Chapter I

Introduction and Literature Review
1.1. Historical background

Lectins the term derived from legere meaning “to choose” are carbohydrate recognizing/binding proteins of non-immune origin and are other than enzymes and antibodies occurring in most living organisms. These molecules were identified as early as 1888, when castor bean extracts were shown to agglutinate red blood cells. Later it was shown that the active component responsible for the agglutinating property is ricin, a protein and this discovery made the beginning of Lectinology. In fact, the first observation of erythrocyte agglutinating property was demonstrated in rattlesnake venom as early as 1860 and that was the first demonstrated example of lectin activity. Lectins are known to occur in organisms ranging from viruses to bacteria, fungi, plants and animals to perform key physiological functions involving molecular and cellular interactions.

Lectins interact with specific carbohydrates either in solution or on cell surface, non-covalently and reversibly without altering the ligand structure. Majority of lectins are polyvalent containing two or more carbohydrate binding sites, hence their interaction with sugars on the surface of erythrocytes results in cross-linking of blood cells and subsequent precipitation. This phenomenon, known as cell agglutination, is a major detectable property exhibited by many lectins. However this feature is no more recognized in defining lectins (Barondes, 1988). The agglutination or
the precipitation by lectins could be inhibited by specific carbohydrate. Because of this property and since they were initially detected in many plant seeds, lectins are also called phytohaemagglutinins. In the present context lectins are defined as “Carbohydrate binding proteins of non immune origin that agglutinate cells and glycoconjugates and exhibit a specific and reversible non covalent binding activity to carbohydrates and sugar containing substances whether free in solution or on cell surfaces without altering covalent structure of any glycosyl ligand”.

1.2. Occurrence and Biological significance

Lectins are ubiquitous and are widely distributed in living organisms including plants, animals, fungi, bacteria and viruses (Gold and Balding, 1975; Simpson et al., 1978; Hart, 1980). In a broader sense, in all the living systems considering the wealth of knowledge accumulated, lectins are the molecules exhibiting the ability to act as recognition molecules inside or outside the cell.

1.2.1. Plant Lectins

Majority of the lectins studied in the past are from plants and much of our knowledge is because of plant lectins. Several hundreds of these proteins have been isolated and many of them are well characterized and also a large number have been sequenced. The three dimensional structures of over 40 plant lectins mainly from legumes, cereals and monocots have
been elucidated. The functional properties of plant lectins are not clearly understood and are even today debated (Rudiger, 1998). Most plant lectins are argued as storage proteins, which acquire a potential role in defense mechanism against insect or fungal pests (Peumans and Van Damme, 1995; Gatehouse et al., 1999; Janzen et al., 1976; Mirelman et al., 1975). They are also shown to mediate symbiotic association of leguminous plants and nitrogen fixing bacteria (Brock and Madigan, 1991).

1.2.2. Animal Lectins

Although occurrence of lectins in animals particularly in all non vertebrates and in many lower vertebrates was known quite early but till 1970 only three lectins, from eel (Springer and Desai, 1971), snail (Hammarstorm and Kabat, 1969) and horse shoe crab (Marchalonis and Edelman, 1968) were isolated. However later to 1980 the number of animal lectins purified grew rapidly and added new dimension to the functional properties of these lectins. It was soon realized that the animal lectins mediate several important physiological functions such as regulation of differentiation and organ formation (Sharon, 1983), in metastasis of cancer cells (Raz and Lotan, 1987), mediating the phagocytosis of micro-organisms (Blackwell et al., 1985; Kan and Bennett, 1988; Speer et al., 1988; Sharon and Lis, 1989), in the migration of lymphocytes from the blood stream to the lymphoid organs (Junqueira et al., 1995) and also some are known to
function as antitumor and immunomodulatory molecules (Lin and Chou, 1984; Kawagishi et al., 1990; Beuth et al., 1992; Wang et al., 1995; 1996).

1.2.3. Bacterial lectins

Bacterial lectins gathered greater significance when it was shown that they are mediating the host-parasite interactions. The first indication of lectin mediated host-parasite interaction emerged, when it was found that the adherence of gram negative bacteria to eukaryotic cells was specifically inhibited by mannose and methyl α-D-mannoside (Ofek et al., 1978). Also at the same time Aronson et al., (1979) demonstrated that the urinary tract infection by *Escherichia coli* could be prevented by methyl α-D-mannoside in mice. Now it is well established fact that majority of the infectious bacteria including human oral pathogens produce surface lectins, which are referred to as adhesins. These play pivotal role in initiation of infection process by mediating adhesion to host cells (Jann and Jann, 1990; Karlsson, 1995; Rostand and Esko, 1997; Mouricout, 1997; Sharon and Ofek, 2000). Hence the current intense interest in microbial lectins gathered significance in the last two decades. Indeed, microbial surface lectins are now considered to be determinants of virulence in infection of both animals and plants (Skvortsov and Ignatov, 1998; Burdman et al., 2000). Majority of bacterial lectins occur on the cell surface in the form of fimbriae through which the bacteria interact with the surface glycoproteins and glycolipids of host cells. The binding involves not only the terminal oligosaccharide
sequence, but also the ceramide moiety of the receptor (Karlsson, 1989, 1995; Hirmo et al., 1999). Like plant lectins, the bacterial lectins with different sugar specificities are reported (Mireman and Ofek, 1986).

Aspects of host-parasite interactions encompass a wide range of phenomena such as adhesion to epithelial surfaces, interactions with cells of immune system, phagocytic cell interactions, colonization of oral bacteria etc. Most of the bacteria, which infect animals and plants, contain mannose or galactose specific fimbrial lectin (Eshdat et al., 1978; Sharon and Ofek, 1986; Leffler and Svanborg-Eden, 1986). Essentially microbial lectins serve as molecules of recognition between bacteria and epithelial cell or phagocytic cell (Scleisinger et al., 1996). Adherence of bacteria to epithelial surfaces appears to be prerequisite for colonization and infection of numerous tissues (Rudiger et al., 2000). These developments with bacterial lectins, have paved the way for anti-adhesion therapy using carbohydrates for infectious diseases (Mulvey et al., 2001).

1.2.4. Fungal lectins

Occurrence of lectins in fungi was known as early as 1907, when Ford demonstrated strong hemagglutinating activity in the extracts of *Amanita solitaria* and also in 40 species of Agaricaceae (Gold and Balding, 1975). Although lectins widely occur in fungi they did not receive much attention compared to their plant counterparts for long time. However after the report of isolation and purification of *Agaricus bisporus* lectin (Preasant
and Kornfeld, 1972; Kawagishi et al., 1988) and finding its remarkable property of reversible antiproliferative activity on epithelial cell lines (Yu et al., 1993), a sudden spurge in the fungal lectin research was seen (Pemberton, 1994; Straver et al., 1994; Konska et al., 1994; Yoshida et al., 1994; Lehr et al., 1995; Kawagishi, 1995; Neethling and Nevalainen, 1996; Guillot and Konaska, 1997).

Majority of the fungal lectins isolated are from the fruiting bodies and rarely from the vegetative mycelia (Giollant et al., 1993; Kellens et al., 1992; Wang et al., 1998; Oda et al., 2003). Contrary to the established roles of bacterial lectins in host parasite interactions, functional roles assigned for fungal lectins are speculative. Many believe that fungal lectins do mediate host-parasite interactions (Rudiger, 1998) similar to bacterial adhesions. Several other roles are also put forth, mainly based on the source selected for isolation and on the location of the lectin in the fungus (Barak and Chet, 1990; Elad et al., 1983; Inbar and Chet, 1994; Kellens and Peumans, 1990). Some of the roles assigned to fungal lectins are storage proteins (Kellens and Peumans, 1990), fungal-fungal interactions (mycoparasitism) and host parasite interactions (Fukazawa and Kagawa, 1997; Hostetter, 1994; Rudiger, 1998). Another argued function which is gaining greater attention is their involvement in morphogenesis and development of the fungus (Yatohgo et al., 1988; Cooper et al., 1997). Recent structural investigations by X-ray crystallography demonstrated that the fungal lectins are unique
class with approximately 140 amino acids with unique folding similar to bacterial "porins", a family of pore-forming toxins (Cooper et al., 1997; Birck et al., 2004). Majority of them are developmentally regulated and galactose specific, hence they are called fungal galectins (Oda et al., 2003; Swamy et al., 2004).

Fungal lectins are receiving greater attention due to their unique structural and carbohydrate binding properties especially in the past five years, as is evident from the recent advances published (Paaventhan et al., 2003; Wimmerova et al., 2003; Walser et al., 2004; Carrizo et al., 2005)

1.3. Properties of Lectins

1.3.1. Carbohydrate binding property

Majority of lectins recognize monosaccharides, indeed exhibit higher specificity towards oligosaccharides and some of the lectins may not show affinity to any of the simple sugars but bind to complex oligosaccharides.

According to monosaccharide ligand towards which they exhibit the highest affinity, lectins are classified into five groups: Glucose/mannose, galactose/N-acetylgalactosamine, N-acetylgalactosamine, N-acetylglucosamine, fucose and N-acetylgalactosamine (GlcNAc). Most plant lectins specific for mannose combine well with glucose and to some extent with N-acetylglucosamine (GlcNAc), but the binding affinities would change.
Thus, these lectins tolerate some variations at the C-2 of the sugar to which they bind (Goldstein and Hayes, 1978). Exceptions are the mannose-specific lectins from Escherichia coli, which do not react at all with glucose (Firon et al., 1987). On the other hand, galactose-specific lectins do not react with glucose, nor do lectins specific for glucose react with galactose, indicating importance of 4-hydroxyl group configuration. This rule does not always apply to the binding of N-acetylhexosamines. For example, wheat germ agglutinin (WGA), specific for GlcNAc and its oligomers, also binds to GalNAc to certain extent (Privat et al., 1974), while the blood group A-specific GalNAc-binding lectin from *Helix pomatia* interacts with GlcNAc (Hammarstrom et al., 1977). In rare cases, lectins bind to apparently unrelated sugars. The best example is WGA, which in addition to reacting with GlcNAc, reacts well also with NeuNAc (Bhavanandan and Katlie, 1979; Peters et al., 1979; Monsigny et al., 1980).

For some lectins, no efficient monosaccharide inhibitors have been found, and these lectins are inhibited only by oligosaccharides. The minimal structural unit that inhibits the leukoagglutinating isolectin of PHA (L-PHA) is the disaccharide GlcNAcβ2Man; the most complimentary structure is the pentasaccharide Galβ4GlcNAcβ2(Galβ4GlcNAcβ6)Man (Hammarstrom et al., 1982). The most potent inhibitor of the potato lectin is the β 1→4 linked pentasaccharide of GlcNAc (Allen and Neuberger, 1973). The lectin from *Datura stramonium* is also inhibited by oligomers of
GlcNAc and not by the monomer (Horejsi and Kocourek, 1978; Kilpatrick and Yeoman, 1978). It is of interest that the *Agaricus bisporus* lectin (Presant and Kornfeld, 1972) and the blood group N-specific lectin of *Vicia graminea* (Duk et al., 1982) appear to recognise carbohydrate sequences together with the amino acid or peptide to which the latter are linked.

### 1.3.2. Lectin-Glycoconjugate interaction

Lectins due to their extraordinary property to selectively bind carbohydrate moieties they can as well interact with specific carbohydrate ligands of glycoproteins or glycolipids on the cell surfaces. Virtually all cell surfaces are endowed with a glycocalyx, carbohydrate extensions which are part of membrane glycoproteins, glycolipids and glycosaminoglycans together referred to as glycans or glycoconjugates (Schauer et al., 1988). These carbohydrate residues of each of glycoconjugates may encode a large repertoire of information, which differ in number and type of sugar residues, sequence of sugar moieties, the anomeric linkage type, presence or absence of branches and amount of sialic acid. These differences contribute to the complex microheterogeneity of cell surface glycoconjugate structures. Because of their structural complexity and variability, cell surface carbohydrates serve as recognition signals (Sharon and Lis, 1993). Structural modification of oligosaccharide domains of cell surface glycoconjugates occurs during normal cell development and is correlated

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with a host of physiologically important functions such as cell-cell recognition, growth, contact inhibition and differentiation (Hakamori et al., 1974).

The structural diversity encodes unique signals that are recognized by divalent or polyvalent lectins on the surface of apposing cell in a complimentary way analogous to ligand-receptor interaction (Sharon and Lis, 1989). In addition, soluble lectins and glycoproteins may act as bridges by binding to carbohydrates on apposing cells and extracellular matrix.

1.4. Cell surface glycoconjugates as markers

A number of studies have shown that cell surface carbohydrates are modified upon malignant transformation, tumor cell differentiation and metastasis (Freizi, 1985; Hakamori, 1989; Yogeeswaran, 1983; Dennis et al., 1989; Dennis and Laferte, 1987; Joshi et al., 1987; Dennis, 1992; Nicolson, 1984; Irimura et al., 1988; Lu and Chaney, 1993; Lu et al., 1994). Altered glycosylation of cell surface glycoconjugates would result from any of the following events:

1. Increased sialylation or desialylation
2. Increased branching
3. Increased fucosylation
4. Altered sugar chain sequence
Also the level of expression of cell surface carbohydrate-binding proteins (endogenous lectins) is altered in cancer and metastasis (Joshi et al., 1987; Lotan and Raz, 1988; Rak et al., 1992). During neoplastic progression, not only are the levels of surface lectins altered, but also levels of subcellular (intracellular) lectins. Tumour associated serum mucin glycoconjugates serve as important tumor markers in breast, ovary, lung, pancreas, bladder, colon, prostate and esophageal squamous cell cancers (Devine et al., 1993; Feller et al., 1990; Kannan et al., 2003).

Thus tumor associated cell surface glycoconjugates and endogenous lectins continue to be explored in vivo and in vitro as markers for screening, diagnosis, prognosis and targeted therapy and to access the risk and response in cancer therapy (Gabius, 1987a; Gabius et al., 1987; Monsigny et al., 1983; Monsigny et al., 1988; Mackay et al., 1992). Lectins are further useful in drug designing (Karlson, 1991), in distinguishing tumours of different classes (Kayser, 1988), in anatomical localization of malignant tissues (Hoslt and Powers, 1990), for immunomodulation (Beuth et al., 1992; Gabius, 1987) and also in anti adhesive therapy (Chiquet-Ehrismann, 1993).

1.5. Lectins as diagnostic probes

The cellular protein glycosylation pattern is influenced by several physiological changes, such as the occurrence of disease. Thus the altered
glycoform population of a given glycoprotein may be diagnostic of the disease responsible for the alteration itself. Abnormal glycosylation has been detected in cancer development (Dennis et al., 1987). Both qualitative and quantitative lectin binding differences were observed for cytosolic glycoproteins in benign and malignant thyroid neoplasms (Krzeslak et al., 2003).

One of the well studied altered glycoform is the increased expression of the blood group Thomsen-Friedenreich (TF) antigen (Itzkowitz et al., 1989; Campbell et al., 1995). TF antigen is often referred to as oncofetal antigen and is a disaccharide, Galβ1-3GlcNAc linked to either serines or threonines. TF antigen is mucin type carbohydrate antigen which is characterized by class 1 core sequence Galβ1 – 3GlcNAc-α-OSer/thr in oligosaccharide chains (Springer et al., 1979a). This antigen occurring in cell surface glycoproteins of healthy cells is masked (cryptic), but exposed in a high percentage on several human carcinomas and other neoplastic cells (Springer, 1984; Springer, 1997). TF antigen is demonstrated to be over expressed on the cell surfaces in variety of cancers and hence is considered as cancer-associated antigen (Osinaga et al., 1994; Ryder et al., 1984).

The carbohydrate binding specificity of lectins for various cell surface glycoconjugates has been exploited in localization studies to identify structural differences on cell membrane and thus have various histochemical
and cytochemical applications (Menghi et al. 1996; Zambenedetti et al., 1996; Triantafyllon et al., 2004). Thus some of the lectins are finding applications as useful tools in monitoring tumor associated cell surface changes and have been utilized in the diagnosis and prognosis of human cancers (Hakomori, 1989; Muramatsu, 1993). Tumor antigen specific lectins have been used to study the expression of different antigens during oncogenesis and metastasis and such lectins could serve in the diagnosis and prognosis of cancer (Mody et al., 1995; Schumacher, 1995; Beuth et al., 1995; Rotman et al., 1996; Lalwani et al., 1996; Jannsen et al., 1996; Saussez et al., 1998; Basu et al., 2003; Kannan et al., 2003).

Since TF antigen is tumor associated and is the carbohydrate antigen specifically over-expressed on different kinds of human tumor cells (Hakamori, 2001), lectins, which bind to this glycosyl moiety, have greater applications in cancer diagnosis and therapy. Some of the notable examples of TF antigen binding lectins, which are already in use, are peanut agglutinin (Lotan et al., 1975), *Agaricus bisporus* lectin (Presant and Kornfeld, 1972), *Amaranthus caudatus* agglutinin (Rinderele et al., 1989), lectin from *Atrocarpus heterophyllus* (Kabir, 1998), *Vicia villosa* B4 isolectin (Tollefsen and Kornfeld, 1983).

Although many of these lectins share common specificity for the TF antigen, they exhibit subtle differences in their finer specificities towards modifications of this glycan structure and hence they bring about markedly
different effects on cancerous cells. For example, it has been shown that ABL, and jacalin (a lectin from *Artocarpus integrifolia* seeds) inhibit the proliferation of HT29 colonic cancer cells, whereas peanut (*Arachis hypogaea*) lectin (PNA) and *Amaranthus caudatus* lectin (ACL), stimulate their proliferation (Ryder et al., 1992; Yu et al., 1993; Yu et al., 1999; Yu et al., 2000; Yu et al., 2001; Yu et al., 2002). In the recent years, such novel lectins attracted considerable interest and are therefore continuously isolated and exploited for their potential applications in cancer biology.

1.6. Prelude to present study

*Sclerotium rolfsii* lectin (SRL) a TF antigen specific lectin was purified recently from the sclerotial bodies of a phytopathogenic fungus *Sclerotium rolfsii* and characterized in this laboratory. SRL is a homodimer with M, 34 kDa, recognizes Galβ1 – 3GalNAc-a-0-ser/thr which is part of TF antigen and also is the core structure of O linked sugar chains of secretory glycoproteins of animal origin (Swamy et al., 2001; Wu et al., 2001). The 3D structure of SRL was determined at 1.1 Å resolution by x-ray diffraction (Leonidas et al., 2003). Recently a conclusive evidence was provided for the first time establishing the functional role for a fungal lectin in development and morphogenesis by isolating putative endogenous
receptor (GIPC, a galactosylinositolphosphoryl ceramide) for SRL (Swamy et al., 2004).

Unlike PNA, SRL also bind to sialylated TF antigen, but similar to Agaricus bisporus lectin (ABL), and Amaranthus cadatus lectin (ACL). However, remarkably it differs from ABL and ACL by having specificity to the oligosaccharide chain when it is part of the peptide chain. Considering this interesting sugar specificity of SRL to recognize TF antigen it was tempting to study its interaction with cancer associated antigens. As part of this objective, results of the preliminary studies carried out for its binding pattern with oral cancer tissues using lectin histochemistry were encouraging. With this objective, recently in this laboratory several studies are initiated to exploit Sclerotium rolfsii lectin to develop it as prognostic and diagnostic tool in cancer. One of the programs of this study is to investigate the expression of TF antigen in the saliva of oral cancer patients.

1.7. Relevance and scope of the present study

Cancer afflicts all communities worldwide, approximately 10 million people are diagnosed with cancer and more than 6 million die of the disease every year. (Park, 2005) Head and neck squamous cell carcinoma (HNSCC) is an aggressive epithelial malignancy that is the sixth most common neoplasm in the world today (Jemal et al., 2004). Currently each year more
than 500,000 new cases are diagnosed and 3,20,000 deaths occur worldwide. Oral cancer is one of the 10 most common cancers in the world and incidence of oral cancer is very high amongst Central and South East Asian countries, which include India, Bangladesh, Sri Lanka, Thailand, Indonesia and Pakistan (Park, 2005). At least 95% of the HNSCC arise most commonly in the oral cavity. This high incidence of cancer is attributed to chronic abuse of tobacco. Oral cancer is a major problem in India, which accounts for 50 to 70% of all cancers diagnosed as compared to 2 to 3% in UK and USA (Park, 2005) This alarming situation warranted for intensive area of medical research in many countries. One of the objectives in this conquest is to develop diagnostic methods for early detection and the prognostic methods for the effective management of cancer therapy. The early diagnosis of manifestation of oral carcinoma tends to be a problem because there are no truly specific early symptoms. It is probable that, even with different therapeutic strategies tumor cells remain in patients resulting in local and / or distant relapse. Therefore, current methods have some limitations in respect of their sensitivity, suggesting that new strategies should be developed.

In this context, the identification of tumor markers is crucial and constitutes a field, which continues to expand progressively. Many glycoproteins and glycolipids have been described as tumor-associated antigens since they may be over expressed or be different to normal as a
consequence in alteration in their synthesis. Increased levels of mucin complex at the cell surface are associated with increased degree of dysplasia (Saussez et al., 1998; Croce et al., 2001; Chimenos et al., 2004). In the past, lectins have been used as histochemical probes for investigating differential expression of TF antigen, and related mucin-type carbohydrate antigens in normal oral mucosa, premalignant lesions and oral SCC (Bryne et al., 1991; Saussez et al., 1998; Remani et al., 1997; Tanda et al., 1996; Pillai et al., 1996; Nitta et al., 2000). However to the best of our knowledge, there are no attempts for the identification of tumor markers in the saliva of cancer patients using lectins as probes. Saliva is emerging as non-invasive diagnostic medium for rapidly widening range of diseases and clinical situations (Tabak, 2001; Kaufaman and Lamster, 2002; Streckfus and Bigler, 2002). Hence the present study aims to identify tumor-associated antigens in the saliva using novel lectin from Sclerotium rolfsii.
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