and gently rinsed with double distilled water. The blots were immediately photographed, as the color developed is stable only for 5 to 10 minutes.

3.2 Results and Discussion

SRL and PNA, required for various salivary glycoprotein-lectin interactions were purified using the protocols. Purification of the SRL from the sclerotial bodies of the fungus Sclerotium rolfsii, involves extraction of the lectin in 50 mM acetate buffer, pH 4.3 containing 140 mM NaCl,
followed by 0-30% methanol precipitation. Methanol precipitation step is particularly helpful in eliminating large amounts of polysaccharide associated with the crude extract apart from unwanted proteins. Methanol precipitate (0-30%) was fractionated on ion exchange chromatography on cation exchanger, CM-Cellulose. Elution profile of the SRL on CM cellulose is given in the figure. 3.1. Lectin activity in the eluting fractions was monitored by hemagglutination assay using trypsinized human O group erythrocytes. Initial washing of the column with the equilibrating buffer facilitated the elution of 75% of the total protein loaded, devoid of lectin activity. Subsequently strongly adsorbed protein having lectin activity constituting almost 22% was eluted on elution with NaCl gradient (0-500mM) in the initial equilibrium buffer. Electrophoresis on SDS-PAGE of this peak showed several associated contaminating proteins. Hence this lectin peak fraction was further subjected to fractionation by gel filtration chromatography on Biogel P60 column. Elution profile of the lectin on Biogel P60 column is given in figure 3.2. Although the lectin was eluted in a major protein peak with the activity, the fraction was associated with a small portion of high molecular weight protein as shown by SDS-PAGE. Final purification of the lectin was achieved by gel filtration chromatography on Sephadex G75 column; the elution profile is given in figure 3.3.
Figure 3.1. Cation exchange chromatography of crude lectin extract (methanol precipitate) of *Sclerotium rolfsii* on CM Cellulose. The column (30 x 1.5 cm) was equilibrated in 50mM acetate buffer (pH 4.3) containing 75 mM NaCl, and 30 ml of the crude lectin extract was passed through the column. The unadsorbed proteins, pigments and polysaccharides were washed with the equilibration buffer & adsorbed lectin peak was eluted with the buffer gradient from 75 mM NaCl to 500mM NaCl. Fractions of 3.0 ml were collected at a flow rate of 20.0 ml/hr.

- - Absorbance at 280nm
- - Hemagglutination activity
- - NaCl gradient (75mM to 500mM)
Figure 3.2. Gel filtration chromatography of the *Sclerotium rolfsii* lectin on Bio Gel P-60. CM Cellulose eluted lectin sample (15mg), in 1.5 ml of 50mM Sodium acetate buffer (pH 4.3) containing 100mM NaCl, was applied onto the column (45 x 2.5cm), equilibrated in the same buffer, and eluted at a flow rate of 12.0ml/hr. Fractions of 3.0ml were collected.

- - - Absorbance at 280nm  - - - Hemagglutination activity
Figure 3.3. Gel filtration chromatography of the *Sclerotium rolfsii* lectin on Sephadex G-75. Bio Gel eluted lectin sample (10mg), in 1.5 ml of 50mM Sodium acetate buffer (pH 4.3) containing 100mM NaCl, was applied onto the column (100 x 1.5cm), equilibrated in the same buffer, and eluted at a flow rate of 12.0ml/hr. Fractions of 3.0ml were collected.

Absorbance at 280 nm

Hemagglutinating activity

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0.5

0.4

0.3

0.2

0.1

0

0

10

20

30

40

50

Fraction number

Hemagglutination activity X 10^3

Absorbance at 280 nm

Hemagglutinating activity
Lectin peak eluted as single symmetrical peak, indicating the homogeneity of the lectin preparation, which was further confirmed by SDS PAGE figure. 3.4. Peanut agglutinin (PNA) purified by affinity chromatography on lactose-sepharose column essentially according to the method described by Lotan et al., (1975). Purified PNA sample showed single band on SDS PAGE indicating the homogeneity of the preparation (figure 3.5).

Precipitation assays were carried out in order to detect the glycoprotein species, which react specifically with SRL, in the saliva of normal individuals and cancer patients. Precipitation assays were performed by incubating 80 μg (fixed) of SRL with varying amounts of salivary glycoproteins whose concentration was expressed as carbohydrate content. The amount of protein precipitated against each concentration of the glycoprotein added is plotted. Results of the precipitation assays carried out with different normal saliva samples (plates N1 to N10) are presented in figure 3.6.

Results of the precipitation assay obtained with the salivary glycoprotein samples of cancer patients (plates C1 to C10) are presented in figure 3.7. The protein precipitated against each concentration of salivary glycoprotein used was nil or negligibly low with all the 10 normal samples, indicating the absence of any SRL binding glycoprotein in these saliva samples. On the contrary, results obtained with salivary glycoproteins of
Figure 3.4. SDS-PAGE of purified SRL in 12 % gel showing the homogeneity of the lectin: lane A; Crude extract of the lectin (50 µg), lane B; Purified lectin (10 µg), lane C; Standard molecular weight marker proteins: BSA (66 kDa), Ovalbumin (45 kDa), Carbonic anhydrase (29 kDa), Trypsinogen (24 kDa), β-lactoglobulin (18.4 kDa), Lysozyme (14 kDa).
Figure 3.5. Homogeneity of purified PNA. SDS-PAGE of purified peanut agglutinin (PNA) in 7.5% gel showing the homogeneity of the lectin: lane A; Ammonium sulfate precipitate (0-60%) of crude extract of the lectin (40 µg), lane B; Purified lectin (8.0 µg).
Figure 3.6: Precipitation of SRL by salivary glycoproteins. Plates N1 to N6 are the salivary glycoproteins of saliva from normal individuals.
Figure 3.6: Precipitation of SRL by salivary glycoproteins. Plates N7 to N10 are the salivary glycoproteins of saliva from normal individuals.
Figure 3.7: Precipitation of SRL by salivary glycoproteins. Plates C1 to C6 are the salivary glycoproteins of saliva from patients of oral SCC.
Figure 3.7: Precipitation of SRL by salivary glycoproteins. Plates C7 to C10 are the salivary glycoproteins of saliva from patients of oral SCC.
cancer patients showed effective precipitation of protein against each concentration of the salivary glycoprotein added. Precipitation indicated the occurrence of the SRL interacting glycoprotein component(s) in all the samples. However, the amount of protein precipitated for any particular concentration varied remarkably for individual cancer patient saliva. That is, the ratio of the concentration of glycoprotein added to the maximum protein precipitated (point of maximum precipitation) varied significantly (a minimum of 0.53 for C6 to a maximum of 2.0 for C1) for each of the sample. Lower the value for point of maximum precipitation, better is the hapten efficacy of the glycan to bind to the lectin. It also implies that the quantity of interacting glycoprotein component could be occurring in high concentration in a mixture. Such observations are shown earlier by precipitation assays demonstrating the formation of Glycoprotein-lectin cross-linked complexes in mixed precipitation systems (Manadal and Brewer, 1992). From these results it was evident that specific glycoproteins, which interact with SRL occur in the saliva of SCC patients but not in the saliva of normal healthy individuals.

Since SRL is known to bind specifically to TF antigen, it may be concluded that TF antigen is expressed in the salivary mucins. Since these salivary glycoproteins are heterogeneous complex mixture, lectin precipitation studies cannot identify whether a specific component or multiple components that occur in saliva bind and precipitate SRL.
In order to investigate and identify the glycoprotein components specifically binding to SRL, further studies were carried out by lectin blot studies. Prior to lectin blot investigations, SDS-PAGE analysis of the salivary glycoproteins of healthy individuals and cancer patients was performed in order to determine complexity. Electrophoretic separation pattern on SDS-PAGE of salivary glycoproteins of 9 healthy individuals (N1 to N9) along with the standard molecular weight markers is presented in Figure 3.8.

The results showed remarkable qualitative and quantitative differences in the salivary glycoprotein components of normal individuals. A high molecular weight band corresponding to 98 kDa could be seen only in samples N1, N6, N7, N8 and N9 but not in N2, N3, N4 and N5. However, second major component corresponding to 77 kDa occur in all the samples except in N1, similarly a low molecular weight band corresponding to 14 kDa is seen in all the samples except in N1. Distinct pattern seen with the sample N1 could not explained however it was observed that this sample when dissolved in the sample buffer large portion of the sample did not go in the solution even after boiling for 15 minutes. Another major group falling in the range of 66 to 45 kDa is consistently observed with all the individuals.

SDS-PAGE analysis of the salivary glycoproteins of 10 SCC patients was performed. Electrophoretic separation pattern of 8 saliva samples from
cancer patients (C1 to C8) is shown in figure 3.9 along with the Std molecular weight markers. Unlike normal samples, several distinctly resolved and prominent bands were seen with these saliva samples. Many of the bands are common in all the samples, with a few differences associated with C4 and C8. Major bands common to all the samples are 58 kDa, 38 kDa and 34 kDa. Indeed the bands in the range of 30 kDa and 14 kDa are also common to all the samples. These results indicate the presence of several glycoproteins, which are unique to oral SCC and considering the intensity of the bands, they are expressed specifically in large amounts.

In order to identify the occurrence of TF antigen, a well-known cancer associated antigen on these glycoproteins, lectin blot analysis following SDS PAGE in 10% gel was carried out using biotinylated SRL. In another set, lectin blot analysis was carried out with the same saliva samples using biotinylated PNA, as control, which is well known for its specificity towards TF antigen. Results of the lectin blot assays carried out with saliva from 10 normal healthy individuals showed no binding by either SRL or PNA. But the lectin blot of 10 SCC saliva samples all the 10 investigated, showed specific binding to three bands corresponding to 45 kDa, 29 kDa and 27 kDa by SRL as seen in figure 3.10. Surprisingly, PNA showed binding to only one band corresponding to 45 kDa, but not to 29 kDa and 27 kDa bands (figure 3.11.) for all the 10 SCC saliva samples.
Figure 3.8. Glycoproteins of the saliva samples of normal healthy individuals separated on SDS-PAGE in 10% gels after staining with silver staining; lane M; standard molecular weight markers, phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20.1 kDa) and lysozyme (14.3 kDa), lanes N1 to N9 are the saliva samples of normal healthy individuals.
Figure 3.9. Glycoproteins of the saliva samples from squamous cell carcinoma patients separated on SDS-PAGE in 10% gels after staining with silver staining; lane M; standard molecular weight markers, phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20.1 kDa) and lysozyme (14.3 kDa), lanes C 1 to C8 are the saliva samples of SCC patients.
Figure 3.10. Biotinylated SRL binds to salivary glycoprotein band of Mr 45kDa, 29kDa and 27kDa expressed in the saliva of squamous cell carcinoma patients. Lectin blot analysis of SCC salivary glycoproteins, after SDS-PAGE in 10 % gel, the gel was incubated with the biotinylated SRL followed by interaction with streptavidin-HRP and the color was developed by TMB/H$_2$O$_2$ reaction.
Figure 3.11. Biotinylated PNA binds to salivary glycoprotein band of Mr 45 kDa, expressed in the saliva of squamous cell carcinoma patients. Lectin blot analysis of SCC salivary glycoproteins, after SDS-PAGE in 10 % gel, the gel was incubated with the biotinylated PNA followed by interaction with streptavidin-HRP and the color was developed by TMB/H$_2$O$_2$ reaction.
Although, SRL is known to recognize TF antigen but it has certain subtle differences in its sugar specificity, which may have resulted in binding to additional two bands. For example SRL does not bind to free galactose like PNA and also it binds to sialylated TF antigen. Another important difference is SRL binds to TF antigen only when it is a part of peptide (Swamy et al., 2001). Probably these differences could be responsible for it's binding to two additional bands corresponding to 29kDa and 27 kDa, in addition to 45 kDa band compared to PNA. It is likely that these additional bands could be the variant forms of TF antigen due to aberrant glycosylation. Mucosal glycosylation abnormalities involving decreased mucin sulfation, increased expressions of TF antigen and sialyl-Tn antigen have been demonstrated in colon cancer (Rhodes et al., 1995; Cortfield et al., 1996). Thus our results with PNA and SRL may be interpreted as SRL apart from binding to well chaacterized PNA binding TF antigen, also binds to two other, probably, novel cancer associated antigens. These interesting findings warrant for extensive investigations involving large number of samples, which could lead to identification of new cancer markers. These findings revealing the use of PNA and SRL to identify novel cancer markers in the saliva samples are shown for the first time. To the best of our knowledge there are no reports on the identification of cancer-associated antigen in saliva of oral SCC patients using lectins. Although there are reports of identification of cancer-associated antigens in
serum using lectins (Kannan et al., 2003), our findings are first of its kind. Findings with 10 SCC saliva samples investigated, showed all of them contain specifically SRL and PNA interacting glycoproteins. Thus the study could be of greater significance for diagnostic purpose and also could open new perspectives for the investigation of new cancer-associated antigens.

The altered expression of blood group antigens by many human tumors is well known (Kuhns, 1980; Hakamori and Kannagi, 1983; Dennis, 1992; Campbell et al., 2001). Of particular importance is the increased expression of TF and Tn antigens, which have been identified in more than 90% of the cancers (Springer; 1984; Fukuda, 1996; Kim and Varki, 1997). The expression of this antigen in various human carcinomas led to propose that the TF antigen represents a general carcinoma antigen (Summers et al., 1981; Coon et al., 1982; Ghazizadeh et al., 1984; Ohoka et al., 1985; Langkilde et al., 1989; Wolf et al., 1988; Cao et al., 1995;). Lectins, which bind to this antigen are known to have greater applications (Wu, 1984). TF antigen-binding lectins have been used to investigate the expression of this antigen during malignancy, which leads to the identification of variant forms of the antigen.

Lectins, capable of binding to cancer-associated antigens are known to elicit signaling events relevant for various aspects of cell physiology. Although all lectins interact with almost all kinds of cells, not
all of them elicit similar effects. The biological activities of lectins serve as basis for the manifold applications of lectins in cancer biology. Notable examples are peanut agglutinin, PNA (Lotan et al., 1975), Amaranthus caudatus agglutinin, ACA (Rinderle et al., 1989), a lectin from Agaricus bisporus, ABL (Presant and Kornfeld, 1972) and Vicia villosa B4 isolectin (Puri et al., 1992; Tollefsen and Kornfeld, 1983) which are commonly used in cancer research.

Sclerotium rolfsii lectin has a complex sugar specificity that recognizes DGalβ1→3GalNAcα-O-ser/thr only when it has an intact peptide backbone. Thus SRL differs from other TF antigen-binding lectins in its requirement for additional intact peptide backbone for its recognition (Swamy et al., 2001; Wu et al., 2001). It does not bind to disaccharide or Gal or GalNAc. Thus SRL with its unique TF antigen binding property would be promising tool for probing aberrant glycosylation and the changes associated with cancer.
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