Physicochemical studies on lectins from *Agrobacterium radiobacter* and *Xanthomonas campestris.*

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C O N T E N T S

DECLARATION

Certified that the work incorporated in the thesis entitled "**Physicochemical studies on lectins from *Agrobacterium radiobacter* and *Xanthomonas campestris* "** submitted by Ms. Bhagyashree Joshi was carried out under my supervision. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

H SivaRaman

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Research Guide

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ABBREVIATIONS

HA :	Haemagglutination
TBS :	Tris Buffer Saline
TNBS :	2,4,6 trinitrobenzenesulphonic acid
DTNB :	5,5' -dithiobis 2nitrobenzoic acid
NBS :	N-bromosuccinimide
SDS-PAGE :	Sodium dodecyl sulphate poly acrylamide gel electrophoresis
EDTA :	Ethylene diamine tetraacetate
glc :	D-Glucose
G, gal :	D-Galactose
M, man :	D-Mannose
F :	L(-) Fucose
GN, glcNAc :	N-acetyl glucosamine
galNAc :	N-acetyl galactosamine
N, NeuNAc :	N-acetyl neuraminic acid
Me :	Methyl
IEF :	Isoelectric Focussing
TCA :	Trichloroacetic acid
ND :	Nondefined
C. W. :	Cell wall

SUMMARY

Lectins are nonenzyme, nonimmunoglobulin proteins that have at least one carbohydrate binding domain. Lectins comprise of a structurally diverse class of proteins. They are found in organisms ranging from viruses and plants to humans and serve to mediate biological recognition events. Microbial lectins are known for more than 80 years and still only a few bacterial lectins are fully characterized for their structure and functions. These lectins are very diverse in their molecular properties as well as in their sugar specificities. They are involved mostly in cellular adhesion, which may or may not exhibit cell agglutinating activity and may or may not be inhibited by simple monosaccharides.

Microbial lectins include bacterial, fungal, viral as well as protozoal lectins. Among bacteria, *E. coli* lectins are the most studied ones. However, many other bacteria also produce lectins most of which are in the form of pili which are filamentous rod like surface appendages. Nonpilus adhesins are also exhibited by some of the microorganisms. Microbial adhesion to the specific tissue through these lectins plays an important role in the infection (parasitism), symbiosis as well as commensalism.

In the last decade, interest in microbial lectins has increased tremendously. Studies on the structure and specificities and their biogenesis has led to a better understanding of the proteins as such, as well as their biological

functions.

Although much work has been done on human and animal pathogens and lectins produced by them, plant pathogens have not received much attention. Hence the present investigation was carried out to screen, purify and characterize lectins from plant pathogens.

Chapter 1 : General Introduction

This part comprises of literature survey with reference to 1. microbial lectins including bacterial, fungal and viral lectins and 2. plant pathogenic bacteria and their specific attachment to the hosts.

Chapter 2 : Purification and characterization of lectins from *Agrobacterium radiobacter* NCIM 2443 (ATCC 6466).

Crown gall tumor disease of dicotyledonous plants is known to be harmful and is produced by *Agrobacterium* strains. These are Gram negative short rods. The very first essential step in the disease production is the site specific attachment of the microbe to its host plant and this attachment is belived to be through lectin carbohydrate interactions and hence the lectins produced by *Agrobacterium* were taken up in the present study.

Ele ven isolates of Agrobacteria were screened for extracellular lectin production and *Agrobacterium radiobacter* was selected because of its highest lectin producing capacity. *Agrobacterium radiobacter* NCIM 2443 (ATCC 6466) is the only known nonpathogenic microorganism of the genus. This microorganism produced 2 different kinds of lectins.

1. Extracellular lectin (Lectin I) : *Agrobacterium radiobacter* NCIM 2443 produced a lectin extracellularly within 48 hours of fermentation. The protein was a monomer ofrelative molecular mass M_r 37000. It is not a glycoprotein itself but is strongly associated with the polysaccharide produced by the organism just like the *Xanthomonas* strain. The lectin showed a pI of 4 and was very specific for the oligosaccharide glcNAc-glcNAc-Man. Tobacco plant extracts inhibited the lectin activity. The protein was purified by conventional chromatographies involving ion exchange (DEAE) and hydrophobic (phenyl Sepharose) chromatographies after a step of ammo nium sulphate precipitation. The lectin was most stable at pH 5.0 and it retained the haemagglutinating activity as well as its monomeric form only in the presence of EDTA (1mM). The amino acid composition showed that it had a large proportion of acidic amino acids and no cysteine.

2. *Agrobacterium radiobacter* NCIM 2443 produced another lectin (Lectin II) which was situated on the surface. It could be the pilus protein. The extraction procedure involved shaking a (5 % w/v) suspension of *Agrobacterium radiobacter* cells at 60°C in presence of 6 M urea for 30 min. The cell debris was removed by centrifugation (9770 Xg, 20 min.). The extract was then subjected to 60 % ammonium sulphate precipitation and loaded onto a Sepharose 4B column in presence of 6 M urea. The first fraction just after the void volume showed lectin activity. The purified lectin was of relat ive molecular mass M_r 40000 and it formed aggregates after dialysis against plain buffer. The amino acid composition

showed that it had 44 % hydrophobic amino acids. It showed specificity for D- glucosamine and its pI was 9.15. Its activity also was inhibited by Tobacco plant tissue extracts. Among plant polysaccharides tested for haemagglutination inhibition of the lectin, Locust gum inhibited the lectin activity strongly.

Chapter 3 : Chemical modification studies on lectins from *Agrobacterium radiobacter* NCIM 2443 (ATCC 6466).

To determine the amino acid residues involved in sugar binding, both of the lectins from *Agrobacterium radiobacter* NCIM 2443 were subjected to chemical modification.

1. Extracellular lectin (Lectin I) : Chemical modification studies on purified extracellular lectin revealed that tryptophan and carboxyl groups are involved in sugar binding.

The protein is associated with polysaccharide produced by the microorganism and it could protect the protein from chemical modification reagents. To get rid of excess of polysaccharide, the purified lectin was passed through hydroxyapatite column, in presence of 5mM phosphate buffer pH 6.8. Further studies revealed that number of tryptophan residues involved in binding was 2 and modification of tryptophan caused more than 85 % loss of activity. Modification of carboxyl groups resulted loss of 60 % loss of haemagglutination activity.

2. Surface lectin (Lectin II): Chemical modification studies of surface lectin revealed that histidine and carboxyl groups were involved in sugar binding. With modification

of tryptophan 50 % loss of activity was obtained whereas modification of histidine residue caused 100 % loss of lectin activity and with Woodward's reagent K, the activity loss was 75 %.

Chapter 4 : Purification and characterization of an extracellular lectin from *Xathomonas campestris* NCIM 5028 (ATCC 29497).

In all 25 isolates of *Xanthomonas* were screened for extracellular lectin activity. Among them, culture No. 25 showed promising results and therefore was selected for further studies. The time course of the production showed that the lectin was produced always along with an acidic polysaccharide popularly known as xanthan gum. Reduced polysaccharide production hampered lectin production also.
A lectin from *Xanthomonas campestris* NCIM 5028 was isolated which was produced extracellularly by the organism within 48 hours of fermentation. The purification procedure of the lectin was very simple and it involved only one chromatographic step (hydrophobic chromatography with phenyl Sepharose) after ammonium sulphate fractionation step. The lectin was a protein of relative molecular mass 70000; a dimer consisting of subunits of M_r 28000 and 30000. It showed specificity only towards fetuin glycoprotein and its o-linked glycopeptide but not with other mono or oligosaccharides. Isoelectric focussing revealed that the strong association between polysaccharide and protein did not involve covalent bonding. All of the polysaccharide remained on the acidic side whereas the lectin concentrated itself at pH 7.2.

The lectin itself was not a glycoprotein and it must be attached to the polysaccharide through hydrophobic and ionic interactions. Lectin molecule alone (without any attached polysaccharide) remained stable only for a few hours only. *Xathomonas campestris* var. *campestris* NCIM 5028 used in this study was pathogenic for the *Brassica oleracea* plant. *Brassica oleracea* tissue extracts inhibited the lectin activity strongly. Inhibition of lectin activity by host plant polysaccharides is supposed to be a clue for its specific attachment to the host plant tissue.

Chapter 5 : General Discussion

In this part of the thesis, lectins produced by plant pathogenic microorganisms, their role in pathogenicity and disease production is discussed.

CHAPTER 1

General Introduction

1.1 : DEFINITION

Till recently, lectins were generally defined as carbohydrate binding proteins of nonimmune origin that agglutinate or precipitate polysaccharides and glycoproteins (1,2). This definition implies that lectins are multivalent. This is true specifically for plant lectins, which were the first to be discovered and studied in detail but not so for the other lectins (including some bacterial lectins). Lectins are now defined as follows :

"Lectins are nonenzyme non-immunoglobulin proteins that have at least one carbohydrate binding domain." They are not necessarily multivalent for carbohydrates nor must they act as agglutinins as required in an earlier definition (3).

Lectins comprise of structurally diverse proteins characterized by their ability to bind carbohydrates with considerable specificity. They are ubiquitous in nature and are found in organisms ranging from viruses and plants to humans and serve to mediate biological recognition events. This is one of the reasons why, during the last decade or two, lectins have become the focus of intense interest.

The main function of lectins is cell surface recognition, a central event in a variety of biological phenomena, such as fertilization, organ formation or immune defence and therefore more attention is being paid to the question of how lectins bind carbohydrates and their physiological role.

1.2 : HISTORY

In 1954, Boyd (14) proposed that blood group specific plant agglutinins be called lectins from latin 'legere' i.e. to pick up or to choose. This term was generalized in 1972 to include all sugar binding or cell agglutinating proteins of nonimmune origin, from plant, animals and microorganisms whether blood group specific or not (2).

Lectin research started around 1888 when Stillmark (4) made a startling observation that extracts of bean (*Ricinus communis*) agglutinated erythrocytes (Table 1.1).

The first report on a fungal haemagglutinin was that of Kobert in 1891 (5). The first demonstration of bacterial haemagglutinins was by Kraus and Ludwig (6), who demonstrated the haemagglutination of rabbit erythrocytes by culture filtrates of *Staphylococcus aureus* and two *Vibrio* strains. Presence of weak haemagglutinins in filtrates of *Pseudomonas aeruginosa*, *Salmonella typhi* and *Staphylococcus aureus* was also shown (6). In 1903, Kayser reported haemagglutination by a culture filtrate of *E. coli* and Pearce and Winne added *Vibrio cholerae* and *Shigella desenteriae* to the list (5). In 1908, Guyot (7) reported that *E.coli* cells agglutinated blood cells.

In 1941, Hirst (8) and McClelland and Hare (9) independently, described haemagglutination of influenza A and B viruses.

Rosenthal (10) found that *E. coli* haemagglutinin agglutinated leukocytes, thrombocytes, yeasts, spermatozoa,spores

of molds and pollens in 1943.

Haemagglutinins of *Haemophilus* were demonstrated in culture filtrates as well as intact cells by Keogh *et al.* in 1947 (11) and were purified by Warburton and Fisher in 1951 (12). This was probably the first report on a bacterial haemagglutinin purification. Lamanna (13) found that toxic culture filtrates of *Clostridium botulinum* contained several haemagglutinins (A-E).

The first report on inhibition of bacterial haemagglutinin by a simple sugar was by Collier and de Miranda (15), who showed that D-mannose strongly inhibited the haemagglutination activity of *E. coli*. Duguid *et al* . (16) observed that in various enterobacteria, there are haemagglutinins that are intimately associated with filaments called fimbriae. Subsequently it was found that most *Salmonella* strains possess type 1 (MS) fimbriae (17). Brinton used the term pili for the same and showed "phase variation" in their production depending upon the cultural growth conditions. In 1972, Gilboa - Garber (18) described the purification and characterization of *Pseudomonas aeruginosa* lectin which was galactose specific.

Ofek *et al.* (19) studied the interaction of *E. coli* with human buccal epithelial cells and were first to propose that mannose sensitive adherence is mediated by lectins on bacterial surface which bind to mannose residues on the animal cells. Direct evidence for the involvement of these lectins in initiation of infection was first obtained in 1979

Table 1.1 : **Microbial lectins : A historical overview**

Year	Investigators	Discovery
1888	Stillmark	*Ricinus communis* extract has haemagglutinating property (4).
1891	Kobert	First report on fungal haemagglutinin (5).
1902	Kraus & Ludwig & Flexner	First report on bacterial haemagglutinin (6).
1903	Kayser	First report on haemagglutination by *E. coli* culture filtrate (5).
1908	Guyot	Report on haemagglutination by *E. coli* cells (7).
1941	Hirst, McClelland & Hare	Haemagglutination by Influenza A and B viruses (8,9).
1943	Rosenthal	Agglutination of yeasts, leukocytes and thrombocytes by *E. coli* cells (10).
1947	Keogh *et al*	Haemagglutination by intact cells and culture filtrates of *Haemophilus* sp. (11).
1951	Warburton & Fisher	First report on purification of bacterial haemagglutinin; from *Haemophilus* sp. (12).
1948	Lamanna	Report on hamagglutination by cult ure filtrates of *Clostridium* (13).
1954	Boyd & Shapleigh	The term "lectin" proposed for haemagglutinins (14).
1955	Collier & de Miranda	First report on inhibition of *E. coli* haemagglutination by simple sugar - D mannose (15).
1957	Duguid & Gillies	Correlation of lectin and adhesin of *E. coli* (16).
1966	Duguid & Brinton	The term fimbriae or pili suggested for *E. coli* adhesins; characterization of *Salmonella* pilus as type 1 pilus.

Table 1.1 continued

Year	Investigators	Discovery
1972	Gilboa-Garber	Purification of galactose specific lectin from *Pseudomonas* cells(18).
1977	Ofek, Mirelman & and Sharon	Report on binding of *E. coli* cells to human buccal epithelial cells through mannose specific lectins (19).
1979	Aronson	Report on urinary tract infection in mice by *E. coli* could be prevented by methylamannoside (20)
1981	Wilson, Skehel & Wiley	Crystal structure of lectins from influenza viruses A and B (21).
1994	Stein, Boodhoo & Armstrong	Crystal structure of pertussis toxin (22).

when it was shown that urinary tract infection in mice by mannose specific *E.coli* could be prevented by Methyl α mannoside (20). These studies raised the possibility that sugar inhibitors of lectin mediated bacterial adherence may prove to be useful against natural infection.

Hundreds of microbial lectins have now been reported, some of them are well characterized and the number is growing fast (Table 1.2).

1.3 :CLASSIFICATION

Wide variety of lectins have been isolated and characterized from nearly all kinds of living organisms but grossly they can be classified under three categories a) plant lectins b) animal lectins and c) microbial lectins.

1.3.1 Plant lectins

Lectins are found in plant seeds as well as stems, roots and leaves. These were the first lectins to be studied in detail. Many plant lectins are well characterized in terms of their primary sequence, 3 D structure and other molecular properties (23). They have found enormous applications in biological and medical research (24).

1.3.2 Animal lectins

The field of animal lectins is also expanding rapidly. These proteins have the ability to recognize carbohydrates endogenous to the animal or those that are presented to it by microbial invaders.

Among animal lectins, some 'C' type (Ca^{++} requiring) and 'S' type (thiol group requiring) lectins have been extensively

studied. The 3D structures of following the lectins are available. (1) CRD (carbohydrate recognition domain) of rat mannose binding protein A (2) E selectin (3) galectin 1 and (4) galectin 2 (23).

1.3.3 Microbial lectins

Microbial lectins are very diverse in their molecular properties such as molecular mass, subunits and sugar specificities. They are produced by all kinds of microorganisms like bacteria, fungi, viruses as well as protozoa.

1.3.3.1 : Bacterial lectins

Bacterial lectins are defined as carbohydrate binding proteins involved in cellular adhesion, which may or may not exhibit cell agglutinating activity and which may or may not be inhibited by simple monosaccharides (25). The majority of bacterial lectins appear to recognize complex oligomeric carbohydrate structures.

Location : The location of microbial lectins and adhesins is highly variable. Many of them are associated with fimbriae, flagella and cell surface or are present in periplasmic space or internally. Others are found in microbial culture filtrates (26,27).

Assay : The haemagglutination assay has most commonly been used to demonstrate bacterial lectins. Participation of "lectin" in agglutination is demonstrated by inhibition of haemagglutination by monosaccharides, oligosaccharides or complex carbohydrates (28).

A variety of other assay techniques have been employed to

examine the bacterial attachment to many different cell types. They are mucosal scrapings in the oral cavity, desquamated urine sedimented cells, gastrointestinal single cell preparations and mucosal brush borders (29). Porcine brush borders have been used with porcine enterotoxigenic *E. coli* with K88 surface fimbrial lectin (30). More refined procedures have been introduced, using synthetic glycoconjugates and inert carriers that bear glycoconjugates. Techniques like

equilibrium dialysis as well as more accurate physicochemical measurements of the interactions of lectins with sugars are also employed.

Purification : Many of the microbial lectins have been purified by use of ammonium sulphate precipitation, heating (for removal of nonlectin heat sensitive proteins), molecular sieving and affinity chromatography using insoluble, naturally occuring or chemically linked sugar binding supports such as Sepharose, agarose, Sephadex and latex beads (31).

Properties : The relative molecular mass of microbial lectins ranges from 11kDa (for monomeric human blood group B specific, galactose binding lectin of *Streptomyces* species which contains two sugar binding sites) (32,33) and PAII lectin of *Pseudomonas aeruginosa* (34), to 800 kDa *Streptomyces* sf1100 fucose/mannose specific lectin which consists of 12 identical subunits, each of 68 kDa and possesses only two sugar binding sites (35,36). All microbial lectins, like most of the plant lectins are generally poor in sulfur containing amino acids and are relatively rich in

acidic and hydroxyl amino acids. There is a great variation in the specificities of the haemagglutinins obtained from different microbial species or strains of the same species.

Fimbrial lectins of bacteria

Fimbriae are proteinaceous appendages that protrude from the surface of bacteria, and have been implicated as bacterial ligands, binding to the host surface receptors. Often, fimbriae and pili are considered as synonyms. These are the thin, nonflagellar protein filaments found on the surface of many types of bacteria. They are variable in length (0.5 - 10 µM) and their number (per cell) varies from 1 to several hundred. These allow them to bind to other bacteria, bacteriophages, mammalian cells and inert surfaces. Piliated bacteria that adhere to mammalian cells are often more pathogenic than their nonpiliated counterparts because the pili enable them to become anchored to the host tissue and resist elimination by body fluids.

Escherichia coli

Much of the knowledge on bacterial surface lectins is based on pioneering studies on pili of *E.coli* in 1950's and 60's by Old and Brinton (17). In early stages of fimbrial research, fimbrial adhesins were classified in two groups *viz* MS and MR, according to their receptor specificity. For example, Type 1 fimbriae of *E. coli* strains, mannose sensitive (MS) and the fimbrial lectins, specific for sugars other than mannose, like Pap pili of *E. coli*, (MR).

Bacteria like *E. coli* that carry genes coding for certain types of lectins may not always express them. Due to the phenomenon of phase variation, bacteria switch back and forth from one phenotype that expresses the lectin to one that does not. Outgrowth of one phenotype over the other may occur in a bacterial population growing under specific conditions. The frequency of phase variation is affected by the conditions in which the bacterium is growing. *In vivo*, this selection is the outcome of the rate of elimination by the host defense mechanisms and the rate of proliferation of each of the phenotypes at a particular site.

Type 1 pili : These are nonconjugative, chromosomally encoded thin rigid rods (7nm diameter). The number varies from 100 to 500 per cell and their length varies from 0.2 to 1 μm. They are peritrichously arranged on the surface. They cause agglutination of guinea pig erythrocytes as well as yeast cells, buccal epithelial cells and mannose containing urinary glycoprotein Tamm-Horsefall protein. It is a typical mannose sensitive lectin. It is sensitive to branched mannose oligosaccharides as well as hydrophobic mannose derivatives such as p nitrophenyl α mannoside (37). The genetic analysis of Type 1 fimbrial gene cluster revealed that the *pilE* gene is responsible for attachment of Type 1 fimbriae to the erythrocytes of guinea pigs (38,39). Krogfelt *et al* (40) proved that the PilE protein is a mannose specific adhesin of Type 1 fimbriae of *E. coli* by direct binding of the PilE protein to D-mannose attached to a carrier protein. By immunoelectron microscopy, it was shown that the *PilE* was located

laterally in the structure of the Type 1 fimbriae.

All strains of *Klebsiella pneumoniae* and several *Salmonella* species also exhibit type 1 pilus. Purified type 1 fimbriae of *E. coli* consist of stable arrays of identical protein subunits of 163 amino acids (M_r 16600) (41). The corresponding subunit from *Salmonella typhimurium* has M_r of 21000 and that of *Klebsiella pneumoniae* has M_r of 21500. The subunits contain high proportion of hydrophobic amino acids (approx. 50 %) and they tend to aggregate to form parallel bundles.

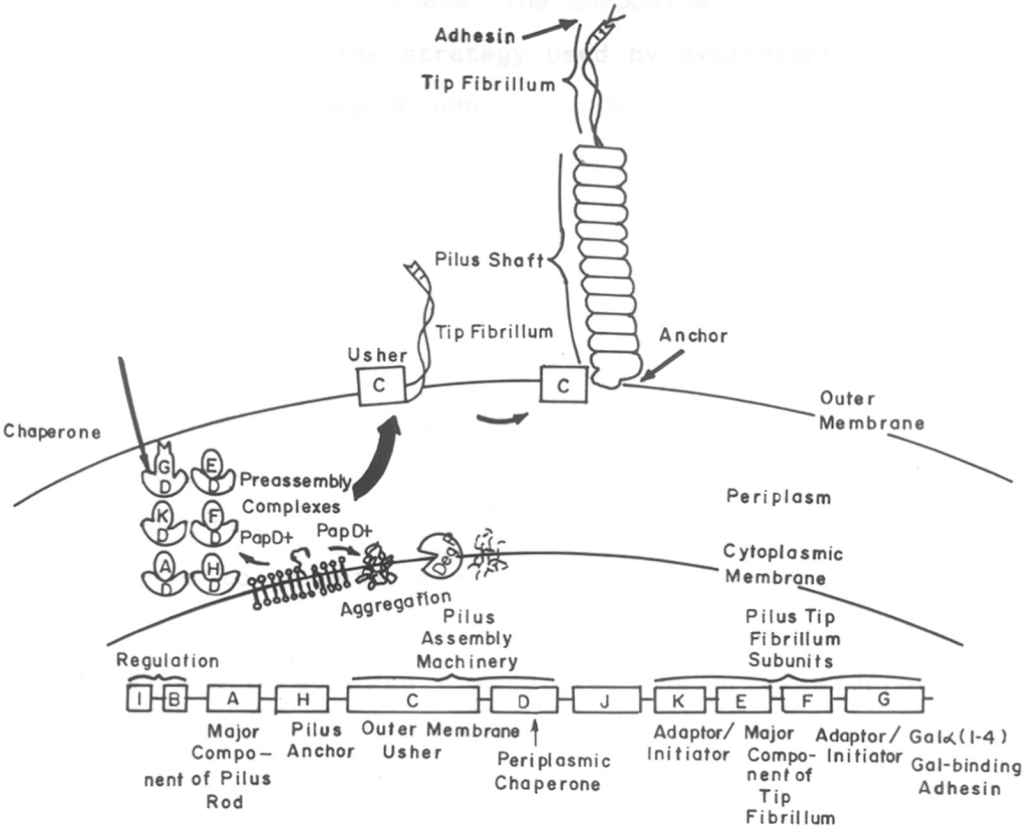
Firon *et al* (42) examined many branched mannosylated oligosaccharides and showed that Man-α6[Man-α3]Man-oMe(Manα3)Manα-oMe and Man-α6[Man-α3]Man-α6[Manα2-Manα3]Manα oMe, as well as the trisaccharide Man-α3Manβ-4glcNAc and aromatic glycoside p-nitrophenyl-α-Man are strong inhibitors of the yeast agglutination by type 1 fimbriae of *E. coli* 346 (43,44). The pattern of this agglutination is different from that of mannose binding plant lectins of Con A - Lentil - Pea group. It also differs from the mannose binding lectins of *Pseudomonas aeruginosa* PA II and *Streptomyces* which exhibit more specificity for fucose than for mannose.

Mannose specific lectins are a key factor in the ability of various bacteria to cause infections by mediating the attachment of bacteria to the surfaces of target tissues. It is also possible to prevent infection by suitable inhibitors of adherence.

Type P pili : P fimbriae are specific for Gal-α1,4-gal globotetraosylceramide. Pyelonephritic *E.coli* produce these

Figure 1.1 - Model of P pilus biogenesis

Adapted from (45).


Adhesin
Tip Fibrillum
Pilus Shaft
Tip Fibrillum
Anchor
Usher
C
C
Outer Membrane
Chaperone
Preassembly Complexes
PapD+
PapD+
Periplasm
Cytoplasmic Membrane
Aggregation
Pilus Assembly Machinery
Pilus Tip Fibrillum Subunits
Regulation
I
B
A
H
C
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J
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F
G
Major Compo- nent of Pilus Rod
Pilus Anchor
Outer Membrane Usher
Periplasmic Chaperone
Adaptor/ Initiator
Major Compo- nent of Tip Fibrillum
Adaptor/ Initiator
Galα(1-4) Gal-binding Adhesin

type of fimbriae. These are less hydrophobic and show mutiple bands on SDS-PAGE, the largest one has M_r 19500. These pili contribute to bacterial infectivity. (Fig. 1.1)

Assembly of pili by Gram negative bacteria seems to be a highly controlled process. The composite architecture of the P pilus reveals the strategy used by pyelonephritic *E. coli* to present the Pap G adhesin to eukaryotic receptors (45 - 49). The rigid Pap A rod distances the adhesin from lipopolysaccharide while the flexible fimbrillum allows Pap G steric freedom to recognize and bind to the digalactoside moiety on the uroepithelium. Pap F is an adaptor protein and Pap K regulates the length of the tip fibrillum. Pap D is a chaperon required for the assembly of P pilus. Its function is to bind a group of proteins (Pap A, Pap H, Pap K, Pap E, Pap F and the adhesin Pap G) and escort them from the cytoplasmic membrane to an outer membrane usher, allowing their correct folding and assembly into pili, without being a part of the final stucture. Pap C regulates the ordered targetng of chaperone subunit complexes to an outer membrane assembly site where the chaperone is dissociated from the respective subunits allowing their polymerization into pili. Pap G is an adhesin molecule which binds specifically to gal-β1,4gal. The shiga toxin is also specific for the same disaccharide but is more selective in relation to substituents indicating that uropathogenic *E. coli* adhesin and Shiga toxin recognize different epitopes of this disaccharide (50).

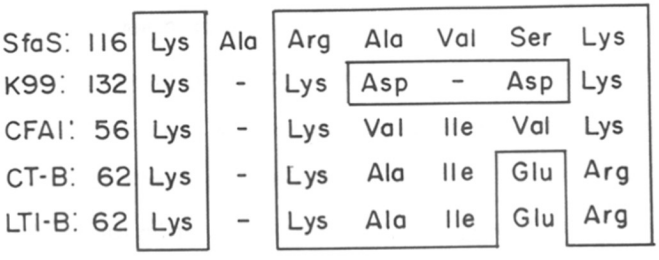
There are P related pili which exhibit carbohydrate specific-

ity slightly different from that of P fimbriae. They are specific for GalNAcß 1-3gal.

Sialic acid specific lectins : Some strains of *E. coli*, isolated from human and farm animals have been found to express fimbrial haemagglutinins specific for glycoconjugates containing sialic acid. The haemagglutination caused by fimbriated bacteria is decreased or completely abolished after sialidase treatment of erythrocytes. Several fimbrial lectins of this type are known. They include K99 and F41, the adhesins expressed by human enterotoxigenic *E. coli* strains isolated from piglets, calves and lambs suffering from diarrhoea, CFA I/II expressed by human enterotoxigenic *E. coli* isolates and type S expressed by *E. coli* strains frequently isolated from newborn infants suffering from sepsis and meningitis. Sialic acid specific lectins are also present in *Mycoplasma, Pseudomonas aeruginosa* and *Bordetella bronchiseptica* (51-53). The major adhesin of *Mycoplasma pneumoniae* (M_r 190000) is present at the cell surface. Sialic acid specific bacterial lectins seem to have a common motif in their amino acid sequences. Morschhauser *et al* (54) analysed the sequences of several sialic acid binding adhesins and found that the basic amino acids lysine and arginine were common in putative active sites of K99 adhesin, CFA I adhesin, cholera B subunit, *E.coli* labile toxin B subunit and fimbrial sfa S adhesin (Fig. 1.2). K99 fimbrial lectin is specific for N-glycolylneuraminic acid and 2-benzyl-N-acetyl neuraminic acid. Smit *et al* (55) isolated glycolipids from horse erythrocytes that contain attachment sites for K99 lectin. It's

Figure 1.2 - **Comparison of the amino acid sequence segment between Lys-116 and Lys-122 of Sfa S to amino acid sequences of sialic acid specific K99 adhesin, CFA I adhesin, Cholera B subunit, and *E. coli* LT1 B subunit. Identical or functionally identical amino acids are in boxes.**

Adapted from : J. Hacker (1990) Curr. Top Microbiol. Immunol. 151 : 1-27


SfaS: 116 Lys Ala Arg Ala Val Ser Lys
K99: 132 Lys - Lys Asp - Asp Lys
CFA1: 56 Lys - Lys Val Ile Val Lys
CT-B: 62 Lys - Lys Ala Ile Glu Arg
LTI-B: 62 Lys - Lys Ala Ile Glu Arg

structure is NeuAcα2-3galβ1-4glcβ1-ceramide. Receptor structure of the piglet glycolipid is NeuGcα1-3galβ1-4glcβ1-ceramide (56). Other inhibitors isolated were -NeuGc2-3galβ1-4glcβ1-ceramide and NeuGc2-3galβ1-4glcNAcβ1-3galβ1-4glcβ1-ceramide (57).

Neeser *et al* (58) and Pieroni *et al* (59) showed that CFA I and CFA II are specific for complex type N- linked or human milk oligosaccharides, sialylated glycoproteins from human erythrocyte membranes and LacNAc. Only a limited number of studies have been performed on *E.coli* strains bearing F41 adhesin. The F41 strains produce shiga like toxin and the receptor probably contains both sialic acid and galNAc. Virtually nothing is known about the linkages, size of combining site or contribution of hydrophobicity.

Salmonella typhimurium

Lectins are in form of Type 1 pili and the pilus filaments are 6 nm in diameter and over 1 μm long. It has M_r 21000, isoelectric point 4.1 and contains 40.3% hydrophobic amino acids. This lectin is specific for α methyl mannoside (60).

Shigella flexneri

Pili were detected in clinical isolates using electron microscopy. Pili are 2 -5 μM long and 3 - 5nm in diameter. They are supposed to be type 3 pili (61). The strains agglutinate fresh erythrocytes of fowl and tannic acid treated horse erythrocytes.

Vibrio lectins

Cholera vibrios produce a variety of haemagglutinins (62). Agglutination of human blood group O erythrocytes and adhesion of the organism to brush borders of epithelial cells is specifically inhibited by L (-) fucose. The lectin has been purified to apparent homogeneity. It has M_r 32000 and pI of 6.3, 5.3 and 4.7. The lectin possessed proteolytic activity that was inhibited by antiserum against the lectin. It was active at 4°C but the protease activity was lost indicating a bifunctional role *viz.* haemagglutination and proteolysis. *Vibrio* El-Tor biotype produces a cell associated mannose specific haemagglutinin of M_r 17000 which is also inhibited by fructose (63). There are two other forms of lectins produced by *Vibrio cholerae*, one of them is produced as a protein of molecular mass 34000, which on storage gets converted to lectin of M_r 32000 because of its own proteolytic activity (64). A pilus protein of M_r 20000 with haemagglutination activity and intestinal adherence properties expressed by a clinical isolate of non-01 *Vibrio cholerae* has been reported (65). A chitin binding lectin from *Vibrio parahaemolyticus* has also been reported (66). Its activity is calcium dependent and pI is 3.6. It is a high molecular mass protein (M_r 134000). It is thermotolerant and is active in presence of 0-4 M NaCl.

Mycoplasma pnaumoniae lectin

Studies on *Mycoplasma* adherence to animal cells have special appeal because they have no cell wall and no surface append-

ages. Work on *Mycoplasma gallisepticum* indicated the protein nature of the adhesin (67). It has been shown that two proteins, designated P1 (M_r 190000) and P2 (M_r 78000) are responsible for adhesion (68). It expresses a lectin specific for NeuNAc attached by NeuAcα2-3galβ- linkage to terminal galactose residue of the poly N-acetyl lactosamine sequence of blood type I/i antigen (69,70). Sialic acid linked α 2-3 to galactose is the most preferred receptor sequence (71). It was shown that *Mycoplasma pneumoniae* as well as other Mycoplasma species could bind to Gal($3SO_4$) β1- residues. *Mycoplasma pneumoniae* contains at least two separate lectins, one specific for sialyl glycoproteins and the other for sulphated glycolipids.

Neisseria lectins

Neisseria gonorrhoeae belongs to a group of microorganisms that bind to lacto and ganglio series of glycolipids (72). It was found that the organisms specifically bind to terminal and internal GlcNAcβ1-3galβ1-4glc and GalNAcβ1-4galβ1-4glc sequences in the lacto and ganglio series of glycolipids. (73). Perhaps the best illustration of the ability of a single strain to produce different fimbriae is *Neisseria gonorrhoeae*, strain P9 (74,75). Isogenic variants of P9 have been shown to produce fimbriae with M_r 19500, 20500, 21000 and 18500. These fimbriae are designated a, B, D and G respectively. Though a and B subunits have regions of considerable homology, they do not recognize the same epitope on animal cell surface. Another lectin produced by it known as

protein II also appears to mediate attachment. It comprises a family of proteins with molecular masses ranging from 27500 to 29000.

Pseudomonas aeruginosa lectins

Pseudomonas aeruginosa is an opportunistic pathogen causing hospital infections especially to eye, lungs, skin and other parts of the body. It produces fimbriae specific for sialic acids, fucose, galactose, mannose and ManNAc. The galactophilic lectin PA I from *Pseudomonas aeruginosa* ATCC 33347 was purified by Gilboa-Garber *et al* in 1972 (77). Its haemagglutination activity is much stronger after papain or sialidase treatment of human and animal erythrocytes. The lectin (M_r 12000) is stable to heating upto 80°C, proteolysis and to extreme pH values. It requires divalent cations for activity. Only one cysteine is present in the protein and is involved in sugar binding. The PA II lectin exhibits fucose/mannose specificity (34). It has M_r of 11000, association constant (Ka) for fucose 1.5×10^6 M^{-1} and one fucose binding site per subunit. Both of these proteins are internal proteins. There are some reports saying that adhesion of the organism is through fimbriae (78). Lectins specific for sialic acid have also been reported (79). Wentworth *et al* (1991) (80) have proposed an entirely new mechanism to account for lectin dependent adhesion of *Pseudomonas aeruginosa*. This organism produces two lectins PAI and PAII, the former specific for galactose and the latter specific for L (-) fucose, mannose and their hydrophobic derivatives. Proton motive force dissi-

pating agents caused partial lysis of bacteria with the concomittant release of the lectins. The released lectins in turn could bind to intact bacteria giving them the ability to bind to the cultured rabbit corneal epithelial cells. The synthesis of PAI and PAII is dependent on the cell age. Differential rates of expression of PAI and PAII could explain the selectivity for glycoconjugates (81).

Lectins from gram positive bacteria

There seems to be an increasing awareness that in Gram positive bacteria, surface lectins also play a role in adhesion. Members of genus *Streptococcus* namely *Streptococcus pneumoniae* and *Streptococcus sobrinus* possess carbohydrate binding proteins on their surface. Staphylococci and members of genus *Actinomyces* also express surface lectins.

Staphylococcus saprophyticus

Many strains of *Staphlococcus saprophyticus* cause direct haemagglutination of sheep erythrocytes. A 160 kDa surface polypeptide is a major component required for haemagglutination. It requires EDTA in micromolar quantities during growth (82).

Streptococcus pneumoniae

It possesses a surface lectin specific for galNAcβ1-3gal. The receptor probably belongs to neolacto and lacto series of glycolipids containing this disaccharide unit. This lectin is also inhibited strongly by Galβ1-4glcNAcβ1-3galβ1-4glc tetrasaccharide. This tetrasaccharide is present in human milk and

it has been suggested that it protects breast feeding infants from pneumococcal infections (83).

Streptococcus cricetus

It produces a lectin which is specific for glucan and the α 1-6 linkage in glucan promotes strong aggregation. This strain requires Mn^{++} ion for growth and expression of glucan binding lectin (84,85).

Actinomyces viscosus T14V

Lectin recognizes the carbohydrate structure present on cell surface of *Streptococcus sanguis* 34 and is involved in the coaggregation of oral actinomyces and streptococci. The Actinomyces lectin activity is increased by sialidase treatment. The most effective inhibitor is Galβ1-3galNAc. (86).

Members of genus *Propionibacterium* also seem to be able to bind lactosylceramides. *Propionibacterium freudenreichii* binds to Galβ1-4 glcβ1-ceramide. *Propionibacterium granulosum* has, however, no affinity for this structure.

Non pilus adhesins

Many bacteria produce nonpilus adhesins. The architecture of non pilus adhesins is not known but most of them are presumably linked to the cell surface as monomers or simple oligomers. The recognition of host receptor structures by both pilus and non pilus adhesins is extremely fine tuned allowing for selective interactions with the host.

Goldhar *et al* (87) purified the NFA-1 and NFA-2 nonfimbrial haemagglutinins from *E. coli*. Both of the proteins tend to

form aggregates and have subunit molecular masses of 21000 (NFA-1) and 19000 (NFA-2). Electron microscopic examinations showed the presence of an extracellular capsule like layer in adhering *E. coli* but not in nonadhering bacteria. Both proteins are antigenically distinct but recognize a common receptor. Darfeuille *et al* (88) identified a nonfimbrial bacterial surface protein of 16000 (AFA-1) isolated from a patient with acute infantile diarrhea. The lectin has 22 % nonpolar residues and 2 to 3 cysteines per subunit. Hoschutzky *et al* (89) characterized the nonfimbrial adhesin NFA-4 from uropathogenic *E. coli* 07:K98. This adhesin consisted of noncovalently linked subunits (M_r 28000) which formed aggregates of molecular mass greater than 10^6. Electron microscopic analysis of thin sections of *E. coli* expressing NFA-1, NFA-2 and NFA-4 revealed that these adhesins surround the bacterial cell, like a capsule (90). Kroncke *et al* (91) performed electron microscopic studies of coexpression of adhesive protein capsules and polysaccharides and found that the bacteria expressed composite capsules with the adhesin as recognition peptide at the cell distal outer region and the K antigen at the cell proximal inner region indicating that the adhesion part of the capsule was necessary for interaction with the eukaryotic receptor.

Haemophilus influenzae colonizes the respiratory tract. It lacks visible pili but is capable of binding to eukaryotic cells. Most strains of this genus express one or two adhesins. Based on their amino acid sequence, both the proteins show homology with filamentous haemagglutinin of *Bordetella*

Table 1.2 : Bacterial lectins

Organism	Location	Specificity	Ref.
Actinomyces viscosus	fimbriae	galactose	103
Actinomyces naeslundii	fimbriae	galactose	153
Aeromonas hydrophila	fimbriae	fuc,man,gal	154
Aeromonas liquifaciens	fimbriae	mannose	155
Aeromonas sobria	fimbriae	gal, man	156
Arizona spp.	fimbriae	mannose	157
Bacillus thuringiensis	fimbriae	MR	152
Bacillus polymyxa	surface	-	158
Bacteroides fragilis	fimbriae	MR	159
Bacteroidesmelaninogenicus	fimbriae	glucosamine galactosamine sialic acids	160
Bacteroides gingivalis	fimbriae	galNAc	161
	& surface		162
Bradyrhizobium japonicum	surface	galactose	163
Bacilliformis bartonella	fimbriae	glcNAc	164
Bordetella pertussis	outer membrane	glcNAc	165
Chlamydia trachomatis	-	glcNAc	166
Citrobacter freundii	-	mannose	157

Table 1.2 continued

Organism	Location	Specificity	Ref.
Citrobacter halleaupensis	-	mannose	157
Clostridium botulinum	non defined	-	167
Corynebacterium diphtheriae	fimbriae	mannose	168
Corynebacterium parvum	fimbriae	-	-
Eikenella corrodans	nondefined	gal,galNAc	156
Enterobacter amnigenus	fimbriae	mannose	169
Enterobacter aglomerans	fimbriae	MS & MR	169
Enterobacter aerogenes	fimbriae	MS & MR	169
Enterobacter cloacae	fimbriae	mannose	169
Enterobacter intermedium	fimbriae	-	170
Enterobacter sakazaki	fimbriae	mannose	171
Erwinia caratovora	fimbriae	mannose	172
Erwinia rhapontici	fimbriae	ß gal	139
Escherichia coli	fimbriae	mannose	37
Escherichia coli	fim.(K99)		55
Escherichia coli	fim.(CFAI/CFAII)	-	58
Escherichia coli	fim.P	galα1-4gal	50
Escherichia coli	surface	NeuNAcα2-3gal	173
Escherichia coli	flagellar	MR	102
Escherichia coli	nondefined	mannose	175
Escherichia coli	fim.(ss142)	galß1-6glc	176

Table 1.2 continued

Organism	Location	Specificity	Ref.
Escherichia coli	fim(K88)	ß gal, fucose	177
Fusobacterium nucleatum	nondefined	gal,lac	178
Haemophilus influenzae	nondefined	-	86
Haemophilus paragallinarum	ND	-	92
Haemophilus ducreyi	surface	MR	179
Kingella denitrificans	fimbriae	galactose	180
Kingella kingae	fimbriae	galactose	180
Klebsiella pneumoniae	fimbriae	MS & MR	181
Klebsiella aerogenes	fimbriae	MS & MR	182
Lactobacillus acidophilus	-	complex	183
Leptotrica buccalis	ND	galNAc	184
Moraxella bovis	fimbriae	-	185
Morganella morganii	fimbriae	MR	186
Mycobacterium smegmatis	surface	arabinose	95
Mycoplasma pneumoniae	-	-	67
Myxococcus xanthus	ND	galß1-3galNAc	187
Neisseria gonorrhoeae	fimbriae	galß1-3galNAc	72
Neisseria meningitidis	fimbriae	MR	188
Pasteurella multosida	ND	glcNAc	189
Proteus mirabilis	fimbriae	MR mannose	186
Proteus myxofaciens	fimbriae	MR	186
Providencia stuartii	fimbriae	MR	186
Pseudomonas aeruginosa	fimbriae	sialic acid	80

Table 1.2 continued

Organism	Location	Specificity	Ref.
Pseudomonas aeruginosa	internal	gal,man	75
Pseudomonas multivorans	fimbriae	mannose	155,190
Pseudomonas echinoides	fimbriae	mannose	191
Salmonella typhi	fimbriae	mannose	26
Salmonella typhimurium	fimbriae	mannose	60
Salmonella spp	fimbriae	MR	192
Serratia marinorubra	fimbriae	mannose	193
Shigella flexneri	fimbriae	mannose	61
Staphylococcus saprophyticus	ND	galß1-4glcNAc	81
streptococcus pneumoniae	ND	galNAcß1-3gal	82
Streptococcus mutans	ND	dextran	194
Streptococcus salivarius	ND	galNAc &lac	196
Streptococcus mutans	ND	galactose	195
Streptococcus sanguis	ND	NeuNAcα2-3gal	197
*Streptomyces*spp	ND	gal,fucose	198
Vibrio anguillarum	C.W.	mannose	199
Vibrio cholerae	ND	fucose,mannose	62
Vibrio cholerae	ND	MR	200
Vibrio ordalli	C.W.	MS & MR	201
Vibrio parahaemolyticus	ND	MS & MR	66
Xanthomonas campestris	fimbriae	-	142
Yersinia enterolytica	fimbriae	MR	202
*Yersinia*spp.	ND	MR	203

pertussis that appears to play a critical role in adherence and colonization. Therefore it has been suggested that these non pilus adhesins facilitate the adhesion and colonization of human respiratory tract (92).

Haemophilus paragallinarum also produces a surface nonpilus lectin (93). It was purified using monoclonal antibody. It agglutinates chicken erythrocytes and is probably a protective antigen.

Strptococcus pyogenes is a causative agent of suppurative disease of the pharynx which is sometimes followed by the developmant of rheumatic fever or acute glomerulonephritis. This microorganism is also a common cause of skin infections. To cause this broad range of disease, it should adhere to epithelial cells of pharynx or skin. It does not produce pili but produces two proteins called M protein anf F protein. Both of them are surface lectins and are dominant factors involved in binding. These lectins are environmentally regulated in responce to alterations in concentrations of oxygen and carbon dioxide (94).

Helicobacter pylori also produces a nonpilus adhesin on its surface which is specific for fucose (95).

Mycobacterium smegmatis produces a lectin (M_r12000) specific for arabinose. It's isoelectric point is 5.5 and it is rich in aspartic and glutamic acid residues. The protein is supposed to be a mediator of attachment between the organism and host cell surface (96).

Bacterial toxins : ADP ribosyl transferases are emerging as

important tools for understanding the pathways of signal transduction which are involved in the modification of cellular response (97). They are critical exogenous virulence factors and are represented by exotoxins of *Bordetella pertussis, Vibrio cholerae, E. coli, Corynebacterium diphtheriae,* and *Pseudomonas aeruginosa.* Pertussis toxin (PT), Cholera toxin (CT) and heat labile toxins (LT) of enteropathogenic *E. coli* contribute to pathology of pertussis (whooping cough), catastrophic diarrhea and dehydration and traveler's diarrhea, respectively. These toxins can be structurally classified as AB_5 multimeric proteins composed of a single catalytic protomer "A" and a pentameric oligomer "B". The crystal structures of B oligomers of PT,CT and LT have been determined (98,22). These oligomer subunits are typical lectins and interact with the carbohydrate moieties of the receptor. The CT B oligomer specifically binds to cells possessing G_{M1} ganglioside on their surfaces, including cells that line the epithelium of gut, the major anatomical target of *Vibrio cholerae* pathogenicity. The pentasaccharide of the CT B - G_{M1} receptor can be represented by galß1-3galNAcß1-4{NeuNAc(α2-3)}galß1-4glcß1-1 ceramide (99). The crystal structure of recombinant CT B oligomer complexed to this pentasaccharide has revealed the receptor recognition domain and the mechanism of its interaction. The varied bacterial toxins, show structural congruence, which indicates that nature controls diversity through conservation of like structures for correspondent biological activities.

1.3.3.2 Fungal lectins

Lectins or agglutinins are common in fungi. They are found mainly in the fruiting bodies of higher fungi but they have also been detected in mycelia of lower fungi. (Table 1.3)

1.3.3.3 Viral lectins

Although the ability of viruses to agglutinate cells was established 4 decades ago, their use as lectins with a defined specificity has come up only recently.

Virus attachment protein (VAP) : There are two mechanisms through which viruses induce haemagglutination: (a) by a structural component of the virus particle itself or (b) by a haemagglutinin induced in the host cell as a result of infection which is required by the virus during its maturation and release from the cell. For example, haemagglutination is shown by extracts of variola and vaccinia virus infected cells but not by the viral particles themselves. The extracts contain a glycoprotein (M_r 89000) which apparently is the haemagglutinin (113). Both enveloped and nonenveloped viruses display haemagglutinating ability. For nonenveloped viruses VAP is a capsid protein and in enveloped viruses it is a component of the envelope often projecting from the surface of the virus (Table 1.4).

Influenza virus haemagglutinin, a paradigm of viral lectins, is a surface glycoprotein specific for sialic acid. The subunit of lectin is composed of two polypeptides, covalently linked by a single disulfide bond and it associates noncovalently to form trimers. The haemagglutinin is a homotrimer

Table 1.3 : Fungal Lectins

Organism	Lectin Molecular Properties	Sugar specificity	Biological Role	Ref.
Phytophthora cinnamoni	-	*Zea mays* root cap slime	Binding to host roots.	105
Ulex europeus zoospores	-	L(-)fucose	to bind to host plant	106
Arthrobotrys oligospora zoospores	M_r 20000 Ca^{++} dependent	galNAc	Nematode Nematophagocity	107
Arthrobotrys conoides	-	glc	--"--	107
Monacrosporium eudermatum	-	man	--"--	108
Monacrosporium rutgeriensis	-	fuc	--"--	108
Trichoderma harzianum	-	-	-	110
Sclerotium rolfsii	M_r48000	gal	To attach to *Trichoderma*	109
Microbotryum violaceum	M_r74000 fimbrial glyco-protein	man arabinose glc	mating	111

Table 1.4 : Viral lectins

Major virus group	Type	Erythrocyte type	conditions of HA	VAP	Reference
NONENVELOPED VIRUSES					
Adenovirus	I II III IV	Rhesus Rat Rat Rat	20°C to 30°C & Nautral pH	Fiber of penton isomer	116
Papovavirus	Polyoma	Guinea pig	4°C,pH 7.0	Capsid protein	116
Parvovirus	-	Rodents	4°-37°C pH 7.0	Capsid protein	116
Picornavirus	-	Guinea pig, sheep, human type O	4°C pH 7.0	Capsid protein	116
Reovirus	-	human type A	20°C pH 7.0	σ1 capsid protein	114
Rotavirus	-	-	-	-	115
ENVELOPED VIRUSES					
Bunyavirus	-	Goose	20°C acidic pH	G1 glyco protein	117
Coronavirus	-	Human,Rat mouse	4°C pH 7.0	E2 spike	121
Myxovirus	influ-enza	Chicken human '0' Guinea pig	4°C pH 7.0	HA spike	117
Paramyxovirus	Sendai	--"--	4°C pH 7.0	HN spike	117
Retrovirus	murine leukemia	Guinea pig	4°C narrow range	gp70	-
Togavirus	-	1 day old chicken	37°C Ca^{++}	E1 spike	-

which has 3 long stretches of α helices (118). Molecular structure of the VAP of influenza virus is the best characterized of all glycoproteins. X- ray crystallographic studies revealed that a globular region containing residues of haemagglutination activity sits on the top of fibrous stem and includes an 8 stranded ß sheet arrangement. Within the globular part, is a highly conserved region of amino acids in a pocket (119). A similar type of multichain parallel arrangement of a homotrimer has been proposed for the G protein spike of the vesicular stomatitis virus on the basis of crosslinking data (120). Human influenza strains bind preferentially to the disaccharide NeuAc(α2-6)gal found predominantly on human cells. Avian and equine strains prefer isomeric compound NeuAc(α2-3)gal which is more abundant in animals. The lectin mediates attachment of virus cells by combining with sialic acid containing carbohydrates on their surface. This is followed by fusion of viral and cellular membranes, allowing release of the viral genome into the cytoplasm and subsequent replication. Removal of sialic acid from cell membranes abolishes binding and prevents infection.

1.3.3.4 Protozoal lectins

Protozoa are unicellular animals that exist singly or in colony. Parasitic protozoa adapt themselves to an altered existance inside the host. The adaptation generally involves an interaction of the parasite with the host cell. There are several lines of evidence indicating that this interaction is

Table 1.5 **Protozoal Lectins**

Organism	Lectin Molecular prop.	Sugar Specificity	Biological Role	Ref.
Giardia lamblia	Taglin, membrane bound, M_r 56000 divalent cation dependent	phospho-mannose	To attach to intestinal lumen in Giardiasis	123
Entamoeba histolytica	M_r260000 subunits M_r 170000 35000 and 31000	gal galNAc	To bind to colonic mucosa in amebiasis mitogenesis	125
Trichomonas	High mol. wt. glycoprt.	man glcNAc sialic acid	cytopathy	126
Plasmodium falsiparum	-	glcNAc galNAc NeuNAc	To bind to host cells	127
Dictyostelium discoidium	M_r 25000 26000 & 26500	gal galNAc	mediating intercellular adhesion	128

mediated by specific recognition mechanisms involving sugar-lectin interaction (Table 1.5)

Adhesive properties of microbes

All living cells express surface carbohydrates that participate in intercellular interactions. Because of their abundance on the cell surface, carbohydrates are preferentially selected as receptors rather than peptides. This form of adhesion depends upon lectins on the surface of the microorganisms. It is considered to be highly specific and responsible for tissue tropism of pathogenic and symbiotic microorganisms, e.g. microbial colonization of gastrointestinal tract by *Vibrio cholerae*, of lungs and meninges by *Streptococcus pneumoniae*, of urinary tract by *Staphylococcus saprophyticus*.

Recent work has demonstrated that carbohydrate specific bacterial lectins may recognize the receptor in both internal and terminal position. Membranes from different tissues differ in their composition. Accordingly, two lectins recognizing different epitopes on the same oligosaccharide may have a considerably different tissue binding distribution. Such interactions may have clinical relevance also. The receptor of one bacterium may be (totally or partially) identical with the receptor of the other bacterium. Thus *E.coli*, *Salmonella*, and *Klebsiella pneumoniae* adhesins all compete for mannose residues. The infection with certain microorganisms may destroy or uncover receptors for others. For example, influenza virus neuraminidase destroyes myxovi-

rus receptors, MN blood group receptors and receptors for certain *Mycoplasma* strains whereas blood group I/i receptors are uncovered and thus represent secondary uncovered receptors for *Mycoplasma pneumoniae* (100). Myxovirus neuraminidase uncovers cryptic ß-Dgal(1-3) N- acetyl D galactosamine residues which serve as receptors for *Actinomyces* strain or for certain strains of *Pseudomonas aeruginosa* (101). Myxovirus has been shown to destroy human blood group M antigen (102) which specifically binds certain pyelonephritic *E.coli* strains. Microbial lectin receptors of glycolipid and glycoprotein nature can be destroyed or uncovered by bacterial proteases as shown for MR pyelonephritic *E. coli* strains (103).

1.4 PLANT PATHOGENIC BACTERIA AND THEIR ATTACHMENT TO PLANT SURFACES

For many years, the study of plant pathogenic bacteria was the province of a small group of biologists. Since 1970's the interest has broadened due to developments in molecular biology and the ease with which plant pathogenic bacteria can be manipulated genetically.

Crop loss caused by bacteria is more harmful than viruses or fungi in some cases because, a bacterial pathogen is too destructive for many seasons. Examples include bacterial wilt (*Pseudomonas solanacearum*) of potato, tomato and other crops and fire blight (*Erwinia amylovora*) of pears. Of the staple food crops, severe losses in some years have been reported for rice (*Xanthomonas campestris* var*oryzae*), wheat,

(*Pseudomonas syringae*), soya bean, (*Pseudomonas syringae* var*glycinea*), cassava (*Pseudomonas syringae* var *mannihotis*) and potato (*Erwinia caratovora*). For maximum pathogenic potential, it is important for invading bacteria to colonize the plant tissue rapidly and to reach high population levels before plant tissue maturation or other plant responses limit bacterial growth. In addition by production of specific virulence factor, successful pathogens are sometimes able to increase the susceptibility of the host and avoid provoking the plant resistance mechanisms. Plant pathogens invading tissue need to be attached to specific sites. Attachment of bacteria to the foliage and to the root surfaces is an early and possibly significant step in certain plant diseases and may represent an important factor in epiphytic colonization which probably increases the capacity of the organism to cause the disease (129).

1.4.1 Attachment to plant surfaces

Initial binding of bacterial cells to plant surfaces is characterized by two steps 1) early reversible interaction and 2) irreversible attachment. As bacterial and plant surfaces both exhibit a net negative charge in an aqueous environment, electrostatic forces alone would tend to keep the cells apart. The negatively charged bacterial cell must therefore overcome the repulsive energy barrier for attachment to the plant cell surface through bridging by divalent cations, ionic bridging, hydrogen bonding and van der Waal's forces. Additionally, hydrophobic surfaces on surface of

plant and hydrophobic regions on the surface of bacteria may bring cells into close contact, facilitating further interaction (130, 131).

In certain plant pathogens, the site of attachment on the plant surface appears to be specifically localized. *Peudomonas syringae* pv. *Phaseolicola* adheres preferrentially to stomata of bean whereas some strains of *Peudomonas syringae* are evenly distributed over the plant surface (132). *Xanthomonas campestris* pv. *hyacinthi* as well as fimbriae isolated from the same attach preferrentially to stomata of hyacinths (133). The nitrogen fixing *Klebsiella* sp. adheres specifically to root hair but not to the epidermal cells elsewhere along the root surface (134).

1.4.2 Bacterial structures involved in attachment

Various bacterial surface structures or molecules exported from the cell have been suggested to function as adhesins. They include 1) polysaccharides, 2) proteins e.g. pili and 3) other molecules such as β1-3 glucan and lipopolysaccharides.

1) **Polysaccharides** : Bacterial EPS (extracellular polysaccharide) is the main molecule suspected to be involved in initial attachment of bacteria to the plant surface (135). It has been described as a thick, continuous, highly ordered, hydrated gel. It absorbs and binds water thus buffering the bacterial cell against dehydration and promoting its survival. Many studies have shown that polysaccharide deficient strains are avirulent (136). It was suggested that bacterial cells not surrounded by EPS come in contact with the plant

cell walls and therefore induce HR (hypersensitive responce). But whether the polysaccharide alone is responsible for attachment or is there any other molecule involved in it is not known.

2) **Bacterial fimbriae or pili**

Bacterial fimbriae is the most probable structure which could be responsible for the bacterial attachment to the plant surface (Table 1.6).

Erwinia rhapontici : It infects Rhubarb (*Rheum rhaponticum*) (137) and also causes pink wheat grains (*Triticum aestivum*) (138). It expresses fimbriae of a novel type (139). Fimbriated bacteria and isolated fimbriae efficiently absorb to the Rhubarb leaf surface and to wheat grains. Isolated fimbriae show haemagglutination activity and it is specific for LacNAc, Galβ1-4 glcNAc and other β galactosides (140). Adhesion and infection rate of the organism is reduced by the treatment of the organism with β gal sugars. Some other *Erwinia* also produce type 1 fimbriae.

Klebsiella spp. : It expresses type 1 and type 3 fimbriae. With the help of these fimbriae, the organism attaches itself to the root hairs of *Poa pratensis* and other grasses. Fimbriated strains adhere to the specific site on root hairs but not to epidermal cells between the root hairs. Nonfimbriated strains fail to attach (134).

Pseudomonas syringae and Pseudomonas solanacearum

Both of them produce pili. They are supposed to mediate binding to leaf cells and their autoagglutination. A major

Table 1.6 : **Plant pathogenic bacteria**

Pathogen disease	Host plant	Location	Specificity	Ref.
Agrobacterium tumefaciens	Dicots ; Crown gall	Surface or extracellular	Fucose	147
Erwinia amylovora	Pears ; Fire blight	Fimbriae	Mannose	129
Erwinia rhapontici	pink wheat grains	Fimbriae	ß - gal	139
Klebsiella	grasses	Fimbriae	-	134
Pesudomonas syringae	Wheat Cassava	Surface lectin & Fimbriae	-	141
Xanthomonas campestris	Rice *Brassica*	Fimbriae	-	142

protein subunit (M_r 9500) has been isolated from *Pseudomonas solanecearum* which is known to be a pilus protein (141, 132).

Xanthomonas campestris : In *Xanthomonas campestris* pv. *vesicatoria*, production of fimbriae is induced in the presence of plant material whereas *Xanthomonas campestris* pv. *hyacinthi* produces fimbriae constitutively (142). The amino terminal sequence of the pilus protein (M_r 17000) of *Xanthomonas campestris* pv. *hyacinthi* shows homology to the so called type 4 fimbriae expressed by *Haemophilus influenzae*, *Neisseria gonorrhoeae* and *Pseudomonas aeruginosa*. The receptor has not been established but the sugar specificity of other type4 fimbriae is galNAc ß 1-4 gal (143). A suspected protein (M_r 32500) is essential for type 4 pili secretion in *Xanthomonas campestris* (144).

Rhizobium : According to Lugtenberg *et al* (145) pea lectins and microorganism surface polysaccharide are responsible for rhizobial attachment to the root surface.

Bradyrhizobium japonicum : It binds to Soya bean root in a polar fashion. This binding is galactose specific suggesting a lectin mediated binding phenomenon. A lectin residing on the bacterial surface has been isolated and purified. It is known as BJ38 and has molecular mass of 38000 and is not a pilus protein (146).

Agrobacterium tumefaciens : Production of a lectin (M_r 36000) has been reported. This lectin is supposed to be a mediator of pathogen and host plant. The lectin is not a pilus protein and is specific for L(-) fucose (147).

3) **Other Molecules** : Among other molecules involved in the attachment of bacterial plant pathogens to host plants, (A) 2- linked ß -D -glucan as well as cell envelope lipopolysaccharides are reported in case of *Agrobacterium tumefaciens* (148, 149). (B) Variety of pectic enzymes are produced by plant pathogenic bacteria, e.g. endo polygalacturonide lyase by *Erwinia* spp. Plant tissue maceration is largely a result of pectic endo enzyme activity. A sequel to plant tissue maceration is cell death with release of water and of utilizable carbon and nitrogen sources for nutrition of the pathogen. Besides *Erwinia*, *Pseudomonas*, *Bacillus subtilis*, *Bacillus polymyxa and Clostridium* also produce pectic enzymes. (C) Toxins - Toxic substances produced by plant pathogens are of low molecular weight and are active at very low concentrations. Toxin production appears to be confined to *Pseudomonas syringae* spp. Toxin molecules attach to and invade host tissue producing local lesions. The toxins produced by *Pseudomonas* are - syringomycin, coronatin, phaseolotoxin, tabtoxin and others.

1.5 BIOLOGICAL FUNCTIONS OF THE MICROBIAL LECTINS

1. **Microbial adhesion to cellular and acellular sugar containing matrix** : Interactions with their own ploysaccharides, mucoid secretions and other glycosylated macromolecules may serve in the organization of cell envelopes and extracellular matrix, and in holding them near the cell. Lectins may also function in intracellular organization. The

slime mold *Dictyostelium discoidium* lectin, discoidin, interacts with its extracellular matrix as well as with foreign microbial glycoconjugates. Microbial adhesion to sugar bearing macromolecules may serve as a recognition signal for microbial migration and settlements in ecosystems. The acellular glycomatrix assures the nutritional requirement of the microorganisms and supplies them with a mechanical support. Mucosal secretions, fibronectin, and Tamm-Horsefall urinary glycoprotein may function as acellular glycomatrix.

2. **Microbial autoaggregation** : Myxobacteria and slime molds exhibit the property of autoagglutination through lectins. This phenomenon is often triggered by an environmental signal such as deficiency of a certain essential nutrient or exposure to certain substance or changed conditions of growth. The aggregation may serve for the formation of a new multicellular structure (150).

3. **Microbial coaggregation in mixed populations** : Coaggregation is a specific bacterial adherence to microorganisms of certain species and genera. e.g. Actinomyces lectins are found to interact with receptors on *Streptococcus*. These interactions contribute to the establishment of mixed bacterial population in human dental system forming dental plaques.

4. **Microbial adhesion in symbiosis** : Extensive literature has been published on the role of plant lectins to the symbiosis between rhizobia and legumes (145). The role of bacterial lectins in its interaction with legume has also been

described. *Bradyrhizobium japonicum* produces a lectins which is suposed to be a mediator of the microorganism and its symbiotic host.

5. Microbial lectins in parasitism : Most of the pathogenic microbes are bound to the host surfaces by their lectins. e.g. *E. coli* enterotoxigenic or uropathogennic, Viruses such as influenza A contains sialophilic lectins linked to sialidase which enable them to affect the host cell, fuse with its membrane and invade it, Exotoxins of *Shigella or Corynebacterium* which are lectins, show specificity for specific type of host tissue.

6. Lectin mediated phagocytosis of microorganisms : The microorganisms may be trapped by their own lectins. The lectin mediated adhesion leads to phagocytosis of the microorganisms. It is termed as lectinophagocytosis.

1.6 : STRUCTURAL BASIS OF LECTIN CARBOHYDRATE INTERACTION

The structural basis for selective sugar recognition by lectins has been investigated by X -ray crystallography of several plant and animal lectins (151). Mechanisms for sugar recognition by lectins have evolved independently, in diverse protein structural frameworks. In spite of their independent origins, the binding sites that have been analysed so far, share a few key features *viz.* They have low affinities for sugar than those of others which include sugar utilizing enzymes or sugar transport proteins, most of the lectins have binding sites on surface and are relatively shallow indentations whereas the others have binding sites which are

often deep clefts in the protein, selectivity in lectin sugar interactions is achieved mainly through hydrogen bonding, van der Waal's forces,hydrophobic interactions and coordinate bonding. Lectins bind to sugar -OH groups by hydrogen bonds and water molecules play a major role in these interactions. High resolution structures of unliganded lectins revealed that discrete water molecules form hydrogen bonds with those polar atoms that form hydrogen bonds with the sugars in lectin sugar complexes.These water molecules serve as mimics of the hydrogen bonding patterns of sugar -OHs. The van der Waal's contacts between lectin and sugar often include packing interactions with aromatic amino acid side chains. Such packing is particularly common in gal specific sites, but it is also observed in legume lectins that bind to mannose and glucose. Hydrophobic interactions are common between acetamido groups in glcNAc, galNAc and NeuNAc and aromatic side chains of the lectins. The coordinate bonds that link sugars to Ca^{++} in C type animal lectins contribute substantially to the affinity of binding.

1.7 PRESENT INVESTIGATION

In the last decade, interest in microbial agglutinins and lectins has increased tremendously. Several microbial lectins have been shown to have an important role in pathogen-host interactions. Studies on the structure, carbohydrate specificity and biogenesis of these lectins has led to a better understanding, at the molecular level, of their possible function in the interaction between microbes and host cell

surfaces.

Although much work has been done on human and animal pathogens and their lectins, plant pathogens have not received much attention. The present work was initiated to isolate, purify and characterize lectins from plant pathogens. Studies on their carbohydrate specificity can lead to a better understanding of the role played by lectins in phytopathogenicity.

REFERENCES

1. I. J. Goldstein, R. C. Hughes, M. Monsigny, T. Osawa and N. Sharon (1980) Nature **285** : 66.
2. N. Sharon and H. Lis (1972) Science **177** : 949 - 959.
3. S. H. Barondes (1988) Trends Biochem. Sci.**13** : 480.
4. H. Stillmark (1888) Ueber Ricin, ein giftiges ferment aus dem samen von Ricinus commun. L. und einigen anderen euphorbia ceen, Inaug. Diss. Dorpat university.
5. E. R. Gold and P. Balding (1975) Receptor specific proteins. Plant and animal lectins,Excerpta Medica, Amsterdam.
6. R. Kraus and S. Ludwig (1902) Wien. Klin. Wochenschr.**15** : 120 - 121.
7. G. Guyot (1908) Zentralbl. Bakteriol. Abt. I. Orig. **47** : 640 -643.
8. G. K. Hirst (1941) Science **94** : 22 - 23.
9. L. McClelland and R. Hare (1941) Can.J.Public Health **32** : 530 - 538.
10. L. Rosenthal (1943) J.Bacteriol. **45** :545 - 550.
11. E. V. Keogh, E. A. North and M. F. Warburton (1947) Nature **160** : 63.
12. M. F. Warburton and S. Fisher (1951)Aust. J. Exp. Biol. Med. Sci.**29** : 265 - 272.
13. C. Lamanna (1948) Proc. Soc. Exp. Biol.**69** : 332 - 335.
14. W. C. Boyd and S. Shapleigh (1954) Science **119** : 419-421.
15. W. A. Collier and J. C. deMiranda (1955) Antonie Leeuwenhoek J. Microbiol. Serol. **21** : 133 - 140.

16. J. P. Duguid and R. R. Gillies (1957) J. Pathol. Bacteriol. 74 : 397 - 411.

17. J. P. Duguid, E. S. Anderson, I. Campbell (1966) J. Pathol. Bacteriol. 92 : 107 - 138.

18. N. Gilboa-Garber (1972) Biochim. Biophys. Acta 273 : 165 - 173.

19. I. Ofek, D. Mirelman and N. Sharon (1977) Nature 265 : 623 - 625.

20. M. Aronson, O. Medalia, L. Schori, D. Mirelman, N. Sharon and I. Ofek. (1979) J. Infect. Dis. 139 : 329 - 332.

21. I. A. Wilson, J. J. Skehel and D. C. Wiley (1981) Nature 289 : 366 - 373.

22. P. E. Stein, A. Boodhoo, G. D. Armstrong, S. A. Cockle *et al* (1994) Structure 2 : 45 - 57.

23. J. M. Rini (1995) Annu. Rev. Biophys. Biomol. Struct. 24 : 551 - 577.

24. M. Slifkin and R. J. Doyle (1990) Clin. Microbiol. Rev. 3 : 197 - 218.

25. W. WadstrÖm, T. J. Trust and D. E. Brooks (1983) in Lectins Vol. III Walter degruyter and Co. Berlin-New-York. PP 479 - 494.

26. S. Stirm, F. ørskov and B. Mansa (1967) J. Bacteriol. 93 : 731 - 739.

27. T. Moch, H. Hoschutzky, J. Hacker, K. D. Kroncke and K. Jann (1987) Proc. Natl. Acad. Sci. USA 84 : 3462 - 3466.

28. J. P. Duguid and D. C. Old (1980) in" Bacterial adherence, receptors and recognition." Ser. B Vol. 6 (E. H.

Beachey ed.) Chapman and Hall, London, pp. 186 - 217.

29. T. Wadstrom, A. Faris, J. H. Freer, D. Habte, D. Hallberg and A. Lungh (1980) Scand. J. Infect. Dis. Suppl. 24 : 148 - 153.

30. R. Sellwood (1980) Biochim. Biophys. Acta. **632** 326 - 335.

31. N. Gilboa-Garber (1982) Methods Enzymol. **83** 378 - 385.

32. Y. Fujita, K. Oishi, K. Suzuki and K. Imahori (1975) Biochemistry **14** : 4465 - 4470.

33. Y. Fujita-Yamaguchi, K. oishi, K. Suzuki and K. Imahori (1982) Biochim. Biophys. Acta **701** 86 - 92.

34. N. Gilboa-Garber, L. Mizrahi, N. Garber (1977) Can. J. Biochem. 55 : 975 - 981.

35. I. Matsui, K. Oishi, K. Kanaya and N. Baba (1985) J. Biochem.97 : 399 - 408.

36. I. Matsui, K. Oishi (1986) J. Biochem. **100** 115 - 121.

37. J. P. Duguid, S. Clegg and M. I. Wilson (1979) J. Med. Microbiol. 12 :213 - 227.

38. L. Maurer and P. Orndoroff (1985) FEMS Microbiol. Lett. 30 : 59 - 66.

39. L. Maurer and P. Orndoroff (1987) J. Bacteriol. **169** : 640 - 645.

40. K. A. Krogfelt, H. Bergmans and P. Klemm (1990) Infect. Immun. 58 : 1995 - 1998.

41. C. C. Brinton Jr. (1965) Trans. N. Y. Acd. Sci. 27 : 1003 - 1054.

42. N. Firon, I. Ofek and N. Sharon (1983) Carbohydr. Res. 120 : 235 - 250.

43. N. Firon, I. Ofek and N. Sharon (1984) Infect. Immun.43 :

1088 -1090.

44. N. Firon, S. Ashkenazi, D. Mirelman, I. Ofek and N. Sharon (1987) Infect. Immun. 55 :472 - 476.

45. S. J. Hultgren, S. Abraham, N. Caparon, P. Falk, J. W. St. Geme III and S. Normark (1993) Cell 73 :887 - 901.

46. S. Normark, D. Lark, R. Hull, M. Norgren, M. Baga, P. O'Henley, G. Schoolrik and S. Falkow (1983) Infect. Immun. 41 : 942 - 949.

47. S. Normark, M. Baga, M. Goransson, F. P. Lindberg, B. lund, M. Norgren, B. E. Uhlin (1986) in " Microbial lectins and agglutinins : Properties and Biological activity" (D. Mirelman Ed.) John Wiley and Sons, New York. pp. 113 - 143.

48. B. E. Uhlin, M. Norgren, M. Baga and S. Normark (1985) Proc. Natl. Acad. Sci.USA 82 : 1800 - 1804.

49. B. Lund (1987) Umea Univ. Med. Diss. New Ser. pp. 194 .

50. H. Leffler, C. Svanborg-Eden (1986) in " Microbial lectins and agglutinins : Properties and Biological activity" (D. Mirelman Ed.) John Wiley and Sons, New York. pp. 83 - 111.

51. J. B. Baseman, M. Banai and I. Kahane (1982) Infect. Immun. 41 : 339 - 344.

52. L. D. Hazzlett, M. Moon and R. S. Berk (1986) Infect. Immun.51 : 687 - 689.

53. H. Ishikawa and Y. Isayama (1987) Infect. Immun. 55 : 1607 - 1609.

54. J. Morschhauser, H. Hoschutzky, K. Jann and J. Hacker

(1990) Infect. Immun. 58 : 2133 - 2138.

55. H. Smit, W. Gaastra, J. P. Kamerling, J. F. G. Vliegenthart and F. K. deGraaf (1984) Infect. Immun. 46 : 578 - 584.

56. S. P. Teneberg, P. Willemsen, F. K. deGraaf and K. A. Karlson (1990) FEBS Lett. 263 : 10 - 14.

57. E. Ono, K. Abe, M. Nakazawa and M. Naiki (1989) Infect. Immun. 57 : 907 - 911.

58. J. R. Neeser, A. Chambaz, K. Y. Hoang and H. Link-Amster (1988) FEMS Microbiol. Lett. 49 : 301 - 307.

59. P. Pieroni, E. A. Worobec, W. Paranchych and G. D. Armstrong (1988) Infect. Immun. 56 : 1334 - 1340.

60. T. K. Korhonen, K. Lounatmaa, H. Ranta and N. Kuusi (1980) J. Bacteriol. 144 : 800 - 805.

61. A. Utsunomiya, T. Naito, M. Ehara, Y. Ichinose and A. Hamamoto (1992) Microbiol. Immunol. 36 : 803 -813.

62. R. A. Finkelstein and L. F. Hanne (1982) Infect. Immun. 36 : 1199 - 1208.

63. J. Osek, A. M. Svennerholm and J. Holmgren (1992) Infect. Immun. 60 : 4961 - 4964.

64. A. Naka, K. Yamamoto, T. Miwatani and T. Honda (1992) FEMS Microbiol. Lett. 98 : 197 - 200.

65. T. K. Sengupta, D. K. Sengupta and A. C. Ghose (1993) FEMS Microbiol. Lett. 112 : 237 - 242.

66. O. S. Gildmeister, B. C. R. Zhu and R. A. Laine (1994) Glycoconjugate J. 11 : 518 -526.

67. I. Kahane, M. Banai, S. Razin and J. Feldner (1982) Rev. Infect. Dis. 4 : s185.

68. P. C. Hu, A. M. Collier and J. B. Baseman (1977) J. Exp.Med. **145** : 1328 -1330.

69. L. M. Loomes, K. Umemura, R. A. Childs, J. C. Paulson, G. N. Rogers, P. R. Scudder, J. C. Michalski, E. F. Hounsell, D. Taylor-Robinson and T. Seizi (1984) Nature 307 : 560 - 563.

70. L. M. Loomes, K. Umemura and T. Seizi (1985) Infect. Immun. **47** : 15 -20.

71. D. D. Roberts, L. D. Olson, M.F. Barile, V. Ginsberg and H. C. Krivan (1989) J. Biol. Chem. **264** : 9289 - 9293.

72. K. A. Karlsson (1989) Annu. Rev. Biochem. **58** : 309 - 350.

73. C. D. Deal and H. C. Krivan (1990) J. Biol. Chem. 265 : 12774 - 12777.

74. P. R. Lambden, G. E. Heckels, L. T. James and P. J. Watt (1979) J. Gen. Microbiol. **114** : 305 - 310.

75. P. R. Lambden, J. N. Robertson and P. J. Watt (1980) J. Bacteriol. **141** : 393 - 396.

76. P. R. Lambden, J. E. Heckels, H. McBride and P. J. Watt (1981) FEMS Microbiol. Lett. **10** : 339 -341.

77. N. Gilboa-Garber, L. Mizrahi and N. Garber (1972) FEBS Lett. **28** : 93 - 95.

78. P. Doig, W. Paranchych, P. A. Sastry and R. T. Irvin (1989) Can. J. Microbiol. **35** : 1141 - 1145.

79. L. D. Hazzlett, M. Moon and R. S. Berk (1986) Infect. Immun. **51** : 687 - 689.

80. J. S. Wentworth, F. E. Austin, N. Garber, N. Gilboa-Garber, C. A. Patterson and R. J. Doyle (1991) Biofoul-

ing 4 : 99 - 104.

81. S. Gatterman, H. G. W. Meyer and G. Wanner (1992) Infect. Immun. 60 : 4127 - 4132.

82. J. Beuth, H. L. Ko, G. Uhlenbruck and G. Pulverer (1987) Eur. J. Clin. Microbiol. Infect. Dis. 6 : 591 - 593.

83. D. Drake, K. G. Taylor, A. S. Bleiweis and R. J. Doyle (1988) Infect. Immun. 56 : 1864 - 1872.

84. D. Drake, K. G. Taylor and R. J. Doyle (1988) Infect. Immun. 56 : 2205 - 2207.

85. N. Stromberg and K, A. Karlsson (1990) J. Biol. Chem. 265 : 11251 - 11258.

86. S. J. Barenkamp and E. Leininger (1992) Infect. Immun. 60 : 1302 - 1313.

87. J. Goldhar, R. Parry, J. R. Golecki, H. Hoschutzky, B. Jann and K. Jann (1987) Infect. Immun. 55 : 1837 - 1842.

88. A. Darfeuille-Michaud,C. Forestier, B. Joly and R. Cluzel (1986) Infect. Immun. 52 : 468 - 475.

89. H. Hoschutzky, W. Nimmich, F. Lottspeich and K. Jann (1989) Microb. Pathog. 6 : 351 - 359.

90. K. Jann and H. Hoschutzky (1990) Current Topics in Microbiology and Immunology 151 : 55 - 70.

91. K. D. Kroncke, I. ørskov, F. ørskov, B. Jann and K. Jann (1990) Infect. Immun. 58 : 2710 - 2714.

92. M. Takagi, N. Hirayama, T. Simazaki, K. Taguchi, R. Yamaoka and S. Ohta (1993) Vet. Microbiol. 34 : 191 - 197.

93. E. Hanski, P. A. Horwitz and M. G. Caparon (1992) Infect. Immun. 60 : 5119 - 5125.

94. H. Ota, T. Katsuyama,K. Ishii, J. Nakayama, T. Shiozawa and Y. Tsukahara (1991) Histochem J. 23 : 22 - 28.

95. M. Kundu, J. Basu and P. Chakrabarti (1989) FEBS Lett. 256 : 207 - 210.

96. B. Baseman, M. Banai and J. Kahane (1982) Infect. Immun. 38 : 389 - 392 .

97. N. Burnette (1994) Structure 2 : 151 - 158.

98. T. K. Sixma *et al* and W. G. J. Hol (1991) Nature 351 : 371 - 377.

99. L. Eidels, R. I. Proia and D. A. Hart (1983) Microbiol. Rev. 47 : 596 - 620.

100. G. Uhlenbruck (1961) Zbl. Bakt. Orig. 81 : 285 -288.

101. H. Lis and N. Sharon (1986) Annu. Rev. Biochem. 55 : 35 -60 .

102. J. Parkkinen, J. Finne, M. Achtmann, V. Vaisanen and T. K. Korhonen (1983) Biochem. Biophys. Res. Commun. 111 : 456 - 468 .

103. M. J. Heeb, A. H. Costello and O. Gabriel (1982) Infect. Immun. 38 : 993 - 996.

104. J. M. Hinch and A. E. Clarke (1980) Physiol. Plant Pathol. 16 : 303 - 307.

105. J. R. Green and D. H. Northcote (1978) Biochem J. 170 : 599 - 604.

106. B. Nordbring-Hertz and I. Chet (1986) in "Microbial lectins and agglutinins, Properties and biological activity" (D. Mirelman, Ed.) John Wiley and Sons, New York, pp 393 - 406.

107. R. Mankau (1980) Annu. Rev. Phytopathol. **18** : 415 - 450.

108. C. A. K. Borrebaeck, B. Maattiasson and B. Nordbring-Hertz (1984) J. Bacteriol. **159** : 53 - 59.

109. R. Barak, Y. Elad, D. Mirelman and I. Chet (1985) Phytopathology **75** : 458 - 462.

110. D. Neethling and H. Nevalainen (1996) Can. J. Microbiol. **42** : 141 - 146.

111. A. J. Castle, N. Stocco and R. Boulianne (1996) Can. J. Microbiol. **42** : 461 - 466.

112. P.Petit (1982) New Phytol. **91** : 705 - 707.

113. L. G. Payne and E. Norrby (1976) J. Gen. Virol. **32** : 63 - 66.

114. H. L. Weiner, D. Drayna, D. R. Averill and B. N. Fields (1977) Proc. Natl. Acad. Sci. USA **74** : 5744 - 5747.

115. A. R. Kalika, J. Flores and H. B. Greenberg (1983) Virology **125** : 194 - 197.

116. G. M. Clinton and M. Hayashi (1976) Virology **74** : 57 - 60.

117. L. Kingsford and D. W. Hill (1983) J. Gen. Virol. **64** : 2147 - 2152.

118. I. A. Wilson, J. J. Skehel and D. C. Wiley (1981) Nature **289** : 366 - 368.

119. C. S. J. Wright (1980) J. Mol. Biol. **141** : 267 - 270.

120. E. J. Dubovi and R. R. wagner (1977) J. Virol.**22** : 500 - 503.

121. L. S. Sturman and K. V. Holmes (1977) Virology **77** : 650 - 655.

122. J. Delarco and G. Todaro (1976) Cell **8** : 365 - 369.

123. E. Gianazza and P. Arnaud (1982) Biochem J. 203 : 637 - 640.

124. W. A. Petri and R. L. Schanaar (1995) Methods Enzymol. 253 : 98 - 103.

125. R. A. Salata and J. I. Ravdin (1985) J. Infect. Dis. 151 : 816 - 819.

126. R. L. Jacobson and R. J. Doyle (1996) Parasitology Today 2 : 55 - 61.

127. L. H. Bannister, G. A. Butcher and G. H. Mitchell (1977) *Bull* WHO 55 : 163 - 169.

128. S. D. Rosen, J. Kaur, D. L. Clark, B. T. Pardos and W. A. Frazier (1979) J. Biol. Chem. 254 : 9408 - 9412.

129. M. Romantschuk (1992) Annu. Rev. Phytopathol. 30 : 225 - 243.

130. G. I. Bell (1978) Science 200 : 618 - 627.

131. J. A. Lippincott and B. B. Lippincott (1984) in " Plant microbe interactions, Molecular and genetic perspectives" (T. Kosuge and E. W. Nester Eds.) Vol. 1, New York, McMillan pp 195 - 214.

132. M. Romantschuk and D. H. Bamford (1986) Micrb. Pathog. 1 : 139 - 148.

133. J. vanDoorn, P. M. Boonekamp and B. Oudega (1991) in FEMS Symp. Mol. Recog. Host-Plant Interact. Abstr. Porvoo, Finland.

134. K. Haahtela, T. Laakso and T. K. Korhonen (1986) Appl. Environ. Microbiol. 52 : 1074 - 1079.

135. I. C. M. Dea, E. R. Morris, D. A. Rees, E. J. Welsh, H.

A. Barnes and J. Price (1977) Carbohydr. Res. 57 : 249 - 272.

136. M. A. Zachowski, F. Ebrahim-Nesbat and K. Rudolph (1990) in " Plant pathog. bacteria, Proc. 7th Int. Conference, Plant pathogenic bacteria, Budapest June 11 - 16 1989 " (Z. Clement Ed.) Akademiai Kiado Budapest.

137. P. Roberts (1974) J. Appl. Bacteriol. 37 : 353 - 358.

138. J. Sellwood, R. A. Lelliot (1978) Plant Pathol. 27 : 120 124.

139. T. K. Korhonnen, K. Haahtela, A. Pirkola and J. Parkkinen (1988) FEBS Lett. 236 : 163 - 166.

140. J. Louhelainen, K. Haahtela, O. Lindroos, E. L. Nurmiaho-Lassila and T. K. Korhonen (1990) Abstr. Int. Symp. Mol. Genet. Plant Microbe Interact. 5th interlaken, Switzerland.

141. W. P. C. Stemmer and L. Sequeira (1987) Phytopathology 77 : 1633 - 1639.

142. M. Romantschuk, E. Roine, T. Ojanen, J. vanDoorn, J. Louhelainen, E. L. Nurmiaholassila and K. Haahtela (1994) in "Molecular mechanisms of bacterial Virulence" (C. I. Kado and J. H. Crosa Eds.) Kluwer Academic Publishers, pp 67 - 77.

143. D. A. Cuppels, A. K. Vidaver and J. L. vanEtten (1979) J. Gen. Virol. 44 : 493 - 504.

144. N. T. Hu, P. F. Lee and C. Chen (1995) Mol. Microbiol. 18 : 769 - 777.

145. B. J. J. Lugtenberg, C. Diaz, G. Smit, S. dePater and J. W. Kijne (1991) Adv. Mol. Genet. Plant-Microbe Interact.

1 : 174 - 181.

146. J. T. Loh, S. C. Ho, A. W. deFeijter, J. L. Wang and M. Schindler (1993) Proc. Natl. Acad. Sci. USA **90** : 3033 - 3037.

147. C. Depierreux, H. C. Kang, B. Guerin, M. Monsigny and F. Delmotte (1991) Glycobiology **1** : 643 - 649.

148. V. Puvanesarajah, F. M. Schell, G. Stacey, C. J. Douglas and E. W. Nester (1985) J. Bacteriol. **164** : 102 -106.

149. M. H. Whatley, J. S. Bodwin, B. B. Lippincott and J. A. Lippincott (1976) Infect. Immun. **13** : 1080 - 1083.

150. S. H. Barondes (1981) Annu. Rev. Biochem. **50** : 207 - 231.

151. W. I. Weis and K. Drickamer (1996) Annu. Rev. Biochem. **65** : 441 - 473.

152. N. Sharon and H. Lis (1995) in " Essays in Biochemistry" (D.K.Apps and K. F. Tipton Eds.) Portant press, Great Britain **30** : 59 - 75.

153. J. M. Saunders and C. H. Miller (1980) Infect. Immun. **29** : 981 - 983 .

154. H. M. Atkinson and T. J. Trust (1980) Infect. Immun. **27** : 938 - 942 .

155. W. A. Pearce and T. H. Buchanan (1980) in " Bacterial Adherence : Receptors and recognition" (E. H. Beachey Ed.) Ser. B, Vol. 6 Chapman and Hall, London pp 289 - 344.

156. M. I. Wanaga and A. Hokama (1992) J. Gen. Microbiol. **138** : 1913 - 1919.

157. H. Nakae, H. Yamamoto, T. Matsuo and S. Ebisu (1994) FEMS Microbiol. Lett. **116** : 349 - 353.

158. J. P. Duguid and D. C. Old (1980) in "Bacterial Adherence : Receptors and recognition " (E. H. Beachey Ed.) Ser. B Vol. 6 Chapman and Hall, London. pp 187 - 217.

159. C. Pruzzo, E. Debbia and G. Sata (1982) Infect. Immun. **36** : 949 - 952.

160. K. Okuda nad I. Tabazoe (1974) Archs. Oral. Biol. **19** : 415 - 418.

161. K. Okuda, J. Slots and R. J. Genco (1981) Curr. Microbiol. **6** : 7 -10 .

162. J. Slots and R. J. Gibbons (1978) Infect. Immun. **19** : 254 - 257.

163. S. C. Ho, M. Schindler and J. L. Wang (1990) J. Cell. Biol. **111** : 1639 - 1643.

164. T. S. Walker and H. H. Winker (1981) Infect. Immun. **31** : 480 -483 .

165. D. A. Beims and B. J. Plotkin (1982) Infect. Immun. **15** : 1120 - 1122.

166. J. W. Gotto, T. Eckhardt, P. A. Reilly, J. V. Scott, J. L. Cowell, T. N. Metcalf, K. Mount-Zouros, J. J. Gibbons and M. Siegel (1993) Infect. Immun. **61** : 2211 - 2215.

167. S. K. Bose, G. B. Smith and R. G. Paul (1983) Infect. Immun.**40** : 1060 - 1065.

168. I. D. Binogradova, R. N> Vuarova, K. K.Ivanov, I. S. Kazjobina, T. I. Bulatova and V. N. Mel'nikov (1983) Biokhimiya **48** : 788 - 790.

169. J. Bagg, I. R. Paxton, J.Goyle, P. W. Ross, D. M. Weir

(1980) in "Microbial Adhesion to Surfaces, Society for Chemical Industry" (R. C. W. Berkeley, J. H. Lynch, P. R. Rutter and D. Vincent Eds.) Ellis Harwood Ltd. Chichester, pp 528 - 530.

170. Y. Yamazaki, S. Ebisu and H. Okada (1981) Infect. Immun. 31 : 21 - 24.

171. R. A. Adegbola and D. C. Old (1983) J. Gen. Microbiol. 129 : 2175 - 2178.

172. R. A. Adegbola and D. C. Old (1982) Infect. Immun. 38 : 306 - 309.

173. T. K. Korhonen, N. Kalkkinen, K. Haahtela and D. C. Old (1987) J. Bacteriol. 169 : 2281 - 2283.

174. J. Goldhar, R. Perry and I. Ofek (1984) Curr. Microbiol. 11 : 49 -53.

175. Y. Eshdat, I. Ofek, Y. Yashouv-Gan, N. Sharon and D. Mirelman (1978) Biochem. Biophys. Res. Commun. 85 : 1551 - 1554.

176. P. S. Cohen, A. D. Elbein, R. Solf, H. Mett, J. Regos, E. B. Menge and K. Vosbeck (1981) FEMS Microbiol. Lett. 12 : 99 - 102.

177. M. J. Kearns and R. A. Gibbons (1979) FEMS Microbiol Lett.6 : 165 - 168.

178. P. Dehazya and R. S. Coles (1980) J. Bacteriol. 143 : 205 - 209.

179. S. M. Spinola, A. Caetellazzo, M. Shero and M. A. Apicella (1990) Microb. Pathog. 9 : 417 - 426.

180. S. Weir and C. F. Marrs (1992) Infect. Immun. 60 : 3437

- 3441.

181. R. C. Fader, L. K. Duffy, C. P. Davis and A. Kurosky (1982) J. Biol. Chem. **257** : 3301 - 3306.

182. T. K. Korhonnen, E. Tarkka, H. Ranta and K. Haahtela (1983) J. Bacteriol. **155** : 860 - 864.

183. T. Mukai, K. Arihara and J. Itoh (1992) FEMS Microbiol. Lett. **98** : 71 - 74.

184. W. A. Falker, C. E. Haweley, J. R. Mongiello (1978) J. Period. Res. **13** : 425 - 429.

185. T. S. Sandho, F. W. White and C. F. Simpson (1974) Amer. J. Vet. Res. **35** : 437 - 439.

186. D. C. Old and R. A. Adegbola (1982) J. Med. Microbiol. **15** : 551 - 553.

187. M. G. Cumsky and D. R. Zusman (1981) J. Biol. Chem. 256 : 12581 - 12585.

188. T. J. Trust, R. M. Gillespie, A. R. Bhatti and L. A. White (1983) Infect. Immun. **41** : 106 - 110.

189. J. C. Glorioso, G. W. Jones, H. G. Rush, L. J. Pentler, C. A. Darif and J. E. Coward (1982) Infect. Immun. 35 : 1103 - 1105.

190. Y. Sharabi and N. Gilboa-Garber (1979) FEMS Lett. **5** ; 273 - 276.

191. W. Heumann and R. Marx (1964) Arch. fur Mikrobiol (Berlin) **47** : 325 - 328.

192. A. Tavendale, C. K. H. Jardine, D. C. Old and J. P. Duguid (1983) Med. Microbiol. **16** : 371 - 373.

193. D. C. Old, R. Adegbola and S. S. Scott (1983) Med. Microbiol. Immunol. **172** : 107 - 110.

194. M. M. McCabe and E. E. Smith (1975) Infect. Immun. 12 : 512 - 516.

195. M. Ramstope, P. Carlsson, D. Bratthall and B. Mattiasson (1982) Caries. Res. 16 : 423 - 425.

196. M. M. McCabe and E. E. Smith (1976) in " Carbohydrate receptors of oral Streptococci in immunological aspects of dental caries" (W. H. Bowen, R. J.Genco and T. C. O'Brien Eds.) Information Retrieval Inc. Washington D C pp 111 - 119.

197. K. Nagata, M. Nakao, S. Shihata, S. Shizukuishi, R. Nabamura and A. Tsunemitsu (1983) J. Periodontol. 54 : 163 - 166.

198. P. A. Murray, M. J. Levine, L. A. Tabak and M. S. Reddy (1982) Biochem. Biophys. Res. Commun.106 : 390 - 394.

199. J. L. Larsen and S. Mellergaard (1984) Appl. Env. Microbiol. 47 : 1261 - 1264.

200. R. A. Finkelstein, M. Finkelstein-Bolsman and P. Holt (1983) Proc. Natl. Acad. Sci. USA 80 : 1092 - 1095.

201. T. J. Trust, E. P. Courtice, A. G. Khouri, J. H.Crosa and M. H. Shiewe (1982) Infect. Immun.34 : 702 - 704.

202. R. M. Maclagan and D. C. Old (1980) J. Appl. Biol. 49 : 353 - 356.

203. G. Kapperud and J. Lassen (1983) Infect. Immun. 42 : 163 - 166.

CHAPTER 2

Purification and characterization of lectins from *Agrobacterium radiobacter*

SUMMARY

Many isolates of *Agrobacterium* obtained from the National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune, (India), were screened for extracellular as well as cell bound lectin production. The microorganisms were grown in liquid media containing ($g.L^{-1}$) glucose 20, peptone 5, yeast extract 3 and malt extract 3, pH 7.0 for 72 hours. Lectin activity in an aliquot of the culture broth was checked every 24 hours by haemagglutination of rabbit

erythrocytes. Out of 11 cultures tested, isolate number 7, i.e. *Agrobacterium radiobacter*, showing consistently higher, extracellular as well as cell bound lectin activity than other isolates was chosen for further studies.

The growth medium for the chosen isolate was optimized by varying the carbon source and pH of the medium. Lectin production in synthetic medium was also checked. The optimized medium which supported highest lectin production contained ($g.L^{-1}$) sucrose 20, peptone 5, yeast extract 3 and beef extract 3, pH 6.5.

The time course of lectin production of *Agrobacterium radiobacter* was followed by monitoring the protein content and lectin activity every 24 hours upto 120 hours. The highest lectin (extracellular and cell bound) activity was found at the end of 48 hours of fermentation and therefore the culture was harvested after every 48 hours.

After optimizing the growth conditions and lectin production,

the extracellular lectin (Lectin I) was purified to homogeneity by ion exchange chromatography on DEAE-cellulose followed by hydrophobic chromatography on phenyl Sepharose. Lectin I was a monomer of relative molecular mass 37000 as determined by denaturing gel electrophoresis as well as size exclusion chromatography on Sephacryl S-300. Its isoelectric point was 4.0. Amino acid analysis revealed that acidic amino acids and glycine were predominant amino acids and cysteine was absent. The lectin was stable under acidic environment and at room temperature for 2 hours. It agglutinated rabbit erythrocytes but not human blood group A, B, or O erythrocytes. The lectin was specific for chitobiose as well as to some extent to mannose. Glycoproteins having high mannose type of carbohydrate structure inhibited the lectin activity strongly.The exact sugar structure which it recognized was glcNAc-glcNAc-man3. Tobacco plant tissue extracts also inhibited the lectin activity.

Agrobacterium radiobacter produced another lectin (Lectin II) which was cell bound. It was extracted in 6M urea at 60°C and purified on Sepharose -4B column in 6M urea after 0.1 % sodium deoxycholate treatment. It was a monomer of M_r 40000 as shown by SDS-PAGE. The lectin had a pI of 9.1 and amino acid composition of the lectin showed that it had 44 % of hydrophobic amino acids. The lectin showed specificity towards D-glucosamine. Fetuin as well as desialylated fetuin and tobacco plant tissue extract inhibited the lectin activity strongly. Locust bean polysaccharide was the best inhibitor among plant polysaccharides.

2.1 INTRODUCTION

It is known that several bacteria produce lectins and lectin like proteins (1). These include exotoxins, surface proteins and small appendages like pili which are involved in specific adhesion of the bacterial cell to eukaryotic cell surface (2). Physiological role of some of these lectins is known as they contribute to microbial pathogenicity. However, bacterial lectins, especially those from plant pathogenic microorganisms have not been studied in much detail. *Agrobacterium* species are gram negative, obligate aerobic soil bacteria capable of saprophytic or parasitic growth and are responsible for the crown gall and hairy root diseases of dicotyledonous plants (3).

The disease production by Agrobacteria, is believed to start with a site specific attachment of the pathogen to its host. Specific constituents for attachment are probably located on the surface of both bacteria and host cells. The genus contains two widely studied pathogenic species : *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*. The genus includes only one nonpathogenic species *viz. Agrobacterium radiobacter* (4). Agrobacteria infect a wide variety of plants and infect only at wound sites. They infect individual cells at the site of infection and cause these cells to proliferate. This is achieved by the transfer of a discrete fragment of bacterial DNA to the nuclei of plant cells, where it is integrated into plant genomic DNA and directs the overproduction of plant

growth hormones. Understanding how *Agrobacterium* species perceive wounded plants and transfer DNA into them has been aimed at by many workers till date.

Agrobaterium is a peritrichous motile organism and the first and foremost step in the infection is supposed to be site specific attachment of the bacterium to the host cell surface. This attachment might involve lectin carbohydrate interaction (5). Although *Agrobacterium radiobacter* is a nonpathogen, there are several reports available on the successful biocontrol of pathogenic Agrobacteria using nonpathogenic ones (6,7).

In case of *Agrobacterium tumefaciens*, Depierreux *et al.* (5) have shown that the lectin resides on the bacterial surface and host plant carbohydrates act as receptors. The nonpathogenic *Agrobacterium radiobacter* probably competes with pathogenic agrobacteria for the same receptor sites on the host and this could be through lectin mediated specific attachment. Therefore, to see whether the nonpathogenic Agrobacteria also produce any lectins was of interest. The present study was aimed at screening of *Agrobacterium* species for extracellular lectin production and optimization of its growth conditions as well as purification and characterization of these lectins.

2.2 MATERIALS AND METHODS

2.2.1 Materials

Various isolates of Agrobacteria were obtained from National Collection of Industrial Microorganisms (NCIM),

National Chemical Laboratory, Pune, (India). Glucose, sucrose, peptone, yeast extract, malt extract, beef extract and agar were obtained from HiMedia Laboratories, Bombay, (India). Other salts required in synthetic medium, like magnesium sulphate, ammonium sulphate, boric acid, citric acid, calcium carbonate etc. were analytical grade and obtained from Qualigens, (India). DEAE- cellulose DE-52 was purchased from Whatman (UK), phenyl Sepharose and Sephacryl S-300 were obtained from Pharmacia (Sweden). Ampholines, SDS-PAGE molecular weight markers, glycoproteins, sugars and gums were obtained from Sigma (USA). Urea and sodium deoxycholate were from Qualigens, (India).

All other chemicals used in this study were of analytical grade.

2.2.2 Maintenance of the microorganisms

All the cultures of *Agrobacterium* were regularly maintained on agar slants containing ($g.L^{-1}$): glucose 20, peptone 5, malt extract 3 and yeast extract 3, pH 6.8.

2.2.3 Screening

All the cultures were grown in 50 ml liquid medium containing ($g.L^{-1}$): glucose 20, peptone 5, malt extract 3 and yeast extract 3, pH 6.8, in 250 ml flasks at 28°C for 72 hours and the lectin activity was checked after every 24 hours. Inall 11 number of isolates were screened.

2.2.4 Medium optimization

Various conditions for growth were tested and the medium

supporting highest lectin production was selected. Sucrose as carbon source was tested instead of glucose; beef extract was supplemented for malt extract; and pH values of the medium were also varied to see its effect on lectin production and growth of the microorganism. Synthetic medium used for growth contained ($g.L^{-1}$) : glucose 10, KH_2PO_4 5, $MgSO_4$ 0.2, $(NH_4)_2SO_4$ 2, citric acid 0.2, H_3BO_3 0.006, ZnO 0.006, $FeCl_3$ 0.002, $CaCO_3$ 0.002, pH 6.8. To check whether PO_4 ions inhibit the polysaccharide production, the synthetic medium was supplemented with 2.2 % (w/v) KH_2PO_4. The selected *Agrobacterium* spp. was also grown in nutrient broth containing ($g.L^{-1}$) : peptone 5 and beef extract 3, pH 7.0. All these experiments were done in duplicates. The flasks were incubated at 28°C for 48 hours under shaking conditions and cells were separated by centrifugation (9770 X g,20 min.) from liquid medium. The lectin activity was checked for both of the lectins, extracellular as well as cell bound lectin.

2.2.5 Time course of production of lectin by Agrobacterium radiobacter

Time course for the production of lectin by *Agrobacterium radiobacter* was followed by monitoring protein as well as haemagglutination activity every 24 hours upto 120 hours. One ml aliquot was removed aseptically for checking the activity. The cells were separated by centrifugation and 100 µl of the culture filtrate was used for haemagglutination activity and 900 µl of the sample was subjected to 12.5 % trichloroacetic acid precipitation. After 30 min. of incubation in ice, the

protein precipitate was separated by centrifugation, washed twice with acetone and then dissolved in minimum volume of distilled water. This sample was used for protein estimation by the method of Lowry *et al.* (6). For determination of cell bound lectin activity, the cell pellet was washed 3 times with TBS (20 mM Tris-HCl, pH 7.2 with 0.15 M NaCl) and suspended in 100 µl of the same buffer. Then haemagglutination activity of the cells was checked.

2.2.6 Purification of lectin I

From one liter culture broth of *Agrobacterium radiobacter* (NCIM 2443) grown at 28°C for 48 hours, bacterial cells were separated by centrifugation (9770 X g for 20 min) and the culture filtrate was subjected to 80 % (w/v) saturation with $(NH_4)_2SO_4$. The precipitate formed was dissolved and dialyzed against 20 mM Tris-HCl buffer, pH 7.2 containing 2 mM EDTA (buffer A), sonicated (Ralsonic sonicator, at 10000 Kcyc for 3 min.) to reduce the viscosity and applied onto a column of DEAE- cellulose DE-52 (2 x 40 cm) preequilibrated with buffer A. The column was then washed with the same buffer till all the unbound protein was removed from the column. Subsequently, the bound protein was eluted with a linear gradient of 0 to 0.5 M NaCl in buffer A. The fractions showing haemagglutination activity were pooled, dialyzed against buffer A and applied on a phenyl Sepharose column preequilibrated with 20 % $(NH_4)_2SO_4$ in buffer A. Elution was carried out with a gradient of 20 % to 0 % $(NH_4)_2SO_4$ in buffer A. Fractions with haemagglutination activity were pooled and dialyzed. The

purity of the protein was checked by SDS-PAGE. To remove nonproteinaceous material i.e. polysaccharide from the purified protein sample, it was passed through hydroxyapatite column (5 ml) in presence of 5 mM potassium phosphate buffer pH 6.8 containing 1 mM EDTA.

2.2.7 Purification of the lectin II

Cell bound lectin was purified according to de Graaf (25). *Agrobacterium radiobacter* cells were harvested after 48 h, by centrifugation (9770 X g) and washed three times with 20 mM Tris-HCl pH 7.0 buffer containing 0.15 M NaCl (TBS). The cells were incubated at 60°C in tris buffer saline containing 6 M urea for 30 min. in a shaker waterbath. The cells were separated from the extraction medium by centrifugation (9770 X g). The supernatant was subjected to 60 % ammonium sulphate precipitation at 4°C for 3 h and centrifuged (9770 X g, 30 min.) to collect the precipitate. The precipitated protein was dissolved in TBS supplemented with 6 M urea and dialyzed against the same. The ammonium sulphate precipitate was then subjected to 0.1 % sodium deoxycholate treatment i.e. dialysis against buffer without 6 M urea followed by dialysis (for 72 h) against tris buffer having 0.1 % sodium deoxycholate. The lipopolysaccharide precipitate formed was centrifuged and the supernatant was again dialyzed against 6 M urea buffer. This sample was loaded on Sepharose -4B column preequilibrated with urea buffer at room temperature. 2 ml fractions were collected and A_{280} was monitored. The first peak just after void volume showed lectin activity after dialysis.

Its purity was checked by SDS-PAGE.

2.2.8 Protein estimation

Protein concentration was determined by the method of Lowry *et al.* (8) using crystalline bovine serum albumin as standard.

2.2.9 Molecular mass determination

The relative molecular masses of lectin I as well as lectin II were estimated by SDS- PAGE according to Laemmli (11) using molecular weight markers (M_r 14000 - 66000) and by gel filtration using a column of Sephacryl S-300 (1x60 cm) equilibrated with buffer A containing 2 % ethylene glycol. The column was calibrated with alcohol dehydrogenase (M_r 153000), bovine serum albumin (M_r 66000), ovalbumin (M_r 45000) and carbonic anhydrase (M_r 29100) in the above buffer.

2.2.10 Determination of isoelectric point

Isoelectric focussing of lectin I in polyacrylamide gels was carried out according to Vesterberg (12), using ampholines of pH range 2.0 to 6.0. The gels were stained with Coomassie Brilliant Blue R 250. Isoelectric focussing of lectin II was carried out according to Sathivel *et al.* (26).

2.2.11 Amino acid analysis

Amino acid composition of the purified lectin I and lectin II was determined by automated amino acid analyzer (Hewlett Packard series 1050, with HP fluorescence detector). The samples were hydrolyzed in 200 µl of 6N HCl for 20 hours at

110°C and then subjected to analysis. Total cysteine was determined according to Habeeb (13) and total tryptophan was determined according to Spande and Witkop (14).

2.2.12 Haemagglutination assay

For haemagglutination activity determination, two fold serial dilutions of lectin solution (50 µl) in a microtiter plate were incubated at room temperature for one hour with equal volume of 3 % (v/v) suspension of rabbit erythrocytes in buffer A containing 0.15 M NaCl and examined. Activity was expressed as titer, i.e. the reciprocal of the highest dilution of the lectin that gave complete agglutination. Specific activity of the lectin is defined as the titer of the lectin per mg of protein. The haemagglutination activity was also checked using untreated and neuraminidase treated human erythrocytes (A, B and O blood groups). Haemagglutination inhibition studies were performed in the same way as HA, but serial dilutions of monosaccharides, disaccharides and glycoproteins (25 µl) were preincubated for 15 minutes with 25 µl of protein of titer 4 at room temperature. 50 µl of rabbit erythrocyte suspension (3 % v/v) was added and plates were read after one hour of incubation at room temperature.

2.2.13 pH and temperature stability

Effect of pH on stability and haemagglutination activity of lectin I was studied in universal buffer of pH range 3 to 11 (15). Similarly effect of temperature on lectin stability was monitored in the range of 0°C to 60°C.

2.2.14 Plant tissue extraction

Tobacco (*Nicotiana tobaccum*) tissue (25 g wet weight) was blended in presence of buffer (50 mM Tris-HCl, pH 7.2 containing 0.15 M NaCl). The blended tissue was separated by centrifugation and the buffer extract was concentrated by Amicon ultrafiltration, fitted with Amicon YM 3 membrane and used for inhibition studies. The homogenized tissue was then boiled for one hour in presence of 1M NaOH (16). The tissue was separated and extract was subjected to alcohol precipitation after neutralization. The alcohol precipitate was dissolved in buffer A and dialyzed against the same buffer. Its inhibitory effect on haemagglutination activity of lectin I and lectin II was checked after concentration.

2.2.15 Effect of plant polysaccharides (plant gums) on haemagglutination activity of Lectin II

Various plant gums were tested for haemagglutination inhibition of Lectin II. The plant gums (10 % suspension in distilled water) were boiled for 2 hours to inactivate the associated proteins and then subjected to alcohol precipitation (1:2 v/v). The samples were then dialyzed against distilled water and used in inhibition studies after checking their total neutral sugar concentrations.

2.3 RESULTS

2.3.1 Screening

A total of eleven *Agrobacterium* isolates were screened for extracellular lectin production by the microorganism. Table

2.1 shows the time and amount of lectin produced by each isolate. Out of 11 cultures, three isolates were good lectin producers. *Agrobacterium tumefaciens* as well as an isolate of *Agrobacterium radiobacter* produced lectin into the medium extracellularly. Culture number 7 *viz Agrobacterium radiobacter* was chosen for further studies because of its higher and consistent production of lectin.

2.3.2 Optimization of growth conditions for Agrobacterium radiobacter

Initially 2 % (w/v) glucose was used in the medium as a carbon source during screening of the Agrobacterial isolates. When the carbon source was shifted to sucrose 2 % (w/v), the organism produced twice the amount of the lectin as compared to glucose in 48 hours. Figure 2.1 shows the concentration of the carbon source used and the amount of lectin produced. The production of lectin seems to be dependent on the type and concentration of the carbon source used. Table 2.2 shows the media designed and lectin produced at the end of 48 hours of culturing. Control medium was the same as used for screening. When there was no carbon source present in the medium as in the case of nutrient broth, 2 times less activity was produced by the same organism. Growth in this medium also was very low as compared to control. In synthetic medium which had just enough carbon source to support the growth, the microorganism produced same amount of lectin as control. Phosphate ions are known to inhibit the polysaccharide production by the organism (9). *Agrobacterium radiobacter* pro-

Table 2.1 : **Screening for production of extracellular and cell bound lectin by Agrobacteria**

No.	Culture	Activity		
		24h	48h	72h
1.	*Agrobacterium tumefaciens* NCIM 2145	0	0	0
2.	*Agrobacterium tumefaciens* NCIM 2146	0	0	0
3.	*Agrobacterium tumefaciens* NCIM 2147	0	20	0
4.	*Agrobacterium tumefaciens* NCIM 2148	0	0	0
5.	*Agrobacterium tumefaciens* NCIM 2232	20	80	40
6.	*Agrobacterium tumefaciens* NCIM 2822	80	160	80
7.	*Agrobacterium radiobacter* NCIM 2443	80	160	160
8.	*Agrobacterium tumefaciens* NCIM 2939	40	80	80
9.	*Agrobacterium tumefaciens* NCIM 2940	30	40	20
10.	*Agrobacterium tumefaciens* NCIM 2941	20	40	20
11.	*Agrobacterium tumefaciens* -	80	160	40

Activity is expressed as units (titer)/ ml.

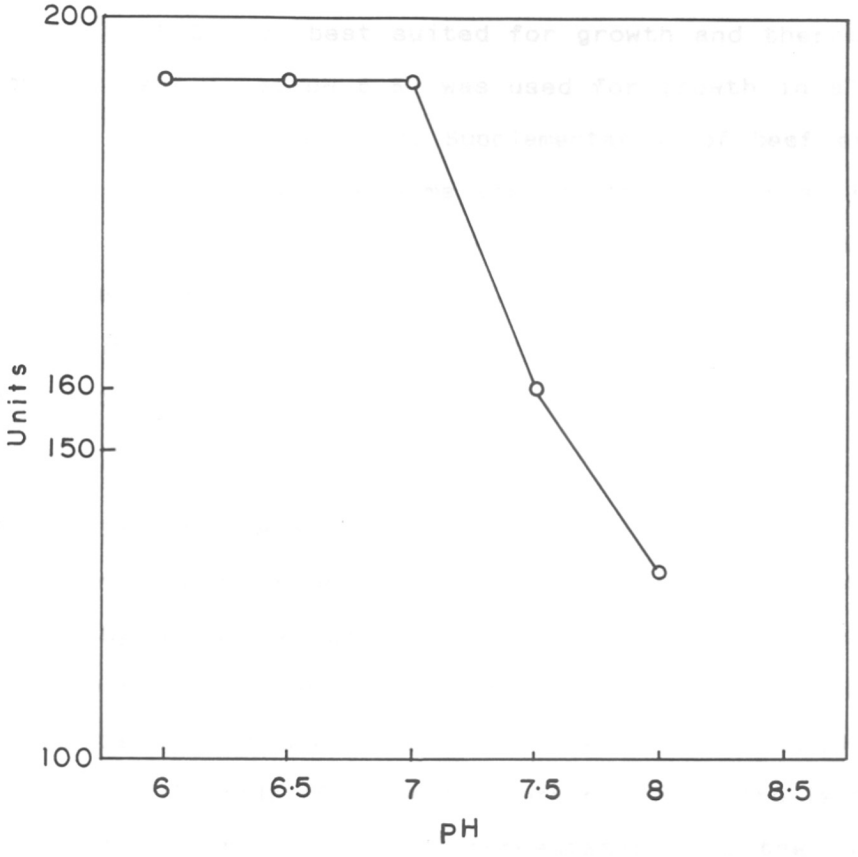
Table 2.2 : **Optimization of culture conditions for production of extracellular and cell bound lectin by *Agrobacterium radiobacter***

No.	Medium	Ingradients	Activity (U/ml)
1.	Control (MGYP)	glucose 2 %, peptone 0.5 % yeast extract 0.3 %, malt extract 0.3 %	160
2.	MSYP	sucrose 2 %, peptone 0.5 % yeast extract 0.3 %, Malt extract 0.3 %	320
3.	BSYP	sucrose 2 %, peptone 0.5 % yeast extract 0.3 %, beef extract	640
4.	Nutrient broth	peptone 0.5 %, beef extract 0.3 %	80
5.	Synthetic medium	glucose 0.1 %, KH_2PO_4 0.5 % $MgSO_4$ 0.02 %, $(NH_4)_2SO_4$ 0.2 %, citric acid 0.2 %, H_3BO_3 0.0006 %, ZnO 0.0006 % $FeCl_3$ 0.0002 % and $CaCO_3$ 0.0002 %	160
6.	Synthetic medium having 125 mM PO_4^{--}	--"-- , 2.2 % KH_2PO_4	80

Figure 2.1 : Effect of carbon source on production of lectins by *Agrobacterium radiobacter*

--O-- glucose, --■-- sucrose

Figure 2.2 : Effect of pH on production of lectin I by *Agrobacterium radiobacter*


200
160
150
100
Units
6
6·5
7
7·5
8
8·5
pH

duces acidic polysaccharide of succinoglycan type (10). When the synthetic medium was supplemented with 125mM KH_2PO_4, it produced 50 % less lectin than in synthetic medium. The production of extracellular lectin activity was checked at various pH values since it is an important factor for growth. pH 6.0 to 6.8 was best suited for growth and therefore a complex medium at pH 6.5 was used for growth in all the further studies (Fig. 2.2). Supplementation of beef extract for malt extract at the same concentration produced better results. Table 2.2 shows the production of lectin was 4 times higher as compared to the control when beef extract was used. Thus a medium containing ($g.L^{-1}$): sucrose 20, peptone 5, beef extract 3 and yeast extract 3, pH 6.5 was used in all further studies.

2.3.3 Time course of lectin production

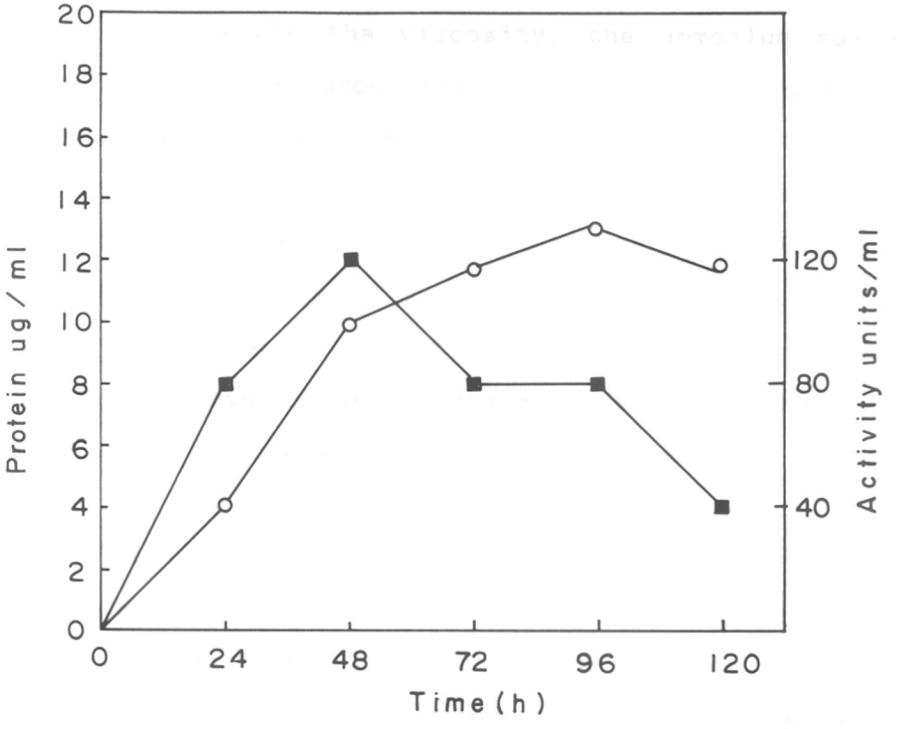
Production of extracellular lectin in the optimized medium by *Agrobacterium radiobacter* NCIM 2443 was monitored for 120 hours. Protein was determined after TCA (12.5 %) precipitation and the activity was checked as haemagglutination with the help of 3 % rabbit erythrocytes. The lectin is produced within 24 hours of fermentation and the highest amount of lectin was produced at 48 hours (Fig. 2.3).

2.3.4 Purification and characterization of Lectin I

Agrobacterium radiobacter NCIM 2443 produced a lectin extracellularly in the culture broth with a titer of 160 units/ml of the culture broth. It was concentrated by ammonium sul-

Figure 2.3 : Time course of production of Lectin I by *Agro-bacterium radiobacter* in the optimized growth medium

-- o -- protein, -- ■ -- activity


20
18
16
14
12
10
8
6
4
2
0
Protein ug / ml
0
24
48
72
96
120
Time (h)
120
80
40
Activity units/ml

phate precipitation of the culture filtrate and purified by two sequential chromatographies. The overall summary of the purification process is represented in Table 2.3. Presence of EDTA (1 mM) was necessary during the whole process of purification as well as for storage of the purified protein. The ammonium sulphate precipitate was a very viscous solution due to the polysaccharide produced by the *Agrobacterium radiobacter*. To reduce the viscosity, the ammonium sulphate precipitate was sonicated (17). Ion exchange and hydrophobic chromatographies could remove contaminating proteins as well as excess amounts of polysaccharide and colouring substances, yielding a single protein which had haemagglutination activity. Some nonproteinaceous material was still associated with the purified protein and it was separated fully from the protein on a hydroxyapatite column. The polysaccharide was bound to the matrix whereas the protein came out in column washing.

The relative subunit molecular mass of the purified lectin as determined by SDS-PAGE (Fig. 2.4) and native molecular mass determined by gel filtration (Fig. 2.5) was 37000 indicating that the lectin is a monomeric protein. Its pI was 4.0 suggesting the acidic nature of the lectin (Fig. 2.6). This observation is also supported by the amino acid composition data which shows abundance of acidic amino acids in the protein. The protein has large amount of glycine, two tryptophans and no cysteine (Table 2.4). The lectin was active when incubated at room temperature for 2 h and was stable at

Table 2.3 : Purification table of *Agrobacterium radiobacter* Lectin I

Step	Volume (ml)	Total Protein (mg)	Total Activity (units)	Specific Activity	Purification % Recovery	Purification fold
Culture filtrate	1000	20	32000	1600	100	0
$(NH_4)2SO_4$ precipitation	70	15.7	25600	1630	80	1
Ion exchange chromatography	50	7	20480	2925.7	64	1.8
Hydrophobic chromatography	2	3	13312	4437.3	41.6	2.7
Hydroxyapatite chromatography	3	2.8	13000	4642.8	40.6	2.9

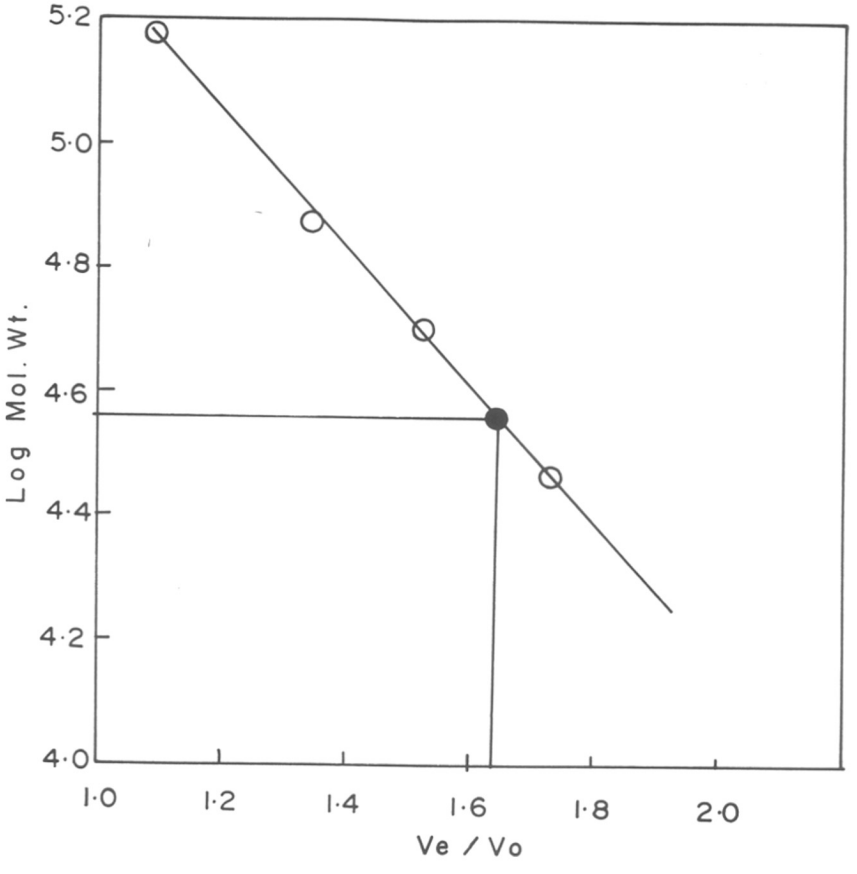

5·2
5·0
4·8
4·6
4·4
4·2
4·0
Log Mol. Wt.
1·0
1·2
1·4
1·6
1·8
2·0
Ve / Vo

Figure 2.4 : Native and SDS PAGE of the *Agrobacterium radiobacte*

Lectin I

a) Native PAGE

b) SDS- PAGE

Lane 1 : Molecular weight markers

bovine serum albumin (66000),ovalbumin (45000)

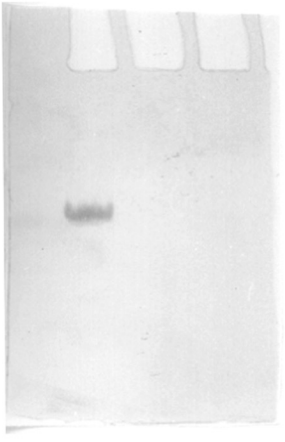
glucose 6 Phosphate dehydrogenase (36000)

carbonic anhydrase (29000), soybean trypsin

inhibitor (20000), lactalbumin (14000)

Lane 2 : Purified Lectin I

(a)



(b)

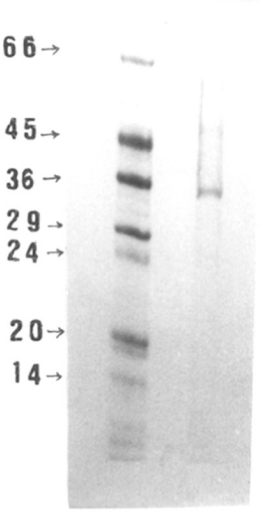
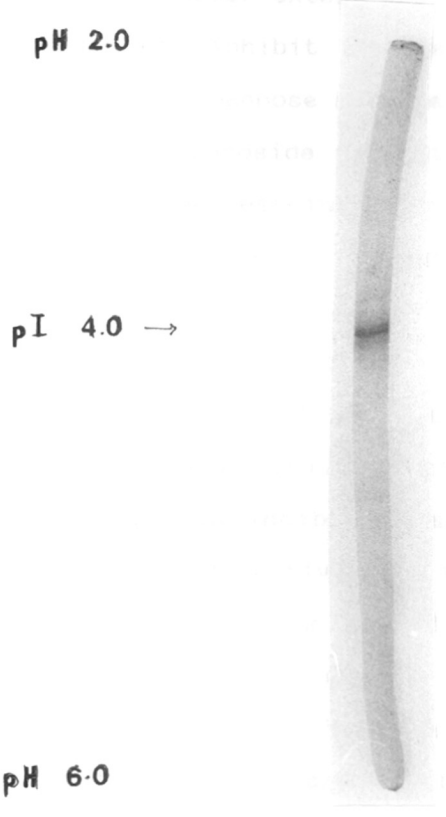


Figure 2.5 : Gel filtration of the *Agrobacterium radiobacter* lectin I

--O-- : Molecular weight markers - alcohol dehydrogenase (153000) bovine serum albumin (66000), ovalbumin (45000), and carbonic anhydrase(29000).

--●-- : Purified lectin

Figure 2.6 : Isoelectric focussing of Lectin I


pH 2.0
pI 4.0 →
pH 6.0

pH 5.0 (4°C). It lost all its activity when stored above pH 7.8 (Fig. 2.7, 2.8). The lectin showed no haemagglutination activity with untreated or neuraminidase treated human RBCs. The HA activity of the lectin was inhibited by N-acetyl glucosamine and chitobiose strongly (Table 2.5), Chitotriose inhibited to a lesser extent whereas chitotetraose and chitopentaose did not inhibit the lectin activity. Lectin I was also inhibited by mannose and its β anomers. The best inhibitor was the trimannoside derivative. The man5-glcNAc oligosaccharide was the best inhibitor among all the mono, di- and oligosaccharides tested. Among glycoproteins tested for haemagglutination inhibition, fibrinogen could inhibit the lectin activity at a concentration of 15 µg whereas fetuin inhibited the lectin activity at slightly higher concentration (30 µg) (Table 2.6). Its sialic acid residues did not have any effect on inhibition as desialylated fetuin also inhibited the lectin activity to the same extent. Orosomucoid inhibited the activity to a still lesser extent than fetuin (50 µg). The lectin showed some affinity towards mannose but very high affinity for also Mannose 9 glycopeptide, yeast invertase, ribonuclease and ovalbumin. Tobacco tissue extracts (buffer and alkali) inhibited the lectin activity. The alkali extract containing polysaccharides from Tobacco plant tissue, strongly inhibited the lectin activity as compared to the buffer extract (Table 2.6).

2.3.5 Purification and characterization of Lectin II

The washed cells of *Agrobacterium radiobacter* (5 g) were

Table 2.4 : **Amino acid composition of the *Agrobacterium radiobacter* lectin I**

Amino acid	res/mol
Asx	42
Thr	12
Ser	19
Glx	51
Pro	23
Gly	50
Ala	26
Cys*	0
Val	15
Met	8
Ile	17
Leu	24
Tyr	13
Phe	15
His	4
Lys	12
Trp*	2
Arg	11

* = Total cysteine and total tryptophan were determined according to Habeeb (13), Spande and Witkop (14).

Figure 2.7 : Temperature stability of purified Lectin I

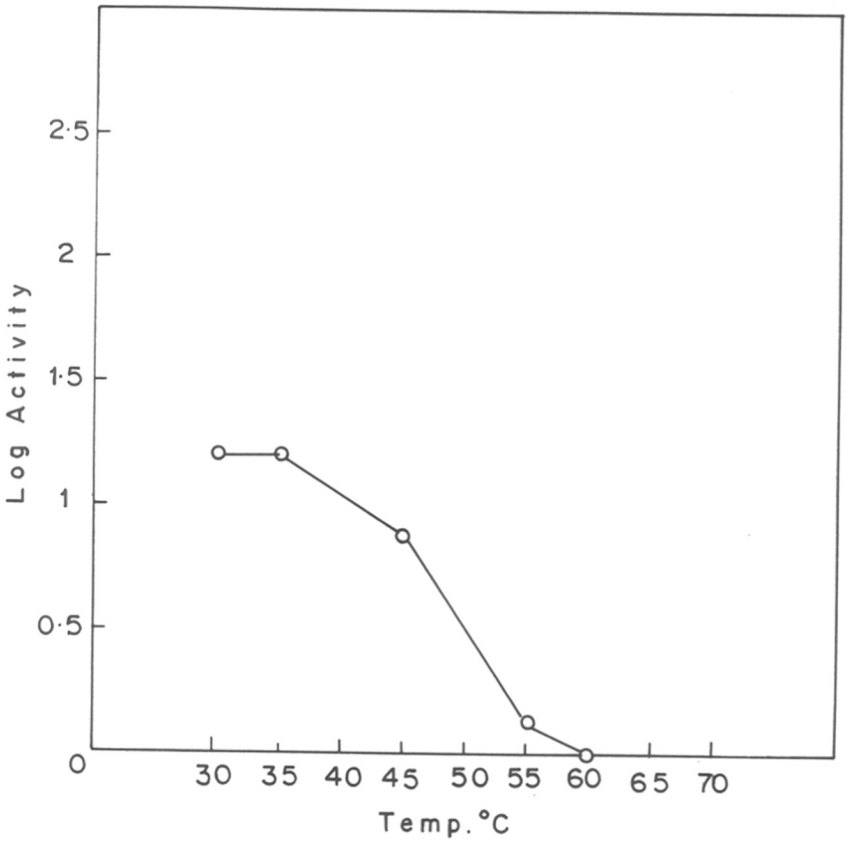

2·5
2
1·5
1
0·5
0
Log Activity
30 35 40 45 50 55 60 65 70
Temp. °C

Figure 2.8 : pH stability of purified Lectin I

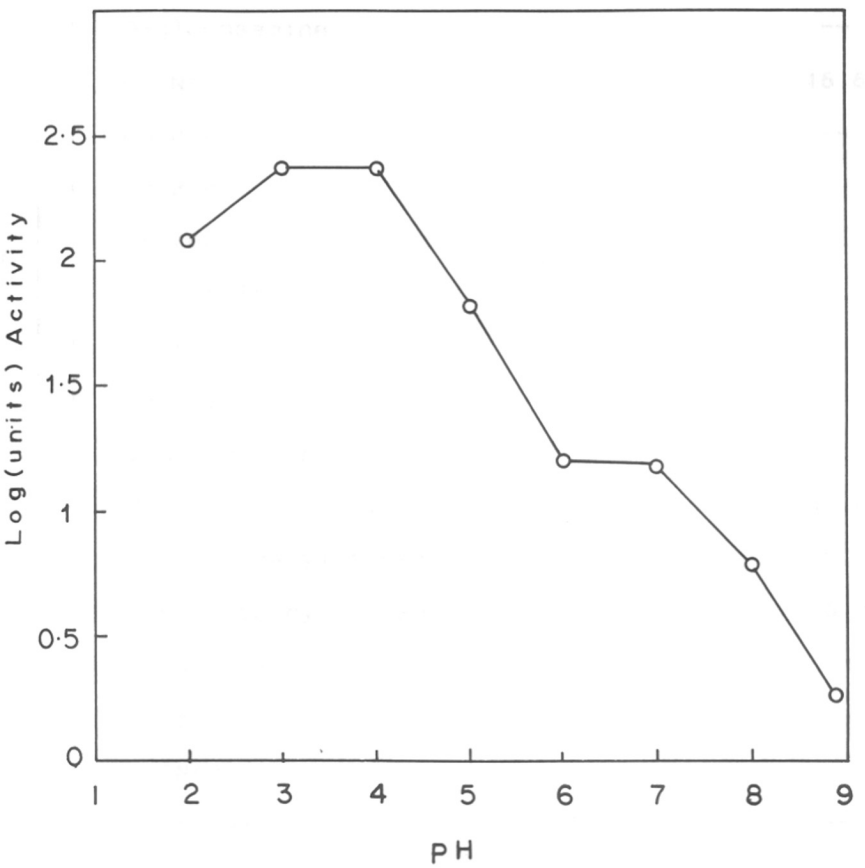

Log (units) Activity
2·5
2
1·5
1
0·5
0
1
2
3
4
5
6
7
8
9
PH

Table 2.5 : Sugar specificity of *Agrobacterium radiobacter* lectin I

Sugar	Inhibitory Concentration (mM)
1. Glucose	--
2. D-glucosamine	--
3. GlcNAc	15.6
4. Me α glc	--
5. Me β glc	--
6. Chitobiose	7.15
7. Chitotriose	12.5
8. Chitotetraose	--
9. Mannose	15.0
10. D-mannosamine	30.0
11. Me α man	30.0
12. p-nitrophenyl α man	12.5
13. p-nitrophenyl β man	6.25
14. Galactose	--
15. D-galactosamine	125.0
16. GalNAc	--
17. Me α gal	--
18. Me β gal	--
19. LacNAc	--
20. L(-)Fucose	--
21. L-Arabinose	125.0

-- = No inhibitory effect at highest (250 mM) concentration.

Table 2.6 : Inhibitory effect of oligosaccharides and glycoproteins on *Agrobacterium radiobacter* Lectin I

Glycoprotein	Inhibitory Concentration (in terms of neutral sugar)
1. Trimannoside	34.3 µg
2. Man5-glcNAc	0.22 µg
3. Man (9) glycopeptide	0.4 µg
4. Fibrinogen	15.0 µg
5. Fetuin	31.0 µg
6. Desialated Fetuin	31.0 µg
7. Orsomucoid	50.0 µg
8. Ovalbumin	7.5 µg
9. Ribonuclease A	0.75 µg
10. Yeast Invertase	0.05 µg
11. Tobacco plant tissue (buffer) extract	25.0 µg
12. Tobacco plant tissue (alkali) extract	3.5 µg

Sugar concentration was determined by phenol-sulphuric acid method.

suspended in 100 ml of urea buffer and the surface lectin was extracted. The deoxycholate treated ammonium sulphate dialysate when passed through sepharose 4B column, yielded a peak after void volume containing lectin activity. Summary of lectin purification is given in Table 2.7. The protein obtained after Sepharose 4B chromatography was homogeneous on SDS-PAGE (Fig. 2.9).

On calibrated column of Sephacryl S300 (1 X 75 cm), the dialyzed protein came out with the void volume but SDS-PAGE showed its molecular mass to be 40000. During isoelectric focussing, the lectin focussed as a single band and showed a pI of 9.1 (Fig. 2.10). The amino acid composition of the lectin showed that it had 44 % of hydrophobic amino acids, one tryptophan and no cysteine (Table 2.8)

The purified lectin agglutinated rabbit erythrocytes but not untreated or neuraminidase treated human erythrocytes of blood group A, B, or O. The lectin was inhibited by D-glucosamine and by methylated derivatives of D-glucose at 10 times higher concentration than D-glucosamine. All other monosaccharides failed to inhibit the activity of Lectin II. Among glycoproteins, fetuin and desialated fetuin inhibited lectin activity at 0.6 µg while fibrinogen inhibited it at 50 times higher concentration and ovalbumin failed to inhibit it. Among the plant polysaccharides, the lectin was inhibited by Gum Ghatti, Gum Guar, Gum Arabic and Locust gum, but the best inhibitor was Locust gum which was 100 fold better than the other gums. Tobacco plant extracts, both buffer as

Table 2.7 : Purification of lectin II

Step	Volume (ml)	Total protein (mg)	Total Activity (units)	Specific Activity	Purification fold	Purification recovery %
Cell Extract	100	125	3000	24	0	100
$(NH_4)_2SO_4$ precipitate	14	99.1	2640	26.6	1.1	88
Sepharose 4B column	8	2.97	2560	861.9	32.4	85

Table 2.8 : Amino acid composition of lectin II

Amino acid	res/mol
Asx	45
Thr	17
Ser	13
Glx	35
Gly	46
Ala	43
Cys*	0
Val	26
Met	9
Ile	21
Leu	31
Tyr	10
Phe	16
His	4
Lys	18
Arg	19
Pro	15
Trp*	1

* = Total cysteine and total tryptophan were determined according to Habeeb (13) and Spande and Witkop (14).

Figure 2.9 : SDS-PAGE of Lectin II

Lane 1 : Molecular weight markers;
bovine serum albumin (66000),ovalbumin (45000)
glucose 6 Phosphate dehydrogenase (36000)
carbonic anhydrase (29000), soybean trypsin
inhibitor (20000), lactalbumin (14000)

Lane 2 : Purified Lectin II

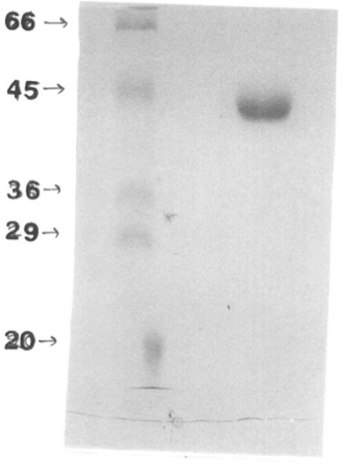

66 →
45 →
36 →
29 →
20 →

Figure 2.10 : Isoelectric focussing of Lectin II

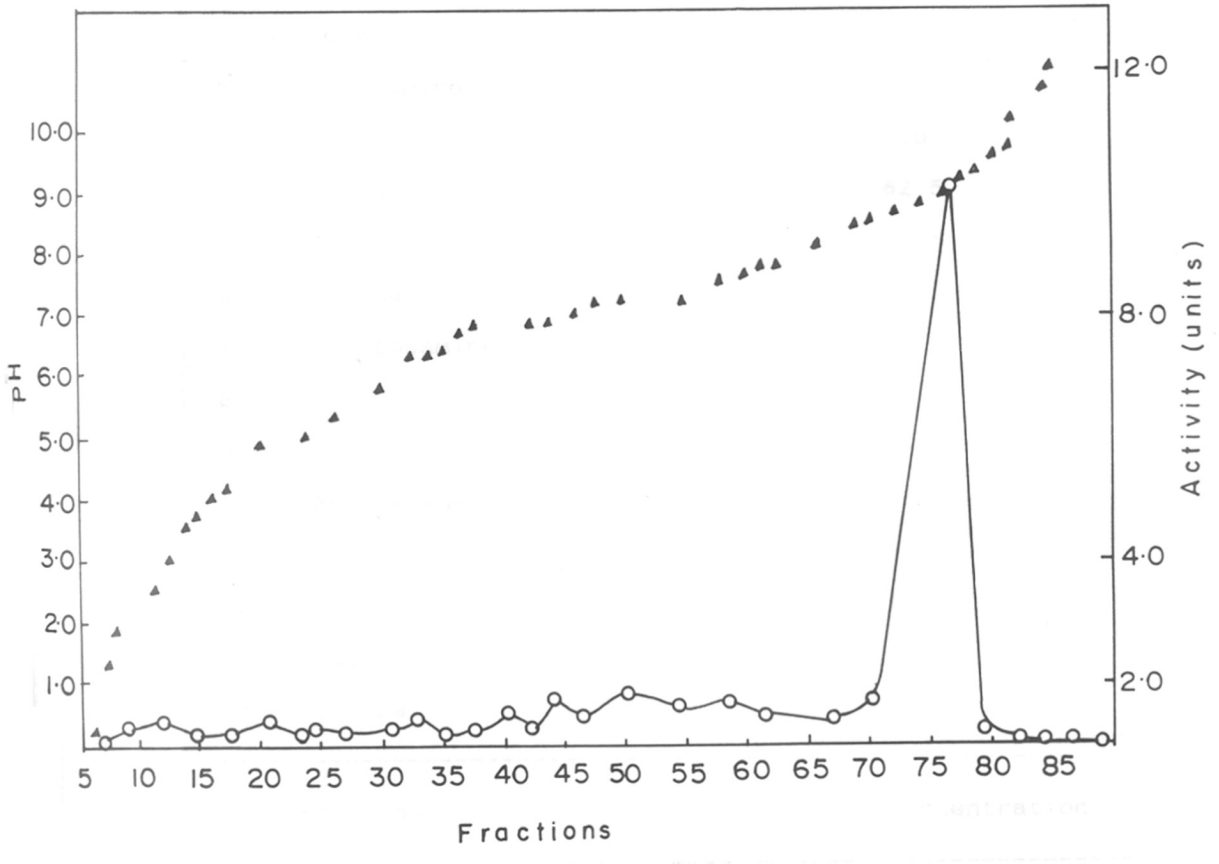

pH
10·0
9·0
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
12·0
8·0
4·0
2·0
Activity (units)
5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85
Fractions

Table 2.9 : Sugar Specificity of Lectin II

Inhibitory Sugar	concentration mM
1. Glucose	--
2. D glucosamine	7.8
3. GlcNAc	250
4. Me α glc	62.5
5. Me β glc	62.5
6. Galactose	--
7. D galactosamine	--
8. GalNAc	--
9. Mannose	--
10.D mannosamine	--
11.Met α man	--
12.Fucose	125
13.Lactose	--
14.Arabinose	125
Glycoproteins	**Concentration**
15.Fetuin	0.6 µg
16.Desialylated fetuin	0.6 µg
17.Fibrinogen	30 µg
18.Ovalbumin	--

-- = No inhibition at highest concentration.

Table 2.10 : Effect of plant polysaccharides on haemagglutination activity of Lectin II

Plant Polysaccharides (Gums)	Inhibitory concentration; µg of neutral sugar
1. Ghatti	17
2. Arabic	--
3. Guar	51
4. Karaya	47
5. Locust	0.5
6. Tobacoco (buffer) extract	25
7. Tobacco (alkali) extract	1.5

-- = No inhibition at 2.5 mg/ml neutral sugar concentration.

well as alkali extract strongly inhibited the lectin activity, the alkali extract being 16 times better than the buffer extract (Table 2.10).

2.4 DISCUSSION

Molecular details of binding of the pathogenic bacteria to the target host cell surface are known only in a few bacterial systems like *E. coli*. The colonization of small intestine by enteropathogenic *E. coli* or of urinary tract by uropathogenic *E. coli* strains, is mediated by cell surface antigens (pili or fimbriae) which enable the bacteria to adhere to the specific site on the host tissue (23). The adhesins involved in this attachment process i.e. pili are often lectins and they are synthesized by large proportion of bacteria in apparently all kinds of environments. Among plant pathogenic bacteria, *Pseudomonas syringae*, *Pseudomonas savastanoi*, *Bradyrhizobium japonicum*, and *Agrobacterium tumefaciens* like organisms are known to produce lectins which are located on the cell surface (24,5).

Agrobacterium radiobacter NCIM 2443 produced two lectins, one which was extracellular (Lectin I) while the other was cell bound (Lectin II). The extracellular lectin was purified by 3 successive chromatographies. Lectin I was associated with the polysaccharide. As a consequence, the polysaccharide protected the protein and it was stable at room temperature. The interaction between polysaccharide and protein did not involve covalent bonding, as hydroxyapatite column step removed most of the associated polysaccharide

from the protein. Presence of EDTA was very essential during the purification process as well as for storage of the protein, otherwise it got fragmented accompanied with loss of activity within 48 hours. The relative inhibitory pattern of the lectin I (Table 2.5) shows that the lectin not only recognizes chitobiose but also the trimannoside with similar affinity. The higher affinity shown for the high mannose oligosaccharide glycoproteins as compared to complex oligosaccharide glycoproteins indicates that antennary structure of complex oligosaccharides sterically interferes in the binding process. The gradation shown in the decrease in affinity from bi-antennary to tetraantennary structures (Tables 2.6 and 2.11) supports this conclusion. Lectin I recognizes a complex carbohydrate structure rather than a simple monosaccharide and the probable structure that can be deduced from inhibition data is glcNAc-glcNAc-man3 core oligosaccharide of Asn linked glycoconjugates.

Tobacco tissue extracts (buffer and alkali) inhibited the lectin I and II activity. Alkali treatment which releases plant polysaccharides from plant tissue, strongly inhibited the lectin activity. This inhibition probably shows a sign of recognition of the receptor molecule on plant surface by lectin.

Lectin II is different from Lectin I which is aparant from their sugar specificity, isoelectric point and amino acid composition. The cell bound lectin (Lectin II) is more hydrophobic than the extracellular lectin (Lectin I). It forms

Table 2.11: **Relative inhibition of the lectin**

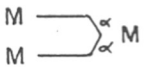
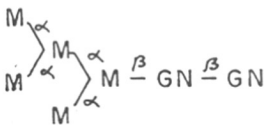
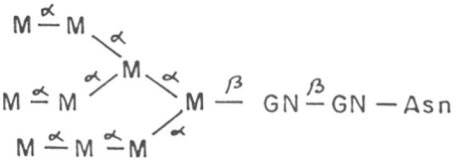
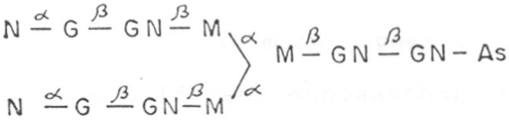
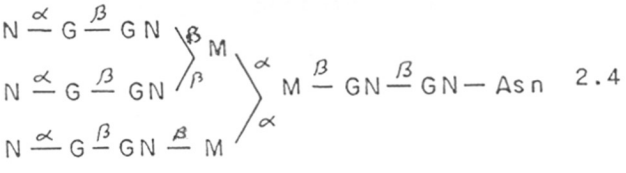
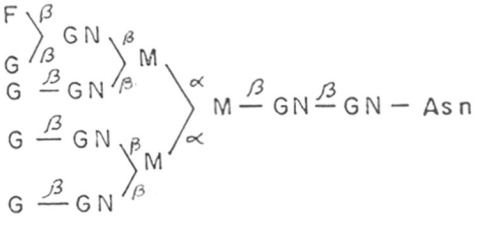
No.	Inhibitory Compound	Structure	Relative inhibition
1.	Chitobiose	GN $\overset{\beta}{-}$ GN	1 (75.7µg)
2.	Trimannoside		2.2
3.	(Man 5-glcNAc)		10^2
4.	Man(9) glycopeptide		10^2
5.	Ribonuclease A	(High mannose type)*	10^2
6.	Yeast invertase	(High mannose type)*	10^3
7.	Ovalbumin	(High mannose type)*	10

Table 2.11 continued

8. Fibrinogen[1]		5
9. Fetuin[2]		2.4
10. Orosomucoid[3]**		1.5

1,2 - Townsend *et al* [25]; 3 - Fournet *et al* [25]

* - Ribonuclease A has man(9)structure; Yeast invertase has more than one man (9) structures and ovalbumin has mixed type of bisected man(5) to man(8) structures.

** - Orosomucoid contains 50 % tetraantennary 30 % triantennary and 20 % biantennary complex oligosaccharides.

aggregates once it is dialysed against plain buffer (without urea). This may be the reason for elution of the protein in void volume of Sephacryl S-300 column. Lectin II shows high affinity for D-glucosamine although it does get inhibited by Me α glc and Me ß glc at much higher concentrations. Lectin II has totally different monosaccharide specificity than Lectin I, which is specific for glcNAc and Man. Lectin II is inhibited by fetuin, to lesser extent by fibrinogen but not by ovalbumin. The glycoprotein specificity of Lectin II is reverse of Lectin I, which shows higher specificity for high mannose glycoproteins such as ovalbumin (Table 2.10). The inhibition of Lectin II at very low concentrations of plant polysaccharides indicates that it is specific for complex oligosaccharide structures.

Agrobacterium tumefaciens lectin (5) as well as *Agrobacterium radiobacter* Lectin I are most active at pH 5.0 . But *Agrobacterium tumefaciens* lectin is specific for L(-) fucose and Lectin I shows a complex sugar specificity. Poplar plant polysaccharides inhibited the *Agrobacterium tumefaciens* lectin activity similar to the inhibition of Lectin I by Tobacco plant polysaccharides. The recognition of plant polysaccharide by these lectins has prompted researchers to put forward protein carbohydrate interaction as a basis for microbial recognition and adhesion. However, other workers have reported several types of molecules to be responsible for this early event of infection. It is known that the disease induction takes place when bacteria attach themselves to wounded plant cells at a specific site (18). It is

selves to wounded plant cells at a specific site (18). It is shown by Shaw et al (19) that bacteria are attracted to the wounds by chemotaxis, where sugars like glucose, galactose, and arabinose, or amino acids like valine and arginine act as chemoattractants. According to Gurlitz (20) a protein may be a part of plant receptor site. It was claimed that bacterial lipopolysaccharides (21) and 2 linked β- D glucan also play an important role in recognition and attachment (22).

Although all members of genus *Agrobacterium* with the exception of *Agrobacterium radiobacter* induce tumors in many plants, successful biological control of this disease has been reported using *Agrobacterium radiobacter* cells (6,7). It is shown that the biological control is achieved as *Agrobacterium radiobacter* attaches itself to the same site as *Agrobacterium tumefaciens*, i.e. the pathogenic bacteria, by competing for the same receptor site. Production of lectins by *Agrobacterium radiobacter*, the affinity shown by the lectin towards plant tissue extracts and plant polysaccharides indicate the possibility of these lectins recognizing the same receptor on the plant cell.

REFERENCES

1. D. Mirelman, and I. Ofek (1986) In 'Microbial lectins and agglutinins' (D. Mirelman, Ed.) Wiley, New York, 1 - 19.
2. N. Sharon (1987) FEBS Lett. 217 : 145 - 157.
3. S. C. Winans (1992) Microbiol. Rev. 56 : 12 - 31.
4. K. Kersters and J. deLey (1984) in " Bergey's Manual of Systematic Bacteriology" Volume 1 (N.R. Kraig and J.G. Holt eds.) The Williums and Wilkins Co. Baltimore pp. 244 - 254.
5. C. Depierreux, H. C. Kang, B. Guerin, M. Monsigny and F. Delmotte (1991) Glycobiology 1 : 643 - 649.
6. M. M. Lopez, M. J. Gorris, C. I. Salcedo, A. M. Montojo and M. Miro (1989) Appl. Environ. Microbiol. 55 : 741 - 746.
7. B. Vicedo, R. Penalver, M. Jose Asins and M. M. Lopez (1993) Appl. Environ. Microbiol. 59 : 309 - 315.
8. O. H. Lowry, R. L. Rosebrough, A. L. Farr and R. J. Randall (1951) J. Biol. Chem. 193 : 165 - 175.
9. P. Souw and A. L. Demain (1979) Appl. Environ. Microbiol. 37 : 1186 - 1192.
10. M. A. O'Neill, P. D. Robison, K. J. Chou, A. G. Darvill and P. Albersheim (1992) Carbohydr. Res. 226 : 131 - 154.
11. V. K. Laemmli (1970) Nature 227 : 680 - 685.
12. O. Vesterberg (1972) Biochim. Biophys. Acta. 257 : 11 - 19.
13. A.F.S.A. Habeeb (1972) Methods Enzymol. 25 : 457 - 464.
14. T.F.Spande and S. Witkop (1967) Methods Enzymol. 11:498- 506.
15. S. J. Rinderle, I. J. Goldstein, K. L. Matta and R. M. Ratcliff (1989) J. Biol. Chem. 264 : 16123 - 16131.
16. G. de Stevens (1956) Methods Enzymol. 3 : 31 -33

17. R. Barak, Y. Elad, D. Mirelman and I. Chet (1985) Phytopathol. 75 : 458 - 462.

18. B. B. Lippincott and J. A. Lippincott (1969) J. Bact. 97 : 620 - 628.

19. C. H. Shaw, A. M. Ashby, A. Brown, C. Royal, G. J. Loake (1988) Molecular Microbiol. 2 : 413 - 418.

20. R. H. G. Gurlitz, P. W. Lamb and A. G. Matthysse (1987) Plant Physiol. 83 : 564 - 568.

21. M. H. Whatley, J. S. Bodwin, B. B. Lippincott and J. A. Lippincott (1976) Infect. Immun. 13 : 1080 - 1083.

22. V. Puvanesarajah, F. M. Schell, G. Stacey, C. J. Douglas and E. W. Nester (1985) J.Bacteriol. 164 : 102 -106.

23. I. Ofek, D. Mirelman and N. Sharon (1977) Nature265:623- 625.

24. M. Romantschuk and D. H. Bamford (1986) Microbiol. Pathog. 1 : 139 - 148.

25. F. K. deGraaf, P. Klemm and W. Gaastra (1980) Infect. Immun. 33 : 877 - 883.

26. C. Sathivel, A. H. Lachke and S. Radhakrishnan (1995) J. Chromatogr. 705 : 400 - 402.

CHAPTER 3

Chemical modification studies on lectins from *Agrobacterium radiobacter*

SUMMARY

Agrobacterium radiobacter produces two lectins (Lectin I and Lectin II). Incubation of Lectin I with N -bromosuccinimide (NBS) resulted in 87 % loss of haemagglutination activity with concomitant modification of two tryptophan residues. Treatment with Woodward's reagent K resulted in 60 % loss of activity. Lectin II was totally inactivated with Diethylpyrocarbonate (DEP) whereas N-bromosuccinimide (NBS) brought about only 50 % loss of the lectin's haemagglutination activity. Lectin II was also inactivated by carboxylate groups modifying reagent, Woodward's reagent K. The loss in activity was 75 %. The results indicate that carboxyl side chains of acidic amino acids and the indole side chain of tryptophan play a role in the saccharide binding activity of Lectin I. However, for Lectin II, histidine, carboxyl groups of acidic amino acids and to a lesser extent tryptophan have a role in it's saccharide binding activity.

3.1 INTRODUCTION

Identification of specific amino acid residues within the active site of a biologically active proteins is important for understanding the relationship between its structure and function as well as the role played by specific amino acid side chains in its biological activity. Chemical modification is among the methods used for identifying at least some of the essential amino acid residues involved in substrate binding. Microbial lectins, in general, have been rarely studied with regards to the amino acid residues involved in their sugar binding activities.

Agrobacterium radiobacter produced two types of lectins, Lectin I and Lectin II. Lectin I is a monomer of M_r 37000 and is specific for oligosaccharide glcNAc-glcNAc-man5. Lectin II is also a monomer of M_r 40000 and it is specific for D glucosamine and complex carbohydrates of plant origin. To identify the residues involved in the sugar binding of Lectin I and Lectin II, they were subjected to modification by various chemical modification reagents. The results of this preliminary study are reported in this chapter.

3.2 MATERIALS AND METHODS

Materials

2,4,6 -trinitrobenzenesulphonic acid (TNBS), diethylpyrocarbonate (DEP), 5, 5'-dithiobis 2-nitrobenzoic acid (DTNB), N -acetyl imidazole, imidazole, Woodward's reagent K, phenylglyoxal and N-bromosuccinimide were purchased from Sigma Chemical Company, U. S. A. All other chemicals used

were of analytical grade.

Methods

Chemical modification reactions were carried out using different chemical reagents under their respective reaction conditions. The residual activity of the lectins (Lectin I and Lectin II) was checked in the following manner : a) after modification, the reaction mixtures were dialyzed thoroughly b) lyophilized to dryness c) dissolved in 100 µl of buffer (Tris-HCl 20 mM, pH 7.2) and haemagglutination activity was checked. The HA activity was determined as described in chapter 2 section 2.2.12.

3.2.1 Reaction with N -bromosuccinimide

This was carried out by incubating both the lectins (250 µg) with different concentrations of NBS at pH 4.5 (0.1M acetate buffer). The number of tryptophan residues getting modified were determined spectrophotometrically by monitoring the decrease at A_{280} after each addition of a small aliquot of 1 mM NBS (1). The residual activities after modification of tryptophan residues, in the protein samples, were monitored by haemagglutination.

3.2.2 Reaction with 2,4,6-trinitrobenzenesulphonic acid (TNBS)

Both the lectins (250 µg) were incubated seperately with varying concentrations of TNBS (0.1 - 1 mM) in 4 % (w/v) sodium bicarbonate at 37°C in the dark. The reaction mixture was dialyzed thoroughly against Tris buffer (20 mM, pH 7.2)

till the pH of the reaction mixture became 7.2. Protein sample incubated with sodium bicarbonate in absence of TNBS served as control (2). The residual activities after modification of lysine residues in the protein were checked by haemagglutination.

3.2.3 Reaction with phenylglyoxal

For modification of arginine residues, 250 µg of both the lectins were incubated with 10 % (w/v) phenylglyoxal in methanol at pH 8.0 and at 30°C for 30 min. Lectin samples in same quantity of methanol without phenylglyoxal, served as control. The residual activities of the lectins were checked after extensive dialysis (3).

3.2.4 Reaction with diethylpyrocarbonate (DEP)

The reaction was carried out by incubating 250 µg of each lectin at pH 7.2 and 30°C for 30 min., with various con centrations of DEP, freshly diluted with absolute ethanol. The concentration of DEP was determined (4,5) using imidazole buffer. Samples incubated under similar conditions except DEP reagent served as control.

3.2.5 Reaction with N - acetyl imidazole

250 µg of both the proteins (Lectin I and Lectin II) at pH 7.5 were incubated with 10 mM N-acetyl-imidazole for 30 min. at room temperature followed by estimation of residual lectin activities. Proteins incubated in the absence of the reagent were taken as control. (6).

3.2.6 Reaction with 5,5'dithiobis 2-nitrobenzoic acid (DTNB)

The proteins were incubated with 10 mM DTNB at room temperature for 30 min. and the residual activities were checked after dialysis. Lectins incubated in absence of DTNB served as control.

3.2.7 Reaction with Woodward's reagent K

The reaction was carried out by incubating 250 µg of each of the lectins at pH 7.2 at 25°C for 30 min., with various concentrations of WRK (upto 30 mM). The residual activities were checked after dialysis. Protein samples incubated in absence of WRK served as control (7).

3.2.8 Determination of protein concentration of Lectins

The protein concentration of Lectin I and Lectin II were determined by the method of Lowry *et al* (8) by using bovine serum albumin as standard.

3.3 RESULTS

3.3.1 Effect of modification of tryptophan residues

Agrobacterium radiobacter Lectin I lost 87 % of its haemagglutination activity when incubated with NBS at a concentration of 28 µM (Table 3.1). A Total of two tryptophan residues were modified. It can be seen from Figure 3.1 and 3.2 that the lectin lost 50 % of it's activity, when the first tryptophan was modified at 15 µM concentration of NBS. Rest of the activity was lost with the modification of the second tryptophan residue.*Agrobacterium radiobacter*

able 3.1 : Chemical modification of Lectin I

hemical reatment	Residues modified	% Residual Activity
one	--	100
-bromosuccinimide	Tryptophan	12
-nitrohenylglyoxal	Arginine	100
-acetyl imidazole	Tyrosine	100
iethylpyrocarbonate	Histidine	100
oodward's reagent K	Carboxyl	40
NBS	Lysine	100
TNB	Cysteine	100

Figure 3.1 : Loss of activity with modification of tryptophan of Lectin I with N-bromo succinimide

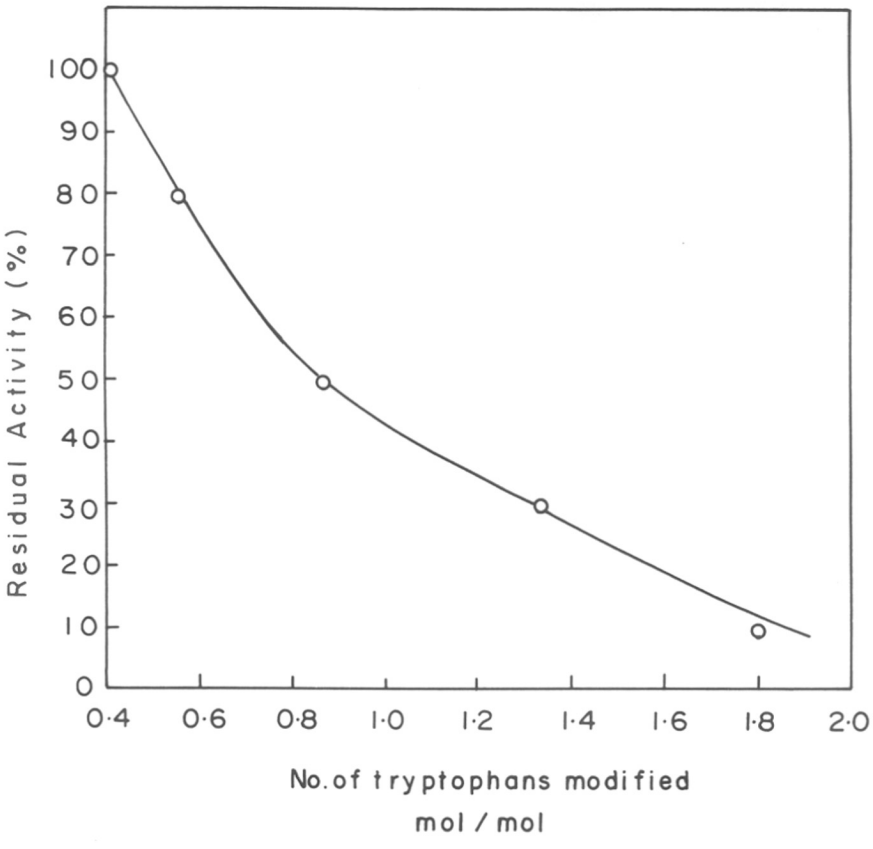

Residual Activity (%)
100
90
80
70
60
50
40
30
20
10
0
0·4
0·6
0·8
1·0
1·2
1·4
1·6
1·8
2·0
No. of tryptophans modified
mol / mol

Figure 3.2 : Concentration dependent loss of HA activity of Lectin I with N-bromosuccinimide

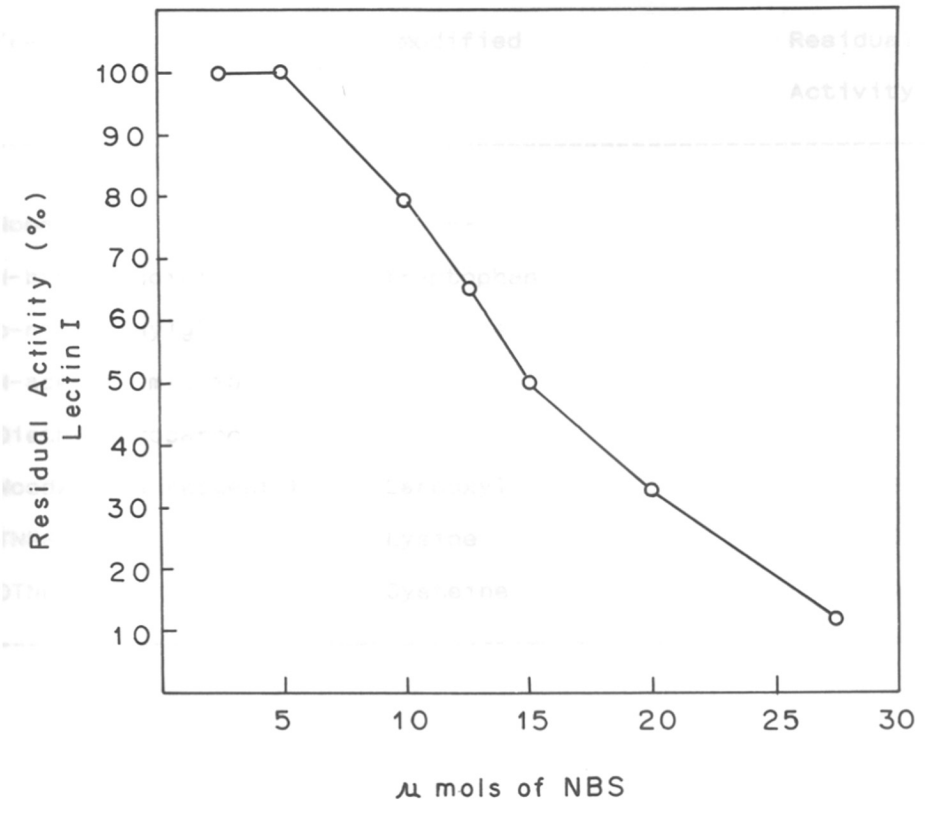

Residual Activity (%)
Lectin I
100
90
80
70
60
50
40
30
20
10
5
10
15
20
25
30
μ mols of NBS

Table 3.2 : **Chemical modification of Lectin II**

Chemical Treatment	Residues modified	% Residual Activity
None	--	100
N-bromosuccinimide	Tryptophan	50
p-nitrohenylglyoxal	Arginine	100
N-acetyl imidazole	Tyrosine	100
Diethylpyrocarbonate	Histidine	0
Woodward's reagent K	Carboxyl	25
TNBS	Lysine	100
DTNB	Cysteine	100

Lectin II lost only 50 % of it's haemagglutination activity with the modification of the only tryptophan present in the lectin. The concentration of NBS required for modification was 20 µM.

3.3.2 Effect of modification of histidine residues

There was no activity loss when Lectin I was treated with Diethylpyrocarbonate (DEP), even when excess reagent was used. Lectin II lost all of it's activity when incubated with the same reagent at 100 µM concentration. At 80 µM concentration of DEP, 50 % loss of activity was observed for Lectin II. (Table 3.2).

3.3.3 Effect of carboxyl groups modification

Carboxyl groups were modified with the help of Woodward's reagent K. Lectin I, when incubated with the reagent, lost 60 % of it's haemagglutination activity. When Lectin II was subjected to Woodward's reagent K modification, it lost almost 75 % of it's haemagglutination activity (Table 3.1, 3.2).

No loss of activity was observed with modification of arginine,lysine,tyrosine or cysteine residues of both of the lectins.

3.4 DISCUSSION

Agrobacterium radiobacter Lectin I is produced extracellularly. This protein is strongly associated with the polysaccharide (EPS) produced by the microorganism. The

lectin was purified by three successive chromatographies and the amount of polysaccharide associated with the lectin decreased gradually with each step. Although after the 2nd step, i.e. hydrophobic chromatography, the sample was pure proteinwise, it had about 10 % of polysaccharide contamination. Only the last step, however, could remove all the polysaccharide associated with it successfully. Therefore the hydroxyapatite chromatography was a very essential step especially for chemical modification studies, as the polysaccharide could have protected the protein from the amino acid modifying chemical reagents.

Lectin I has more acidic amino acid residues. It lost 60 % of it's haemagglutination activity when subjected to carboxyl groups modification. Lectin II however, lost 75 % of it's haemagglutination activity when subjected to Woodward's Reagent K modification. This shows that carboxyl groups in Lectin I and Lectin II contribute in saccharide binding. Hirabayashi *et al.* (9) studied the residues involved in carbohydrate binding of β-gal specific human lectin by site directed mutagenesis. They suggested that the lectin in which cysteine and tryptophan were reported in the sugar binding site, are actually involved in the stabilization of the binding site and the conservative hydrophilic residues, (Asn^{46} and Glu^{71}) are involved in the saccharide binding. In *Saccharomyces cerevisiae* lectin also, hydrophilic amino acid residues like Asn and Glu have been reported to be important in sugar binding (10). The structural basis for selective sugar recognition by lectins has been investigated by X-ray

crystallography of several plant and animal lectins (11) and it has been shown that the selectivity in the lectin sugar interactions is achieved mainly through hydrogen bonding. Hydrophilic residues play important roles in the function of sugar binding because such residues can readily form hydrogen bonds with hydroxyl groups of specific carbohydrate chains directly or indirectly via a water molecule (12,13).

Lectin I has two tryptophan residues and both of them get modified when subjected to NBS treatment with almost complete loss (87 %) of HA activity. The study clearly indicates that both the tryptophans are present at the saccharide binding site of Lectin I and contribute to sugar binding. In case of plant lectin Ricin D as well as in Abrus lectin, tryptophan has been shown to be involved in sugar binding (7,8). In case of other plant lectins like Concanavalin A, Wheat germ agglutinin, *Momordica charantia* lectin and *Pisum sativum* lectin, a tryptophan residue has been shown to be involved in the carbohydrate binding (14,15,16,17). In case of human lectin (18) as well as in mushroom lectin (19) also, tryptophan has been reported to be involved in sugar binding. Lectin II, however, lost only 50 % of it's haemagglutination activity with the modification of the one and only tryptophan residue. It has a histidine in saccharide binding site, as treatment with (DEP) (100 µM) caused total loss of activity. Basu *et al* (20) have shown histidine in sugar binding site of snail haemolymph lectin.

Modification of arginyl, lysyl, cysteinyl and tyrosyl

residues did not lead to any loss in the sugar binding and haemagglutinating activity of both the lectins, ruling out the possibility of involvement of these residues in sugar binding.

REFERENCES

1. T. F. Spande and B. Witkop (1967) Methods Enzymol. 11 : 498 - 506.
2. A. F. S. A. Habeeb (1966) Anal. Biochem. 14 : 328 - 336.
3. R. B. Yamasaki, D. A. Shimer and R. E. Feeney (1981) Anal. Biochem. 111 : 220 - 226.
4. W. B. Melchior Jr. and D. Fahrney (1970) Biochemistry 9 : 251 - 258.
5. G. E. Means and R. E. Feeney (1971) in "Chemical modification of Proteins" (G. E. Means and R. E. Feeney eds.) Holden-Day Inc. San Francisco pp 214 - 230.
6. V. Sinha and J. M. Brewer (1985) Anal. Biochem. 151 : 327 - 333.
7. T. Hatakeyama, N. Yamasaki and G. Funatsu (1986) J. Biochem. 99 : 1049 - 1056.
8. S. R. Patanjali, M. J. Swamy, V. Anantharam, M. I. Khan and A. Surolia (1984) Biochem J. 217 : 773 - 781.
9. J. Hirabayashi and K. Kasai (1991) J. Biol. Chem. 266 : 23648 - 23653.
10. M. Kundu, J. Basu, A. Ghosh and P. Chakrabarti (1987) Biochem. J. 244 : 579 - 584.
11. W. I. Weis and K. Drickamer (1996) Annu. Rev. Biochem. 65 : 441 - 473.
12. F. A. Quiocho (1986) Annu. Rev. Biochem. 55 : 287 - 315.
13. W. Montfort, I. E. Villafranca, A. F. Monzingo, S. R. Ernst,

B. Katzin, E. Rutenber, N. H. Xuong, R. Hamlin and J. D. Robertus (1987) J. Biol. Chem. **262** : 5398 - 5403.

14. G. S. Hassing and I. J. Goldstein (1971) Biochim. Biophys. Acta **243** : 90 - 97.
15. J. P. Privat, R. Lotan, P. Bouchard, N. Sharon and M. Monsigny (1976) Eur. J. Biochem. **68** : 563 - 572.
16. T. Mazumder, N. Gaur and A. Surolia (1981) Eur. J. Biochem. **113** : 463 - 470.
17. M. Cermakova, G. Entlicher and J. Kocurek (1976) Biochim Biophys Acta **420** : 236 - 245.
18. B. Kohnke-Godt and H. J. Gabius (1991) Biochemistry **30** : 55 - 65.
19. H. Kawagishi and H. Mori (1991) Biochim Biophys Acta **1076** : 179 - 186.
20. S. Basu, C. Mandal and A. K. Allen (1988) Biochem J. **254** : 195 - 202.

CHAPTER 4
Purification and characterization of a lectin from *Xanthomonas campestris*

SUMMARY

Several isolates of a typical and well studied bacterial plant pathogen, *Xanthomonas* were screened for extracellular lectin production. Bacteria were grown at 28°C, in a liquid medium containing ($g.L^{-1}$): glucose 20, peptone 5, yeast extract 3 and malt extract 3, at pH 7.0 for 72 hours. Lectin activity was monitored every 12 hours by haemagglutination assay using 3% suspension of freshly prepared rabbit RBC and 100 µl of the culture filtrate (taken out aseptically from the actively growing culture). Out of a total of 25 isolates of *Xanthomonas* cultures, screened for extracellular lectin activity, *Xanthomonas campestris* NCIM 5028 showed the highest and consistent extracellular lectin activity. Hence it was chosen for further studies. The time course of lectin production by *Xanthomonas campestris* NCIM 5028 was followed for 120 hours by monitoring the protein content, viscosity (as a measure of extracellular polysaccharide (EPS) and lectin activity (heamaglutination assay) after every 24 hours of growth. After optimization of the growth conditions for the production of lectin, the lectin was purified to homogeneity using hydrophobic interaction chromatography on a column of phenyl-Sepharose after initial ammonium sulfate precipitation. The lectin was found to be a heterodimer of subunits having relative molecular masses of 30000 and 28000. Gel filtration on Sephacryl S-300 column calibrated with markers, showed its native molecular mass to be approximately 70000. It's isoelectric point was determined to be 7.2. It agglutinated rabbit erythrocytes but did not agglutinate human A, B and O blood group erythro-

ytes. The carbohydrate specificity of the lectin was determined sing simple and complex sugars. Among the sugars tested, the gglutination of rabbit erythrocytes was strongly inhibited by lucosamine, galactosamine and mannosamine. Fetuin glycopeptide O- linked) and host plant (*Brassica oleracea*) polysaccharides ere the best inhibitors among oligo and polysaccharides.

4.1 INTRODUCTION

Microbial agglutinins have elicited considerable attention because of the role they are known to play in adherance to surface colonized by the microorganisms (18, 19). Several reports are available on lectins produced by microorganisms pathogenic especially to animal and human beings. They are well characterized from the point of view of their physiological role. For example, *E. coli*, *Vibrio cholerae*, *Bordetella pertussis* produce lectins which are determinants for host specificity and are known to play a key role in infection. There are very few reports available on lectins produced by plant pathogenic microorganisms and their physiological roles. Though there are several studies on the EPS (extracellular polysaccharide) produced by plant pathogens and their roles in infection (1,2), the lectins associated with EPS are not studied from this point of view. Only a few reports suggest that in plant-microbe interaction, recognition of the host surface could be mediated by lectin carbohydrate interaction, in which a lectin could be produced by the pathogen and plant surface carbohydrates act as receptors (3, 7, 17). Therefore it was of interest to know whether a typical plant pathogen like *Xanthomonas campestris*, produces a lectin and whether this lectin has a role in adhesion. Hence the studies were carried out a) to screen several isolates of *Xanthomonas* for lectin production and b) to isolate and characterize the lectin produced.

4.2 MATERIALS AND METHODS

4.2.1 Microorganism and maintenance of culture

Various isolates of *Xanthomonas* were obtained from National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune, India. They were routinely maintained on agar slants of a medium containing ($g.L^{-1}$) : glucose 20 , peptone 5, yeast extract 3 and malt extract 3 at pH 7.0.

4.2.2 Chemicals

Glucose was purchased from Qualigens, (India), peptone, yeast extract and malt extract were from Hi Media, (India). Sephacryl S300 was from Pharmacia, (Sweden), DEAE cellulose (DE 52) was from Whatman (UK), marker proteins for SDS-PAGE and gel filtration were from Sigma Chemical Company, (USA). All other chemicals used were of analytical grade.

4.2.3 Screening of different Xanthomonas isolates

Xanthomonas cultures were grown in a medium containing (g. L^{-1}): glucose 20, peptone 5, yeast extract 3 and malt extract 3. The pH of the medium was adjusted to 7.0 and was not controlled further. The cells were grown in 1000 ml Erlenmeyer flasks containing 200 ml of medium. The inoculum was built in the same medium for 24 hours. A 10 % inoculum was added to the production medium and the flasks were incubated for 72 hours at 28 °C on a rotary shaker with 200 rev. min^{-1}. Aliquotes of 1 ml were taken out aseptically after every 12 hours and centrifuged (9770 X g, 20 min.) to remove cells. The haemagglutination activi-

y was checked from the supernatant.

.2.4 Time course of the production of lectin by selected Xanhomonas isolate

The time course of lectin production of the selected isolate as followed by monitoring protein content, viscosity (as a easure of EPS production) and haemagglutination activity after very 24 hours of growth upto 120 hours. Protein was measured fter TCA (12.5 %) precipitation. Viscosity of the culture filrate was measured on Ubbelöhde viscometer (14).

.2.5 Purification and characterization of lectin

For purification of the lectin, 48 h grown cells were removed from the fermentation broth by centrifugation (9770 x g, 0 min) and the culture filtrate was concentrated by ultrafilration on Amicon ultrafiltration YM3 membrane. The concentrate as then treated at 4°C with ammonium sulphate upto 80% saturaion, allowed to stand for 3 hours and the precipitate was emoved by centrifugation (9770 x g, 20 min). The supernatant as diluted 1:3 with buffer (Tris-HCl 20 mM, pH 7.2) and subjected to hydrophobic chromatography on phenyl Sepharose (1.5 x 15 m) preequilibrated with 20 mM Tris-HCl buffer, pH 7.2 containing 5% ammonium sulphate. The column was washed with the same buffer till the A_{280} was below 0.03 and the bound lectin was then eluted by the reverse gradient of ammonium sulphate (25% - 0%) in 20mM Tris-HCl buffer of pH 7.2 containing 5% glycerol. The fractions showing haemagglutination activity were pooled and dialyzed against buffer (20mM Tris-HCl, pH 7.2 containing 5% glycerol) concentrated by ultrafiltration and used for further

studies .

4.2.6 Determination of protein concentration

Protein concentration was determined as described by Lowry *et al* (12), using bovine serum albumin as a standard.

4.2.7 Haemagglutination assays

For haemagglutination assays, two-fold serial dilutions of lectin solution (50 µl) in microtiter plate were mixed thoroughly with 50 µl of 3% rabbit erythrocyte suspension in Tris buffer (10 mM, pH 7.2) containing 0.15 M NaCl, at room temperature for one hour. HA (haemagglutination activity) is expressed as titer, i.e. a reciprocal of the highest dilution of the lectin that gave complete agglutination. Specific activity of the lectin was defined as titer of the lectin per mg of protein. Agglutination of erythrocytes by the cells of *Xanthomonas campestris* was carried out similarly.

For HA inhibition, sugars and glycopeptides were serially diluted two - fold in microtiter plates and were incubated with lectin (of titer four) at room temperature. Rabbit erythrocytes (3% suspension in Tris buffer saline) were added after 15 min. and the microtiter plates were read for HA inhibition after an hour of incubation at room temperature.

4.2.8 Determination of molecular mass

The relative molecular mass of pure lectin was estimated by SDS-PAGE as described by Laemmli (9) using the low range protein markers (M_r 14000 - 66000) and by gel filtration using a column of Sephacryl S-300 (1 x 60 cm) equilibrated with 20 mM

Tris buffer, pH 7.2, containing 10% ethylene glycol. The column was calibrated with alcohol dehydrogenase (153000), bovine serum albumin (66000), ovalbumin (45000) and carbonic anhydrase (29000).

4.2.9 Isoelectric focussing

Isoelectric focussing was carried out according to Sathivel *et al* (16), using ampholines of pH range of 3.0 to 10.0.

4.2.10 Plant tissue extraction

Leaves of *Brassica oleracea* var *botrytis* (host for *Xanthomonas campestris* var *campestris* NCIM 5028) were extracted with buffer (Tris-HCl 20 mM, pH 7.2) and centrifuged to separate the insoluble material. The supernatant was termed as 'buffer extract' and used in inhibition studies of lectin. The precipitate was then subjected to alkali extraction for an hour (kept in boiling water bath with 1 M NaOH) and centrifuged. The supernatant was then neutralized with acetic acid and subjected to ethanol precipitation (1:2 v/v). The precipitate was dissolved in buffer (Tris-HCl,20mM, pH 7.2), dialyzed thoroughly against the same buffer and used as 'alkali extract' in inhibition studies (4). Extractions of *Oryza sativa* (Rice) plant were also carried out in a similar manner and extracts were tested for inhibition. Sugar concentrations were measured by phenol-sulphuric acid method in terms of neutral sugar (5), using mannose as standard. Glycopeptides of fetuin and soybean lectin were prepared according to Townsend (20) and Lis and Sharon (10) respectively.

4.2.11 Preparation of Soybean lectin

Soy beans were macerated in phosphate buffer saline (20 mM, pH 7.2) and centrifuged to remove the insoluble material. The supernatant was then subjected to ammonium sulphate precipitation (80 %) and the precipitate was dialyzed against phosphate buffer saline. The soybean lectin was further purified by affinity chromatography using guar gum matrix. The lectin was eluted with 0.2 M lactose and dialysed thoroughly to remove the sugar. The lectin was dried to powder by lyophilization.

4.2.12 Desialylation of fetuin

Fetuin was treated with 0.2 N H_2 SO_4 at 50°C for 2 hours. After this treatment, it was dialysed against distilled water till free from acid and lyophilized to dryness.

4.2.13 Preparation of glycopeptides from fetuin and soybean lectin

Proteins in powder form were mixed with 0.1N HCl and incubated for 2 hours at 60°C. The pH was then brought to 8.0 with 1M NaOH and 40 mg of pronasewas added to the protein solutions. Proteins with pronase were incubated at 37°C for 24 hours and further treatment with 20 and 10 mg of pronase was given in a similar fashion at 24 hours intervals. The digested protein was lyophilized to dryness. The glycopeptides were extracted with minimum amount of 10 mM acetic acid. Pronase digested extract was passed through G-25 and BioGel P-4 columns to remove small digested peptides and amino acids. The peaks with high sugar content were lyophilized and then those glycopeptides were passed through Dowex column to separate them from the oligosaccharides

generated during pronase digestion.

4.3.RESULTS

4.3.1 Screening

Total 25 isolates of *Xanthomonas* were screened for lectin production. Table 4.1 shows the time taken and amount of lectin produced by each isolate.

Out of twenty five isolates screened, nine isolates were good lectin producers (yielding about 320 units/ml of the medium in 48 hours) The lectin activity was dependent mainly on the carbon source used, 2 % glucose being the optimal; decrease in levels of the carbon source, produced very low levels of lectin activity.

Cultures mumbers 1, 3, 4, 5, 14, 23 and 25 were promising, but numbers 4, 5, and 14 showed loss of 50 % activity after 48 hours. It could be due to the increased levels of protease, produced extracellularly by the organism. Culture isolates numbered 1, 3, 23 and 25 did not show any loss of activity. However, culture number 25 was selected for further studies since it was the highest producer and could produce upto 512 units of lectin/ml of the culture broth at the end of 48 hours.

4.3.2 Time course of production of lectin

Xanthomonas campestris NCIM 5028 produced polysaccharide (EPS) in large amounts as well as the lectin within 48 hours. Lectin was not produced if production of polysaccharide was hampered due to repeated subculturing. It can be seen from Fig.4.1, that production of EPS and lectin go hand in hand upto 48 hours and

Table 4.1 : Screening for lectin producing *Xanthomonas campestris*

No.	Strain	Activity at				
		12h	24h	36h	48h	72h
		Units/ml				
1.	*Xanthomonas malvacearum* NCIM 2310	160	320	320	160	160
2.	*X.campestris* pv.*oryzae* Px Co35raceI	40	80	80	80	80
3.	*X.campestris* pv.*oryzae* xCo 8	160	320	640	320	160
4.	*X.campestris* pv.*oryzae* 3849 II	160	320	320	320	320
5.	*X.campestris* pv.*oryzae* xCo 9	0	0	20	0	0
6.	*X.campestris* pv.*oryzae* xCo 14	0	40	40	40	40
7.	*X.campestris* pv.*oryzae* xCo II c	10	320	640	640	640
8.	*X.campestris* pv.*oryzae* 3813	20	40	80	80	80
9.	*X.campestris* pv. *oryzae* IxCo15	0	0	0	20	40
10.	*X.campestris* pv. *oryzae* 3821	0	0	0	0	0
11.	*X.campestris* pv. *oryzae* 3822	0	0	0	0	0
12.	*X.campestris* pv. *oryzae* xCo36	20	20	40	40	40
13.	*X.campestris* pv. *oryzae* 3863 Ia	20	20	20	20	20
14.	*X.campestris* pv. *oryzae* 3844	160	320	320	640	640
15.	*X.campestris* pv. *oryzae* 3841	0	20	40	40	40
16.	*X.campestris* pv. *oryzae* xCoIb	40	40	40	80	80
17.	*X.campestris* pv. *oryzae* 3843	0	0	0	0	0
18.	*X.campestris* pv. *oryzae* xCoIa	20	20	20	20	20
19.	*X.campestris* pv. *oryzae* 3863	0	0	0	0	0
20.	*X.campestris* pv. *oryzae* 3856	0	0	0	0	0

Table 4.1 continued

No.	Strain	Activity at 12h	24h	36h	48h	72h
		Units/ml				
21.	*X.campestris* pv. *oryzae* pxCo86	0	20	40	40	0
22.	*X.campestris* pv. *oryzae* 3858	0	40	40	20	20
23.	*X.campestris* pv. *oryzae* 2954	160	320	320	640	320
24.	*X.campestris* pv. *oryzae* 2956	160	320	320	320	320
25.	*X.campestris* pv. *campestris* NCIM 28	160	320	640	640	640

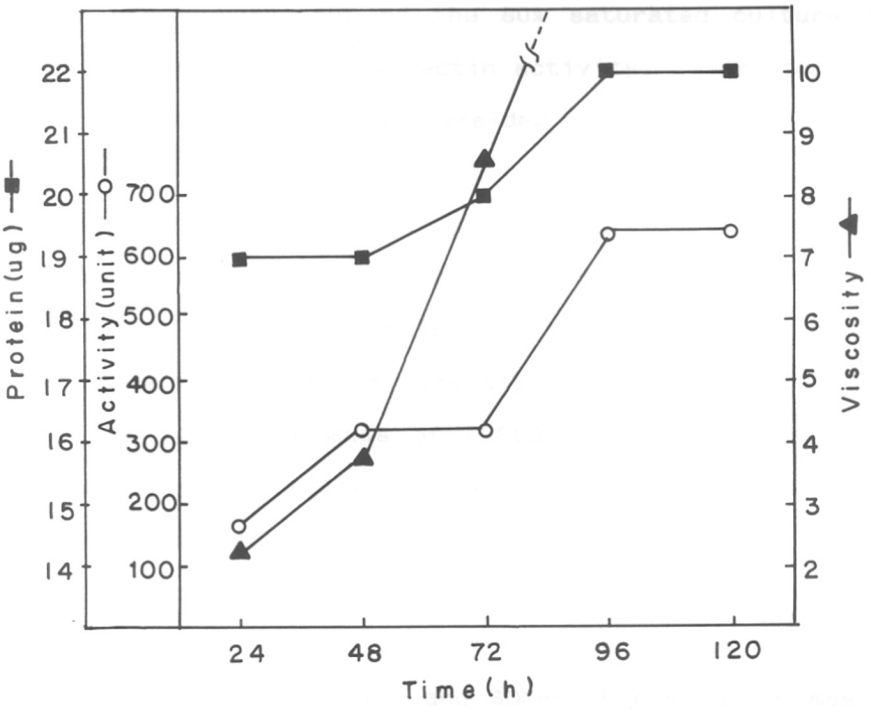
Fig. 4.1 : Time course of production of lectin by *Xanthomonas campestris*

--//-- tremendous viscosity increase after 72 hours of fermentation.

--▲-- viscosity - as a measure of polysaccharide

--■-- protein (TCA precipitable) (µg/ml)

--o-- haemagglutination activity (units/ml)


Protein(ug)
Activity(unit)
Viscosity
Time(h)
22
21
20
19
18
17
16
15
14
700
600
500
400
300
200
100
10
9
8
7
6
5
4
3
2
24
48
72
96
120

there is a tremendous increase in EPS production at 72 hours. For this reason the culture was harvested at the 48th hour, when the viscosity was low and cells could be seperated easily from the broth.

4.3.3 Purification and characterization of lectin

The ammonium sulphate precipitation step removed most of the contaminating proteins as well as a considerable amount of EPS. However, the supernatant of the 80% saturated culture filtrate retained almost 84% of the lectin activity. Hydrophobic chromatography of this supernatant yielded a single protein peak with agglutination activity. The lectin was purified 25 fold with 24% recovery (Table 4.2). It showed two closely placed bands on SDS - PAGE, with relative molecular masses of 28000 and 30000 (Fig. 4.2). The gel filtration of the lectin on Sephacryl S-300 in the presence of 10% ethylene glycol showed a single peak with a relative molecular mass of 70000 (Fig. 4.3). The lectin, therefore, appears to be a heterodimer. The presence of ethylene glycol was necessary because this lectin had a tendency to be retarded on any gel filtration matrix; perhaps because of its hydrophobic nature. The difference in the molecular mass observed for gel filtration and SDS-PAGE cumulative mass of the subunits, could be due to ethylene glycol being used to remove the interaction of the lectin with the gel filtration matrix. On isoelectric focussing, the lectin showed a single peak of activity, and the pI calculated was 7.2 (Fig. 4.4).

The agglutination of rabbit erythrocytes by the purified lectin was inhibited only by aminated sugars among the mono and

Table 4.2 : Purification of lectin from *Xanthomonas campestris* NCIM 5028

Step	Volume ml	Total activity units	Total protein mg	Specific activity units/mg	Purification yield %	fold
Culture filtrate	1000	320000	210[a] (3000*)	1524	100	1
$(NH_4)_2SO_4$ supernatant	150	256000	19.7 (1750*)	13000	84	8.5
phenyl Sepharose	60	76800	2.3	38400	24	25.1

* = protein determined before TCA precipitation.

a = Protein content determined after precipitation with TCA. The sterile medium contains 90 µg/ml TCA precipitable protein.

Fig. 4.2 : Native and SDS-PAGE of the purified *Xanthomonas campestris* lectin

a) Native PAGE

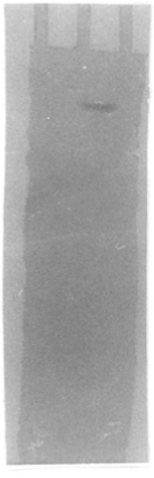
b) SDS PAGE

Lane 1. Purified *Xanthomonas campestris* lectin

Lane 2. Molecular weight markers, from the top:
bovine serum albumin(66000), ovalbumin(45000),
glyceraldehyde-3-phosphate dehydrogenase(36000),
carbonic anhydrase(29000),
soybean trypsin inhibitor(20100),
lactalbumin(14200).

3. *Xanthomonas campestris* lectin after IEF.

(a)



(b)

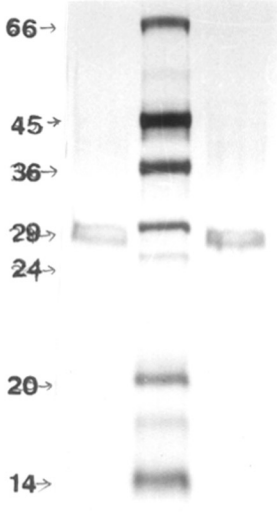


Fig. 4.3 : **Gel filtration of lectin on Sephacryl S-300-**
Markers used are : alcohol dehydrogenase (153000), bovine serum albumin (66000), ovalbumin (45000), carbonic anhydrase (29000) (- o -);
Xanthomonas campestris lectin (- ■ -).

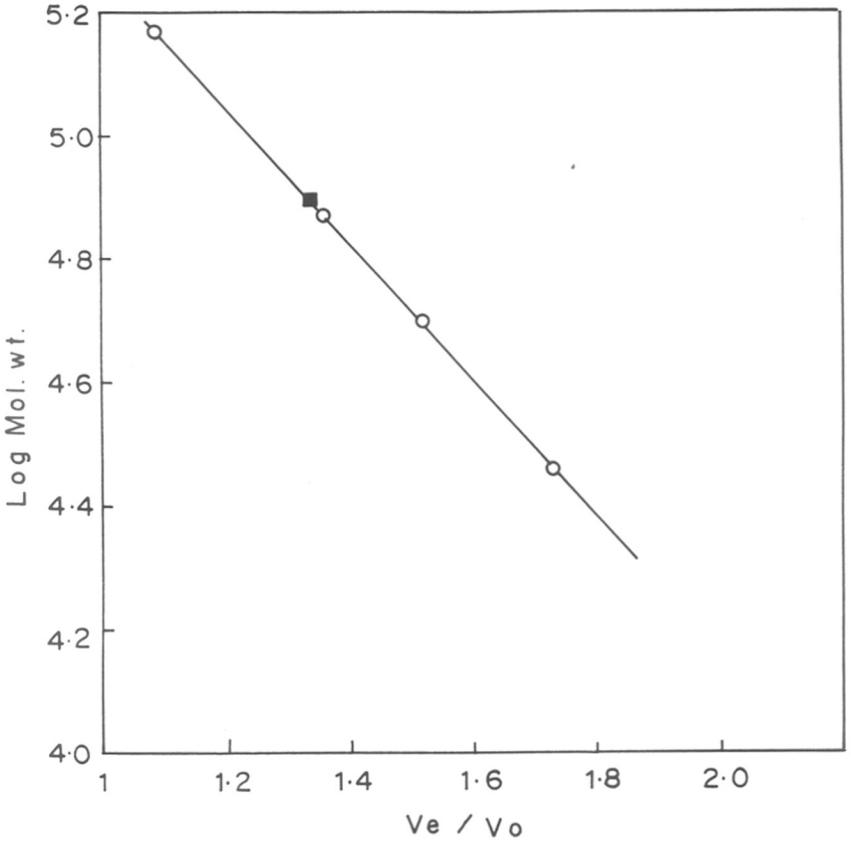

5·2
5·0
4·8
4·6
4·4
4·2
4·0
Log Mol. wt.
1
1·2
1·4
1·6
1·8
2·0
Ve / Vo

Figure 4.4 : Isoelectric Focussing of Purified Lectin Produced by *Xanthomonas campestris* NCIM 5028

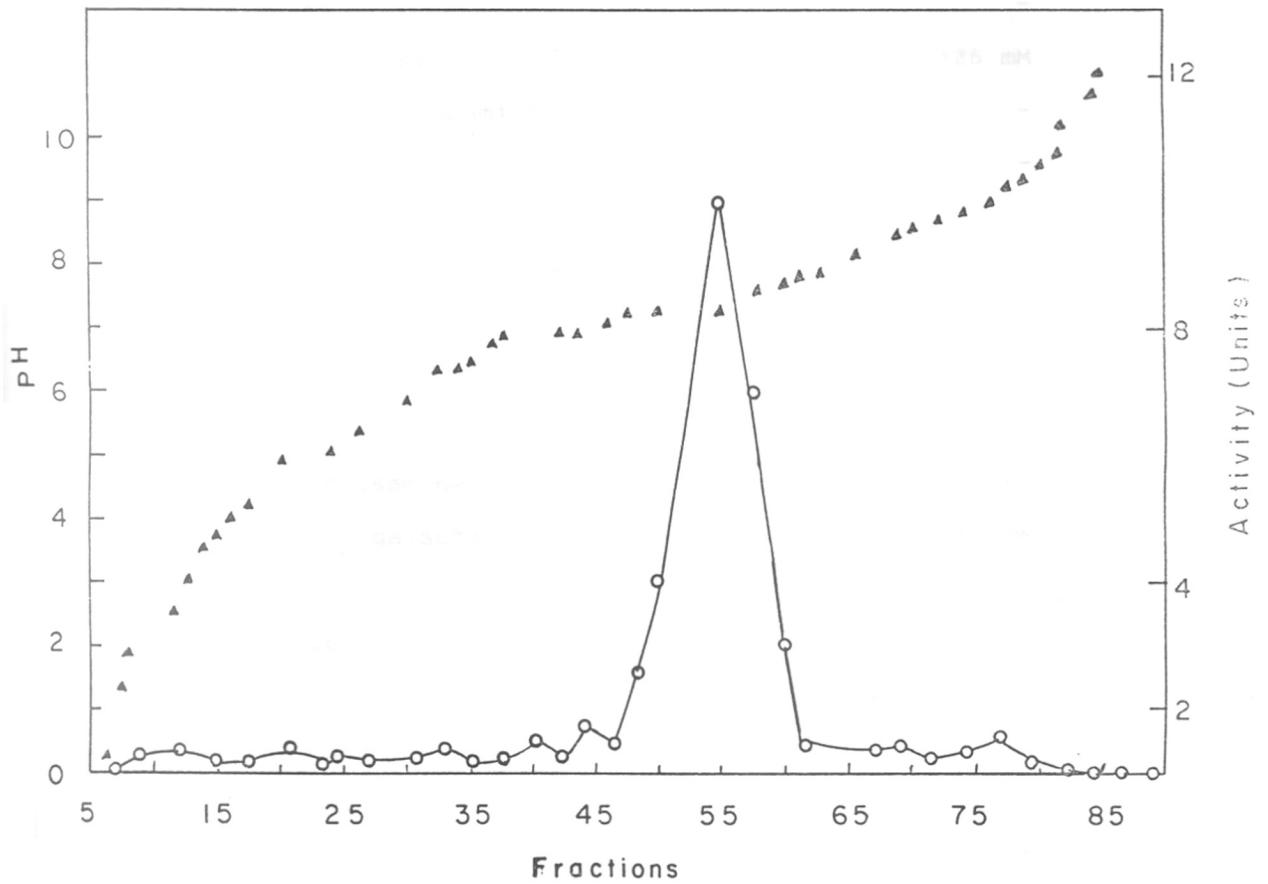

pH
Activity (Units)
Fractions
0
2
4
6
8
10
12
5
15
25
35
45
55
65
75
85

Table 4.3 : Sugar Specificity of *Xanhomonas campestris* lectin

	Sugars	Inhibitory concentration
1.	Glucose	-
2.	D-glucosamine	125 mM
3.	N acetyl glucosamine	-
4.	Me α glc	-
5.	Me β glc	-
6.	Mannose	-
7.	D-mannosamine	125 mM
8.	Me α man	-
9.	Galactose	-
10.	D-galactosamine	125 mM
11.	N acetyl galactosamine	250 mM
12.	Me α gal	-
13.	Me β gal	-
14.	L(-)Fucose	-
15.	Arabinose	-
16.	Rhamnose	-
17.	Melibiose	-
18.	Stachyose	-
19.	Lactose	-
20.	N acetyl lactosamine(50mM)	-

- = No inhibitory effect even at 250 mM concentration.

ble 4.4 : Sugar specificity of *Xanthomonas campestris* lectin

Glycoprotein	Inhibitory concentration
Fetuin	7.5 µg
Desialylated fetuin	3.9 µg
N - linked glycopeptide of fetuin[a]	6.5 µg
O - linked glycopeptide of fetuin[a]	1.87µg
Soybean lectin glycopeptide[b]	NI* (3mg)
Brassica oleracea var*botrytis* buffer extract	5.5µg
Brassica oleracea var*botrytis* alkali extract	2.3µg
Fibrinogen	200µg

NI = noninhibitory at

a = Fetuin glycopeptides were prepared according to Townsend *et al.*(20).

b = Soybean lectin glycopeptides were prepared according to Lis and Sharon,(10).

saccharides tested i.e. N acetyl galactosamine (250 mM) D-
innosamine, D-galactosamine and D-glucosamine (125 mM). (Table
3). Fetuin and glycopeptides of fetuin were the most potent
inhibitors of the HA activity of the lectin. Fibrinogen also
inhibited the agglutination but was less potent than fetuin.
ybean lectin glycopeptide failed to inhibit the agglutination.
linked complex type of glycopeptide of fetuin showed much less
inhibitory activity compared to O- linked glycopeptide of
tuin,which are mucin like structures. The O- linked glycopep-
de was twice as efficient as desialylated fetuin. Host (*Brassi-*
a oleracea var *botrytis*) extracts (buffer as well as alkali)
inhibited the agglutination activity of the lectin (Table
4),indicating that the lectin recognizes carbohydrate struc-
ires on the host surface. The high concentration of simple
gars and the inhibition pattern shown by the glycoproteins and
ycopeptides indicate that this lectin probably recognizes a
mplex sugar structure.

Another important feature of the lectin was its instability
thout polysaccharide (xanthan gum). Although the lectin was
t a glycoprotein itself, it required association with polysac-
aride to remain stable on storage. Other additives such as
ycerol did not stabilize the lectin. Addition of xanthan gum
abilized the lectin for a longer time.

4 DISCUSSION

Xanthomonas campestris cells (prewashed three times with
is buffer,20 mM,pH 7.2, saline) agglutinated rabbit erythro-
tes, therefore the lectin could be the fimbrial lectin which

must have been shed in the medium during growth. It was observed that the production of lectin was always associated with that of the EPS. Production of polysaccharides is supposed to be a major determinant in the ability of a bacterium to colonize a given niche (24). As capsular polysaccharides are highly hydrated, they protect the bacterium from dessication. Additionally, acidic EPSs produced by bacteria are anionic in nature, which may help bacteria to adhere to biological surfaces. EPS^- mutants of *Erwinia, Pseudomonas* and *Xanthomonas* have been shown to have reduced virulence.EPS may have multiple functions in pathogenesis. The acidic polysaccharide produced by *Xanthomonas campestris* is composed of D-glucose, D-mannose, D-glucuronic acid, acetic acid and pyruvic acid. Glucose:mannose:glucuronic acid ratio is 2:2:1.

There are similar reports in literature about other microorganisms, either pathogenic or symbiotic to plants, producing lectins which are associated with the EPS secreted by that organism. For example the fungal plant pathogen - *Sclerotium rolfsii* produces a lectin of M_r 45000 , that is specific for mucin and it is strongly associated with the polysaccharide (1,3-ß-glucan) it produces. The lectin as well as the polysaccharide have a role in pathogenesis (8). Gould and Northcote (6) stated that an integrated fungal molecule that contained both carbohydrate and protein, was responsible for adhesion of *Phialophora radicicola* to the host.

A plant lectin has been shown to be a host range determinant in *Rhizobium* (13); but the symbiotic relationship between *Bradyrhizobium japonicum* and soybean plant has been shown to involve a lectin - carbohydrate interaction in which the lectin

is produced by the microorganism (11). *Agrobacterium tumefaciens* produced a lectin, specific for L(-) fucose, and polysaccharides of Poplar leaves (one of its hosts),inhibits its activity. Depierreux (3) has speculated on its role in specific binding of *Agrobacterium tumefaciens* to the plant host.

The lectin produced by *Xanthomonas campestris* NCIM 5028, is inhibited at high concentrations of aminated sugars and by mucin like structures of fetuin as well as polysaccharides produced by the host plant. It indicates that this lectin has a complex sugar specificity. The agglutination of rabbit erythrocytes by intact cells and identical sugar specificity indicates that this lectin is in all probability a fimbrial lectin.

Bacterial pili or fimbriae which are produced by large proportion of plant pathogenic bacteria also, are mostly sugar binding proteins i.e. lectins. *Erwinia caratovora* produces mannose sensitive as well as mannose resistant lectins (21). The mannose sensitive type of lectin has subunit molecular mass of 16500 whereas the mannose resistant type of fimbrial lectin subunit has the molecular mass of 18000. *Erwinia rhapontici* produces a lectin specific for gal-β 1,4 glcNAc. Other plant pathogens like *Klebsiella* (22) produced fimbriae of type 1 as well as type 3 and nonfimbriated mutants failed to adhere to the host plant surfaces. Plant pathogenic pseudomonads also produce fimbriae but they are mostly useful for epiphytic colonization. *Pseudomonas solanacearum* produced a lectin of subunit molecular mass 9500 which mediated both bacterial binding to tobacco leaf cell walls and bacterial autoagglutination (23). *Xanthomonas*

campestris pv. hyacinthi as well as *Xanthomonas campestris pv. vesicatoria* also produced fimbriae. *Xanthomonas campestris pv. hyacinthi* produced pili type 4 and the sugar specificity of these pili is speculated to be glcNAc β- 1,4 gal (15).

The physiological role played by *Xanthomonas* lectin reported here, is yet unknown although inhibition of the lectin activity by host plant extracts indicate a possible role in adhesion.

REFERENCES

1. D. L. Coplin and D. Cook (1990) Mol. Plant - Microbe Interact. 3: 271 - 279.
2. T. P. Denny, and S. R. Baek (1991) Mol. Plant - Microbe Interact. 4: 198 - 206.
3. C. Depierreux, H. C. Kang, B. Guerin, M. Monsigny and F. Delmotte (1991) Glycobiology 1 : 643 - 649.
4. G. de Stevens (1956) Methods Enzymol.3 : 31-33.
5. M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers and F. Smith (1956) Anal. Chem. 28 : 1747-1748.
6. J. Gould and D. H. Northcote (1986) Biochem. J. 233 : 395 - 405.
7. H. C. Ho, J. L. Wang and M. Schindler (1990) J. Cell Biol. 111 : 1631 - 1638.
8. J.Inbar and I. Chet (1994) Microbiology 140: 651 - 657.
9. U. K. Laemmli (1970) Nature (London) 27 : 680 - 685.
10. H. Lis and N. Sharon (1978) J. Biol.Chem 253:3468- 3476.
11. J. T. Loh, S. C. Ho, A. W. de Feijter, J. L. Wang and M. Schindler (1993) Proc. Natl. Acad. Sci.USA90:3033- 3037.
12. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. Randall, (1951) J. Biol. Chem. 193 : 265 -275.
13. B. J. J. Lugtenberg, C. Diaz, G. Smit, S. de Pater and J. W. Kijne (1991) in Advances in molecular genetics of plant microbe interactions (Kluwer Academic Publications). Vol.1 : 174-181.
14. J. E. McKie and J. F. Brandts (1972) Methods Enzymol.26

: 257-288.

15. M. Romantschuk, E. Roine, T. Ojanen, J. vanDoorn, J. Louhelainen, E . L. Nurmiaho-Lassila, K. Haahtela (1994) Dev.Plant Pathol.3 (Molecular mechanisms of Bacterial Virulence):67-77.

16. C. Sathivel, A. H. Lachke and S. Radhakrishnan (1995) J. Chromatogr. **705** : 400 - 402.

17. L. Sequeira, G. Gaard and G. A. deZoeten (1977) Physiol. Plant Pathol. **10** : 43 - 50.

18. N. Sharon and I. Ofek (1986) in : Microbial lectins and agglutinins ; Properties and Biological Activity (Mirelman D. Ed.) pp 1 - 21.

19. N. Sharon (1987) FEBS Lett. 217 : 145 - 157.

20. R. R. Townsend, M. R. Hardy, T. C. Wong and Y. C. Lee (1986) Biochemistry 25: 5716 - 5725.

21. T. K. Korhonen, N. Kalkkinen, K. Haahtela and D. C. Old (1987) J. Bacteriol. **169** : 2281 - 2283.

22. T. K. Korhonen, K. Haahtela, A. Pirkola and J. Parkkinen (1988) FEBS Lett. **236** : 163 - 166.

23. D. H. Young, W. P. C. Stemmer and L. Sequeira (1985) Appl. Environ. Microbiol. **50** : 605 - 610.

24. I. C. M. Dea, F. R. Morris, D. A. Rees, E. J. Welsh, H. A. Barnes and J. Price (1977) Carbohydr. Res. 57:249- 272.

CHAPTER 5

General Discussion

5.1 Plant - bacterial interactions

The attachment of microorganisms such as bacteria to human, animal or plant host tissues requires adhesion factors (1,2). Interaction of bacteria with eukaryotic structures leads to colonization. The adhesion factors or adhesins can be considered as virulence factors contributing to the pathogenicity of the bacteria since they play a major role in the colonization of ecosystems where bacteria lacking adhesins would not survive. The majority of adhesion factors are of fimbrial nature (3) and since many of the fimbrial as well as nonfimbrial adhesins recognize glycoconjugates on other cells, they are classified as lectins.

Plants coexist with a great number of microorganisms. Plant microbe interaction can be beneficial, as in symbiotic *Rhizobium* - legume and mycorrhizal root associations, but in other cases microbes represent potential pathogens with the ability to parasitize the plants and cause disease. Early in the interactions between plants and microbes, signals are produced that elicit discrete responses in the respective partners, the first step in the cascade that determines the outcome of the symbiosis or pathogenesis. In most cases however, the receptors and signaling mechanisms mediating these responses are ill defined. Signal generation and perception in many of the microbes that interact with plants are understood in greater detail by parallel findings from other prokaryotic and eukaryotic microbes.

In symbiotic bacteria, a relationship has been estab-

lished between *Bradyrhizobium japonicum* and soybean plant. Host plant lectin was proposed to involve in the specific attachment of the homologous *Rhizobium* system to host roots and the initiation of the infection process (4). The same mechanism involved in the attachment of *Rhizobium leguminosarum* pv. *Trifolii* to the white clover root surface has been reviewed (35,36). But in case of *Bradyrhizobium* the available evidence fails to provide a compelling reason to accept the notion that soybean agglutinin plays specific role in surface recognition events. Instead it has been shown that *Bradyrhizobium* produces a lectin which plays a significant role in host surface attachment and symbiosis (5,6).

During their initial association with plant hosts, pathogenic bacteria interact with plant cell walls. The results of this interaction appear to determine whether bacterial multiplication will take place. With one group of bacterial plant pathogens *viz.* - *Erwinia*, attachment to host surface appears to be essential for pathogenesis and it has been shown that it produces a fimbrial lectin which might be responsible for attachment (37,38). With another group of microbes, it is the other way round. Among Pseudomonads, only those strains that do not attach to the host cell wall are able to multiply in the intercellular spaces and infect the host successfully e.g. *Pseudomonas solanacearum* (8). Attachment of *Pseudomonas* strains to tobacco mesophyll cell wall leads to a rapid hypersensitive response (HR) and a drastic reduction in bacterial multiplication.

5.2 Pathogenicity factors of Agrobacterial infection

Site specific attachment of *Agrobacterium tumefaciens* to the plant cell surface is an essential step in tumorigenicity. This statement was based on the observation that avirulent *Agrobacterium tumefaciens* strains which were still able to attach and inhibit initiation of tumor formation by virulent strains, presumably by occupying all available binding sites on the plant cell surface. In addition, specific receptors on the *Agrobacterium tumefaciens* cell surface were supposed to be involved since tumorigenesis could not be blocked by inoculation with heterologous bacteria such as *Rhizobium meliloti or Pseudomonas aeruginosa* (7).

During the last two decades evidence has accumulated that attachment of *Agrobacterium tumefaciens* to plant cells is a two step process (9). In the first step, *Agrobacterium tumefaciens* adheres to plant cell surface as a single cell. In the second phase, in response to plant factors, *Agrobacterium tumefaciens* elaborates cellulose fibrils that entrap bacteria resulting in the formation of bacterial aggregates. These fibrils also cause the bacteria to bind very tightly to the plant cell surface (10).

There are several molecules which are claimed to be involved in the early stages of *Agrobacterium* attachment and infection.

1. **Low molecular weight polysaccharide β 1-2 glucan :** β 1-2 glucan is claimed to be a mediator molecule between plant cell surface and bacterium. *chvA* gene codes for a protein

that is necessary for the transport of ß 1-2 glucan into the periplasm. The *chvB* gene codes for 235 kDa protein which converts glucose into cyclic ß 1-2 glucan (11, 13) and both these genes have been shown to be involved in the bacterial interaction with the plant host.

2. **Lipopolysaccharides** : Lipopolysaccharides produced by Agrobacteria have also been reported to be involved in attachment (14).

3. **Chemotaxis** : Agrobacteria are peritrichous, motile bacteria and possess a highly sensitive chemotaxis system which responds to a wide range of amino acids and sugars. Some of these sugars like galactose, glucose, arabinose, fucose and xylose have been identified as *vir* gene inducers (15). The genes involved in the general chemotactic response towards amino acids and sugars are located on the chromosome. In addition, Ashby (16) reported that *Agrobacterium tumefaciens* C 58 also exhibits chemotaxis towards acetosyringone, one of the major plant phenolic *vir* gene inducers. Broek and Vanderleyden (17) have suggested that the migration towards acetosyringone may constitute the first step in the recognition between *Agrobacterium tumefaciens* and its host plant in soil. Therefore specific chemotactic attraction may guide the bacteria towards plant wounds where the concentration of the inducer is sufficiently high to switch on expression of *vir* genes. However this hypothesis is still in dispute.

4. **Lectins** : Depierreux *et al* (12) have claimed that lectin resides on the surface of the bacterium while plant cell

surface represents the carbohydrate ligand. Lectin produced by *Agrobacterium tumefaciens* has been shown to be an important determinant of infection at very early stage.

As the attachment of *Agrobacterium tumefaciens* to host cells is saturable, presence of specific receptor molecules on the plant cell surface has been postulated for a long time. It is supported by the observation that avirulent strains like *Agrobacterium radiobacter* biologically control the crown gall disease. This may be because both (virulent as well as avirulent strains) of them bind to the same specific receptor molecule on the plant surface (19,20).

It has been shown recently that *Agrobacterium tumefaciens* produces pili which are essential for T DNA transfer from bacterial cell to plant cell. Thus for attachment and channel formation between bacterial and host cell pili are required which may represent lectin carbohydrate interaction (18).

Two different lectins have been isolated from avirulent strain *Agrobacterium radiobacter* ATCC 6466 in the present study. The lectins show different sugar specificities. One of them is produced extracellularly (Lectin I) whereas the other one is cell bound (Lectin II). The cell bound Lectin II was specific for acidic plant polysaccharides whereas the extracellularly produced Lectin I was specific for high mannose type glycosyl moieties of glycoproteins i.e. invertase or ovalbumin. The core structure for which the lectin I is specific, is probably glcNAc-glcNAc-man3. These lectins, produced by avirulent strain of *Agrobacterium*, are

indicative of involvement of a lectin carbohydrate interaction in the adhesion of the microorganism to the host plant.

5.3 Pathogenicity factors of *Xanthomonas*

Many laboratories have used genetic methods to analyze aspects of pathogenicity of bacteria to plants. Many similarities have emerged between sets of "pathogenicity " genes discovered in various pathogens. Daniels *et al.* (21) cloned pathogenicity genes from *Xanthomonas campestris pv. campestris* causing black rot of crucifers. They include *hrp* genes, genes for extracellular enzymes and polysaccharide genes.

1. ***hrp* genes** : *hrp* mutants are defective in their ability to incite hypersensitive response and they have been isolated from *Erwinia*, *Pseudomonas* and *Xanthomonas* (22). The mutations fall in clusters of genes covering at least 20 kb. *hrp* mutants of *X.campestris pv. campestris* were not affected in any enzyme or extracellular polysaccharide production and pathogenicity defect is not caused by ability to produce any of these factors. Starvation promotes *hrp* genes expression. Recent sequence data on *hrp* genes of *Pseudomonas solanacearum* and *Xanthomonas campestris pv. vesicatoria* indicate that some of the gene products are related to some pathogenicity determinants of animal pathogens particularly *Yersinia* (C. Boucher and U. Bonas *et al.* , Abstracts of the sixth international symposium on molecular plant-microbe interactions 1992).

2. **Extracellular enzymes** : *Xanthomonas campestris pv. campestris* produces a number of extracellular enzymes including protease, endoglucanase, polygalacturonate lyase, lipase

and amylase. Since these enzymes have the capacity to degrade plant cell compounds, they are the obvious candidates as pathogenicity factors.

Proteases : Work from several laboratories has suggested that extracellular proteases of phytopathogenic bacteria have a role in disease development. Protease deficient mutants of a number of pathovars of *X.campestris* have been shown to give less severe disease symptoms and lower bacterial numbers in their respective hosts (23,24,25). *X.campestris* produces 2 major proteases. PRT 1 is a serine protease and PRT 2 is a metalloprotease (26). Serine protease is an essential pathogenicity factor at early stages of the disease process, but once infection is well advanced and the bacteria have begun to break out from veins into mesophyll tissue, this enzyme is less significant.

Endoglucanase : It is a major extracellular protein. Mutants lacking endoglucanase however show no reduction in virulence (27). The role of the enzyme is not understood but one possibility is that it contributes to bacterial nutrition during the saprophytic phase of the life cycle.

Pectic lyases : 3 types of lyases are produced, however there is no effect on virulence (28).

3. **Extracellular polysaccharide** : *X.campestris pv. campestris* produces acidic polysaccharide in large amounts popular as xanthan gum. EPS^- mutants have been shown to have reduced virulence (29). However, in some cases, certain strains retained partial or full virulence. The polysaccharide pre-

vents dessication and thereby helps the microorganism in survival in the vicinity of plant and it also helps in rapid spread and multiplication of the bacteria.

4. **Bacterial pilus** : Pilus is a structure that is able to bridge the distance between a bacterial cell and a solid surface such as a plant leaf. These filaments are in many cases hydrophobic, suggesting a rather nonspecific hydrophobic interaction between the fimbriae and plant surface. On the other hand, specific interactions are also known in several cases (30-32). *Xanthomonas campestris pv. campestris* can be found growing epiphytically without causing visible symptoms (33). In suitable conditions, bacteria invade the plant tissue through stomata, hydathodes as well as wounds. van Doorn *et al.* (34) have isolated pili from strains of *Xanthomonas campestris pv. hyacinthi* and *Xanthomonas campestris pv. vesicatoria*. Expression of pili is constitutive and these long flexible pili form bundles. It has been shown that the pili show homology with type 4 pili expressed by *Pseudomonas aeruginosa*. It has been suggested that these pili have a significant role in plant surface attachment and infection, although it has not been confirmed.

A lectin from *Xanthomonas campestris pv. campestris* ATCC 29497 was isolated in this study which was strongly associated with the extracellular polysaccharide produced by the organism. The lectin could be a pilus protein which may have been shed in the medium during growth or it could have been produced extracellularly. The lectin was host plant

tissue specific as its haemagglutination activity could be inhibited by *Brassica* plant tissue extract but not by rice plant tissue extract. This indicates a probable role for lectin - carbohydrate interaction in adherence of *Xanthomonas campestris* to its host.

Although much more work in this area is needed to say anything conclusively, it can be said that lectin carbohydrate interactions could be one of the possible ways for bacterial attachment to plant surfaces where lectin resides on the bacteria and plants present the carbohydrate receptors. More work in this area can put some light on exact mechanisms of pathogenicity of plant pathogenic bacteria and their strategies for infection. Such studies would be very helpful in designing preventive measures against these phytopathogens.

REFERENCES

1. D. C. Old (1972) J. Gen. Microbiol.71 : 149 - 157.
2. L. H. Arp (1988) in " Virulence mechanisms of bacterial pathogens" (J. A. Roth Ed.) pp. 327.
3. S. Clegg and G. F. Gerlach (1987) J. Bacteriol. **169** : 934 - 938.
4. B. J. J. Lugtenberg, C. Diaz, G. Smit, S. de Pater and J. W. Kijne (1991) Adv. Mol. Genet. Plant-microbe interact. 1 : 174 - 181.
5. S. C. Ho, J. L. Wang and M. Schindler (1990) J. Cell Biol. **111** : 1631 - 1638.
6. J. T. Loh, S. C. Ho, A. W. de Feijter, J. L. Wang and M. Schindler (1993) Proc. Natl. Acad. Sci. USA **90** : 3033 - 3037.
7. B. B. Lippincott and J. A. Lippincott (1969) J. Bacteriol. 97 : 620 - 628.
8. L. Sequeira (1985) J. Cell Sci. Suppl. 2 : 301 -316.
9. A. G. Matthysse, K. V. Holmes and R. H. G. Gurlitz (1981) J. Bacteriol. **145** : 583 - 595.
10. A. G. Matthysse (1983) J. Bacteriol. **154** : 906 - 915.
11. G. A. Cangelosi, G. Martinetti, J. A. Leigh, C. Chang Lee, C. Theines and E. W. Nester (1989) J. Bacteriol. **171** : 1609 - 1615.
12. C. Depierreux, H. C. Kang, B. Guerin, M. Monsigny and F. Delmotte (1991) Glycobiol. 1 : 643 - 649.
13. V. Puvanesarajah, F. M. Schell, G. Stacey, C. J. Douglas and E. W. Nester (1985) J. Bacteriol. **164** : 102 - 106.

14. M. H. Whatley, J. S. Bodwin, B. B. Lippincott and J. A. Lippincott (1976) Infect. Immun. **13** : 1080 - 1083.

15. S. C. Winans (1992) Microbiol. Rev. **56** : 12 - 31.

16. A. M. Ashby, M. D. Watson, G. J. Loake and C. H. Shaw (1988) J. Bacteriol. **170** : 4181 - 4187.

17. A. V. Broek and J. Vanderlayden (1995) Molecular Plant Microbe Interact. **8** : 800 - 810.

18. K. J. Fullner, J. C. Lara and E. W. Nester (1996) Science 273 : 1107 - 1109.

19. M. M. Lopez, M.T. Gorris, C. I. Salcedo, A. M. Montojo and M. Miro (1989) Appl. Environ. Microbiol. **55** : 741 - 746.

20. B. Vicedo, R. Penalver, M. J. Asins and M. M. Lopez (1993) Appl. Environ. Microbiol. **59** : 309 - 315.

21. M. J. Daniels, C. E. Barber, J. M. Dow, B. Han, S. A. Liddle, M. A. Newman, J. E. Parker, S. D. Soby and T. G. J. Wilson (1993) in " Mechanisms of Plant Defense Responses" (B. Fritiig and M. Legrand eds.) Kluwer Academic Publishers, Netherlands. pp. 53 - 63.

22. D. K. Willis, J. J. Rich and E. M. Hrabak (1991) Molecular Plant Microbe Interact. **4** : 132 - 135.

23. J. L. Tang, C. L. Gough, C. E. Barber, J. M. Dow and M. J. Daniels (1987) Molecular and General Genetics **210** : 443 - 448.

24. R. K. Gholson, C. Rodgers and M. Pierce (1989) Phytopathology **79** : 1210.

25. G. W. Xu and C. F. Gonzalez (1989) Phytopathology **79** : 1199.

26. J. M. Dow, B. R. Clarke, D. E. Milligan, J. L. Tang, M. J. Daniels (1990) Appl. Environ. Microbiol. 56 : 2994 - 2998.

27. C. L. Gough, J. M. Dow, C. E. Barber and M. J. Daniels (1988) Molecular Plant Microbe Interact. 1 : 275 - 281.

28. A. K. Chatterjee, H. Murata and J. L. McEvoy (1991) in " Biochemistry and Molecular Biology of Plant Pathogen interactions" (C. J. Smith ed.) Clarendon Press, Oxford. pp 177 - 196.

30. D. L. Coplin and D. Cook (1990) Molecular Plant Microbe Interact. 3 : 271 - 279.

31. T. K. Korhonen, N. Kalkinnen, K. Haahtela and D. C. Old (1987) J. Bacteriol. 169 : 2281 - 2283.

32. T. K. Korhonen, K. Haahtela, A. Pirkola and J. Parkkinen (1988) FEBS Lett. 236 : 163 - 166.

33. K. Haahtela, M. Kukkonen, R. Rönkkö, E. L. Nurmiaho-Lassila, R. Karjalainen and T. K. Korhonen (1989) in Z. Clement (ed.) Proc. 7th Int. Conf. Plant Path. Bact. Part B Budapest, Hungary. pp 767 - 772.

34. M. Romantschuk, E. Roine, T. Ojanen, J. vanDoorn, J. Louhelainen, E. L. Nurmiaho-Lassila and K. Haahtela (1994) in" Molecular Mechanisms of Bacterial Virulence" (C. I. Kado and J. H. Crosa eds.) Kluwer Academic Publishers, Netherlands. pp 67 - 77.

35. F. B. Dazzo (1981) J. Supermol. Structure Cell. Biochem. 16 : 29 - 41.

36. F. B. Dazzo and G. Truchet (1983) Membrane Biol.73:1 -16

37. T. K. Korhonnen, K. Haahtela, A. Pirkola and J. Parkkinen (1988) FEBS Lett. **236** : 163 - 166.

38. M. Romantschuk (1992) Annu. Rev. Phytopathol. **30** : 225 - 243.

List of publications

1. B. Joshi, J. M. Khire, H. SivaRaman and M. I. Khan (1996) Purification and chracterization of a lectin from *Xanthomonas campestris* NCIM 5028. Can. J. Microbiol. 42 : 609 - 612.

2. B. Joshi, J. M. Khire, H. SivaRaman and M. I. Khan (1996) Purification and characterization of extracellular lectin (Lectin I) from *Agrobacterium radiobacter* NCIM 2443. Biochim. Biophys. Acta (Communicated).

3. B. Joshi, J. M. Khire, H. SivaRaman and M. I. Khan Purification and characterization of cell bound lectin (Lectin II) from *Agrobacterium radiobacter* NCIM 2443. (Manuscript in preparation).