CHARACTERIZATION OF RHIZOBIAL SURFACE COMPONENTS INVOLVED IN *Rhizobium-Cicer arietinum* L. SYMBIOSIS

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CERTIFICATE

This is to certify that the thesis entitled " **CHARACTERIZATION OF RHIZOBIAL SURFACE COMPONENTS INVOLVED IN *Rhizobium-Cicer arietinum* L. SYMBIOSIS**" submitted by **Mr. RAGHAVENDRA JOSHI** in partial fulfillment of the requirement for the degree of **MASTER OF SCIENCE** in **Agricultural Biochemistry** of the University of Agricultural Sciences, Bangalore, is a record of bona fide research work carried out by him during the period of his study in this University under my guidance and supervision and that no part of the thesis has been submitted for the award of any other degree, diploma, associateship, fellowship or any other similar titles.

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ABBREVIATIONS

ANSA	-	1,2,4-amino napthol sulphonic acid
BSA	-	Bovine serum albumin
CPS	-	Capsular polysaccharides
CTAB	-	Cetyl trimethyl ammonium bromide
DEAE	-	Diethyl amino ethyl
EDTA	-	Ethylene diamine tetra acetic acid
EPS	-	Exopolysaccharides
Glu	-	Glucose
GluA	-	Glucuronic acid
GMD	-	GDP-mannose dehydrogenase
GMP	-	GDP-mannose pyrophosphorylase
KDO	-	2-keto-3-deoxyoctulosonic acid
LPS	-	Lipopolysaccharides
Nod	-	Nodulation
OHBP	-	Ortho hydroxy bi phenyl
PBS	-	Phosphate buffer saline
PMI	-	Phosphomannose isomerase
PMM	-	Phosphomannose mutase
SDS-PAGE	-	Sodium dodecyl sulfate- polyacrylamide gel electrophoresis
TBA	-	2-thiobarbutric acid
TC	-	Total carbohydrates
TEA	-	Triethyl amine
TEMED	-	N,N,N',N'-tetramethylethylenediamine
Tris	-	2- amino 2 (hydroxy methyl) propane-1,3-diol
YEM	-	Yeast extract mannitol
YEMA	-	Yeast extract mannitol agar

INTRODUCTION

I INTRODUCTION

One of the important plant - microbe interactions that has been studied over the years with great interest is the symbiotic interaction between the bacteria of the family Rhizobiaceae, collectively called as rhizobia with the plants of the family Fabaceae, called legumes. In this symbiotic interaction rhizobia form nodules on the legume roots and these nodules fix atmospheric nitrogen into ammonia which is readily available to the host plant for its development.

The *Rhizobium*-legume symbiosis is the result of specific recognition of the host legume by *Rhizobium*. The specificity is conferred by various signal molecules that are produced by both *Rhizobium* and legume namely

1] flavonoids and lectins of the host plant (Innes *et al.*,1985; Philips,1991; Bauer, 1981).

2] bacterial cell surface polysaccharides and nod factors (Denarie *et al.*,1992; Long, 1996).

The mechanism of action of these signal molecules is yet to be fully understood.

As early as 1976, Wolpert and Albersheim observed that lipopolysaccharides from specific rhizobial cells bound to the immobilized host-lectin- agarose column. This was demonstrated in following systems viz *Rhizobium japonicum*-soybean, *Rhizobium leguminosarum*-pea and *Rhizobium phaseoli*-bean.

This observation has led to the confirmation of the earlier hypothesis proposed by Albersheim and Anderson-Prouty(1975), which was based on their work on elicitors of plant defense responses and *Rhizobium*-legume symbiosis. This hypothesis suggested that macromolecules of the pathogen are involved in recognition of specific host; the cell surface recognition being mediated through carbohydrate containing macromolecules and proteins. Furthermore, they proposed that host plant lectins interact selectively with cell surface polysaccharides of the compatible rhizobium.

Similar conclusions were drawn by Maiti and Podder(1989) for *Bradyrhizobium arachis*- groundnut system. It was shown that specific LPS bound the groundnut agglutinin more strongly than the specific exopolysaccharides(EPS) or capsular polysaccharides(CPS) and non specific LPS.

Using DNA recombinant technology, Diaz *et al.*(1989) introduced pea lectin gene into the roots of white clover. The transgenic roots were found to be nodulated by the pea specific *Rhizobium leguminosarum* , thus demonstrating lectin as one of the key molecules involved in host plant specificity in *Rhizobium* - legume symbiosis.

Such studies are mainly limited to the following systems, *Rhizobium japonicum*-soybean, *Rhizobium leguminosarum*-pea *and Rhizobium-meliloti*- clover (Pieternel and Vanderleyden, 1995; Long, 1989; Halverson and Stacey, 1986). In

view of the importance of chickpea for tropical countries, similar studies were initiated in our department.

Chickpea is one of the most important legume crops of semi arid tropics. In Karnataka, it occupies 26% of the total land area under pulses and accounts for 35% of the total production of pulses (Anon,1995). The seeds contain about 18% protein and 60% carbohydrate.(Manay and Shadaksharaswamy, 1997). The crop residue is used as animal fodder.

Earlier a 44kD lectin from chickpea seed has been purified in our department. Chickpea specific rhizobial strain,TAL1148 was shown to be agglutinated by this lectin whereas the non specific strains,TAL1000 and P132 were not agglutinated. In addition the agglutination of TAL1148 was found to be culture age dependent; the maximum agglutination occurring at the exponential phase of bacterial growth. (Shivyogi, 1996).

LPS from specific and non specific strains of chickpea have been isolated and characterized. The total carbohydrate to KDO ratio of LPS of specific strain was found to be nearly half of that of the non specific strains. The banding pattern of LPS of specific and nonspecific strains on polyacrylamide gel was found to be different (Jagadeesh, 1997).

In the present study the characterization of EPS has been undertaken with a view to understand their involvement in *Rhizobium*-chickpea symbiotic interactions. For this purpose the following was done and is described in the thesis.

1] Isolation and purification of EPS from specific and nonspecific rhizobial strains of chickpea.

2] Chemical characterization of the EPS.

3] Inhibition of chickpea seed lectin dependent agglutination of rhizobial cells by EPS and LPS.

REVIEW OF LITERATURE

II REVIEW OF LITERATURE.

Rhizobium-legume interaction is one of the complex interactions that occurs in nature. This interaction requires the complete co-ordination between the multitude of signal molecules that emanate from both the *Rhizobium* and legume plants.

Flavonoids and lectins of the host plant (Innes *et al.*,1985; Philips, 1991; and Bauer, 1981), bacterial cell surface polysaccharides and nod factors(Denarie *et al.*, 1992; Long, 1996) are some of the speculated signal molecules. Surface polysaccharides; lipopolysaccharides, exopolysaccharides, capsular polysaccharides have been shown to be important in the initial stages of symbiosis and the defect in any one of the polysaccharide molecule leads to "empty" or unfunctional nodules on the legume roots (Priefer,1989; Yang *et al.*, 1992).

2.1 Process of nodulation

The process of nodulation or nodule development is multistep and can be divided into the following stages a) preinfection stage, b) infection stage and c) nodule development stage.

2.11 Preinfection stage

The preinfection stage begins even before the host plant and specific rhizobial strain recognize each other as potential symbiotic partners on a cellular basis. Legume

seed coat contains large quantities of diverse array of flavonoids. Flavonoids released by the plants serve as chemoattractants and induce *nod* genes. In free living rhizobia, in the absence of flavonoids, *nod* genes are either not expressed or expressed at very low levels(Long, 1989 and Hirsch, 1992).

The newly emerged root hairs are the most susceptible for the initial attachment of rhizobia (Bhuvaneshwari *et al.,* 1980; Hirsch,1992). The bacterial attachment to root hairs takes place in two steps.First, the rhizobia attach loosely to a plant receptor via rhicadesin, a calcium binding protein that is common among Rhizobiaceae (Smit *et al.,* 1987). Then, tighter adherence occurs by cellulose fibrils or fimbriae (Smit *et al.,* 1987; Vesper and Bauer, 1986).

2.1.2 Infection stage

In the next step, root hairs curl into a characteristic manner called Shepherd's crook. Expression of *nod* genes by rhizobium is essential for the root hair curling. In the following step, the bacteria invade the plant via root hair cells and lead to the formation of infection thread, a tubular structure. The infection thread, penetrating several layers of root tissue is filled with bacteria. The nodule primordium is formed from the cells of root cortex. The infection thread penetrates into the root cortical cells and bacteria are released and get surrounded by peribacteroid membrane. After release, bacteria divide and form bacteroid. Cells infected and uninfected by rhizobia are present in nitrogen fixing root nodules(Vincent, 1980).

2.1.3 Nodule development stage

Two main types of nodules formed on the legume roots are (i) determinate nodules and (ii) indeterminate nodules.

Determinate nodules are commonly developed by tropical legumes such as soybean, frenchbean. Indeterminate nodules are formed on temperate crops like alfalfa, clover, pea and chickpea.

In determinate nodules, meristematic activity occurs only in the beginning of nodule development. Increase in cell size or expansion takes place resulting in the increase in nodule size, after the initial mitiotic activity is over. The nodules are globular in shape. Assimilated nitrogen is in the form of ureides (Pate and Atkins, 1983).

The indeterminate nodules have a persistent meristem resulting in separation of various developmental phases in nodule development within a nodule. The fixed nitrogen in indeterminate nodules is transported as amide. Indeterminate nodules are cylindrical in shape (Pate and Atkins, 1983).

2.2 Genes involved in nodulation

The description of different events in nodule formation at the molecular level has been greatly facilitated by the use of techniques of molecular biology and recombinant DNA technology and transposon mutagenesis. The molecular biology methodologies have identified rhizobial nodulation and nitrogen fixation genes.

The rhizobial genes required for infection and nodule formation can be grouped into two classes (i) nodulation genes (*nod* genes). In most rhizobial species, these *nod* genes reside on a large symbiotic plasmid, whereas in *Rhizobium loti* , *Bradyrhizobium* and *Azorhizobium* species, the symbiosis related genes are located on chromosomes (Pieternel and Vanderleyden, 1995). Some of the *nod* genes (*nod A,B,C*) are interchangeable for nodulation function between different species and biovars, without altering the host range. Therefore, these have been designated as *common nod* genes. In most species *common nod A,B,C* genes form a single operon (Pieternel and Vanderleyden,1995). Mutations in *nod* genes lead to inability of rhizobia to elicit any symbiotic activity in plants such as root hair curling, formation of infection thread, cortical cell division (Kondorosi *et al.*, 1991 and Long, 1992). Some nod genes are involved in the nodulation of a particular host and are therefore called as *host specific nod (hsn)* genes. Host specific nod *(hsn)* genes are not functionally or structurally conserved among rhizobia. Introduction of corresponding genes from other rhizobia

cannot over come the mutations in *hsn* genes.Most often, mutations result in alterations or extension of host range(Pieternel and Vanderleyden, 1995).

The second class of genes include several sets of genes involved in the formation of bacterial cell surface. These comprise genes for determining the synthesis of lipopolysaccharides (*lps* genes), capsular polysaccharides (*cps* genes), β 1-2 glucans (*ndv* genes) and exopolysaccharides (*exo* genes)(Pieternel and Vanderleyden, 1995). These genes are essential for the successful initiation of bacterial infection (Long,1989).

Most rhizobial genes are not expressed in cultured cells but induced in the presence of plant secreted signals; flavonoids. The transcriptional activator protein nod D is also needed for the expression of rhizobial genes. (Fischer and Long, 1992).

Rhizobial LPS defects are pronounced on hosts that form determinate (round) nodules. Selective nodules devoid of bacteria have been observed in *lps* mutants of *Rhizobium leguminosarum* b.v. *phaseoli*(Noel *et al.*, 1986; Diebold and Noel, 1989). Cyclic β 1-2 glucans are also necessary for normal nodule fomation. Rhizobial strains with mutations on *ndv* genes can form nodules, but these nodules are developmentally defective and are unable to fix nitrogen (Dylan *et al.*, 1986). It has been observed that EPS are usually important for legumes that form indeterminate nodules(Chen *et al.*, 1985).

2.3 Occurrence of EPS

EPS can be defined as polysaccharides found external to the structural outer surface of the microbial cell and the term can be applied to carbohydrate polymers of diverse composition and of different physical types(Sutherland, 1990). They are usually in the megadalton range molecular weight and are secreted by a wide variety of microorganisms. For example diatoms(Lind *et al.*, 1997), bacteria in oil rings(Hino *et al.*, 1997), human pathogens *Burkholderia pseudomallei, Pseudomonas aeruginosa* (Nimtz *et al.*, 1997, Eagon, 1956), plant pathogens , *Erwina amylovora, Xanthomonas campestris* (Goodman *et al.*, 1974; Jannson *et al.*, 1975), methanogenic bacteria *Methanobacterium formicium* and *Methanosarcina mazeii*(Veiga *et al.*, 1997), fungi like *Sclerotium rolfsii* and wood rotting basidiomycete *Schiphyllum commune*(Sutherland, 1990).Nitrogen fixing symbiotic bacteria of plants, rhizobia also secrete EPS.In some microorganisms like *Escherechia coli* the EPS may form a capsule around the bacterial cells and in that case it is called as capsular polysaccharide.

2.4 Isolation of EPS

Often used methods that are followed for the isolation of EPS from the rhizobial culture supernatant are described below.The first method was proposed by Robertson *et al.*,(1981).In this method the EPS are precipitated by a known amount of CTAB from the culture fluid after the cells are removed by centrifugation. The CTAB precipitated EPS are dissolved in sodium chloride solution and dialyzed against distilled water. The dialyzate is lyoplilized to get EPS powder.

Another method followed is the precipitation of EPS by ethanol. The rhizobial culture supernatant is usually concentrated to lower volumes (about 1/5th the orginal volume) and 3 volumes of cold ethanol are added. The precipitated EPS collected by centrifugation (Zevenhuizen, 1981; Corzo *et al.*, 1994).

2.5 Purification of EPS

EPS can be purified by either ionexchange chromatography or gel filtration chromatography. An anion exchanger such as Sephadex A-25 is used. The negatively charged EPS binds to the exchanger and can be eluted by a linear gradient of sodium chloride or potassium chloride(Robertson *et al.*, 1981; Kim *et al.*, 1989).

Exclusion chromatography can also be used to purify EPS. The main contaminants of CTAB precipitated EPS are sodium chloride and CTAB which are LMW components.

2.6 Detection and Assay

The EPS can be detected by their carbohydrate content by phenol-sulfuric acid method of Dubois *et al.*, (1956). The glucuronic acid can be estimated by the O-hydroxy-bi-phenol method of Blumenkrantz and Asboe-Hansen,(1973).

2.7 Chemical nature of EPS

EPS can either be homopolysaccharides or heteropolysaccharides. Cellulose is one of the important homopolysaccharides produced by plant associated bacteria such as *Agrobacterium* and *Rhizobium* (Matthysse, 1983; Smit, 1987) whereas levan is produced by *Erwinia* and *Pseudomonas* (Bennet and Billing, 1980; Fett *et al.*, 1986).

Heteropolysaccharides are polymers composed of repeating units containing neutral sugars and uronic acid and a variety of non carbohydrate substituents such as

acetate, pyruvate, hydroxybutryrate and succinate (Sutherland, 1988; Whitefield, 1988). The list of noncarbohydrate substituents in EPS of different microorganisms are listed in Table 2.1. The carbohydrate constituents of EPS of different microorganisms are listed in Table 2.2.

Studies on various *exo* mutants of *Rhizobium* that produce defective EPS have revealed that several species of rhizobia produce EPS of a given repeat unit structure in two major molecular weight ranges, a high molecular weight polymer (HMW) and a low molecular weight oligomer(LMW) both of which are released in the culture supernatant(Amemura *et al.*, 1983, Djordjevic *et al.*, 1986, Leigh and Lee,1988). Battisti *et al.*(1992) have fractionated a LMW succinoglycan of *Rhizobium meliloti* into repeat unit monomers, trimers and tetramers. It was also detected that each class has a varying degree of anionic character.

Rhizobium sp strain NGR234 produces an EPS which is composed of glucose, galactose, glucuronic acid in the ratio 5:2:2 with pyruvate and acetyl groups on the terminal galactose linked to the glucuronic acid residues. [Figure2.1(a)]. *Rhizobium meliloti* strain SU47 produces an EPS, a succinoglycan, known as EPSI. It contains acetyl and succinyl groups at undetermined sites[Figure 2.1(b)](Aman *et al.*, 1981). In addition it has been observed that *Rhizobium meliloti* strain SU47 can also produce a different EPS called EPSII or EPS b that contains glucose and galactose in the ratio 1:1 and acetyl and pyruvate are attached to galactose and glucose[Figure2.1(c)] (Her *et al.*,1990 and Levery *et al.*,1991). The EPS of three biovars of *Rhizobium*

Table 2.1 Carbohydrate substituents of different EPS

Microorganism	Name of the EPS	EPS sugar composition						Reference
		Glu	Gal	Glu A	Man	Xyl		
Pseudomonas fluoresence	1. Marginalin	+	+	-	-	-		Fett *et al.*, (1989)
	2. Another novel EPS	+	-	-	+	-	Rhm	Fett *et al.*, (1989)
	3. Levan	-	-	-	-	-	Fru	Fett *et al.*, (1989)
Erwinia amylovora	Amylovoran	-	+	+	-	-		Smith *et al.*, (1990)
Acetobacter	-	+	+	-	+	-		MacCormic *et al.*, (1996)
Burkholderia pseudomallei	-	-	+	-	-	-	KDO	Nimtz *et al.*, (1997)
Pseudomonads of oil rigs	-	+	+	-	+	+	NAGlu NAGal Rbl	Hino *et al.*, (1997)
Xanthomonas campestris.	Xanthan gum	+	-	+	+	-		Jannson *et al.*, (1975)
Rhizobium sp NGR234.	-	+	+	+	-	-		Leigh and Coplin, (1992)
Rhizobium meliloti SU 47	1. EPS I	+	+	-	-	-		
	2. EPS II	+	+	-	-	-		Aman *et al.*, (1981) Her *et al.*, (1990)
Rhizobium leguminosarum	-	+	+	+	-	-		Canter-Cremers *et al.*, (1991)

Abbreviations: Glu: Glucose, Gal: Galactose, GluA: Glucuronic acid, Man: Mannose, Xyl: Xylose, Rbl: Ribulose, Rhm: Rhamnose, Fru: Fructose, NAGlu: N-Acetyl-Glucosamine. NAGal: N-Acetyl-Galactosamine, KDO:2-keto-3-deoxy-octulosonic acid

Table 2.2 Non carbohydrate substituents of EPS

Substituent	Linkage	EPS producing bacteria.
ORGANIC ACIDS		
Acetate	Ester	Very common in all EPS
Pyruvate	Ketal	Very common in all EPS
Glycerate	Ester	*Pseudomonas elodea*
Propionate	Ester	*Escherichia coli*
Hydroxybutanoate	Ester	*Rhizobium trifolii* *Rhizobium leguminosarum*
Succinate	Ester	*Rhizobium spp* *Agrobacterium spp*
AMINO ACIDS		
L-glutamate		*Klebsiella aerogenes K82*
Serine		*Escherichia coli K40*
INORGANIC ACIDS		
Phosphate		Common to cyanobacteria.
Sulfate		

Ref : Sutherland 1990

leguminosarum i.e *viciae, trifolii and phaseoli* have the same basic structure. [Figure 2.1(d)]. Additional O-acetyl and 3 hydroxybutanoyl substituents were also found. (Canter-Cremers *et al.*, 1991).The compositional analysis of EPS of *Rhizobium fredii* USDA191 have revealed that it contains glucose, galactose, mannose, glucuronic acid in an approximate ratio of 1:1:1:1 and were also found to be pyruvated.(Ko and Gayda, 1990).

2.8 Biosynthesis of EPS

The biosynthesis of homopolymeric EPS by plant pathogens such as *Pseudomonas* and *Erwinia amylovora* is much simpler than the biosynthesis of heteropolysaccharides. Levan and alginate are synthesized by *Erwinia* and *Pseudomonas*, respectively. Levan is synthesized from sucrose (Gross *et al.*, 1989).

Deretic *et al.*(1991) have described the alginate biosynthesis in *Pseudomonas aeruginosa.* Alginate is first synthesized as a homopolymer of mannnuronic acid and variably acetylated and epimerized. Some enzymes that are involved are phosphomannose isomerase (PMI), phosphomannomutase(PMM) and GDP- mannose pyrophosphorylase (GMP).GDP-mannose dehydrogenase(GMD) is a specific enzyme to this pathway which catalyzes the oxidation of GDP -mannose to GDP- mannuronic acid . The mechanisms of polymerization, acetylation and transport of alginate to the cell surface are yet to be known.

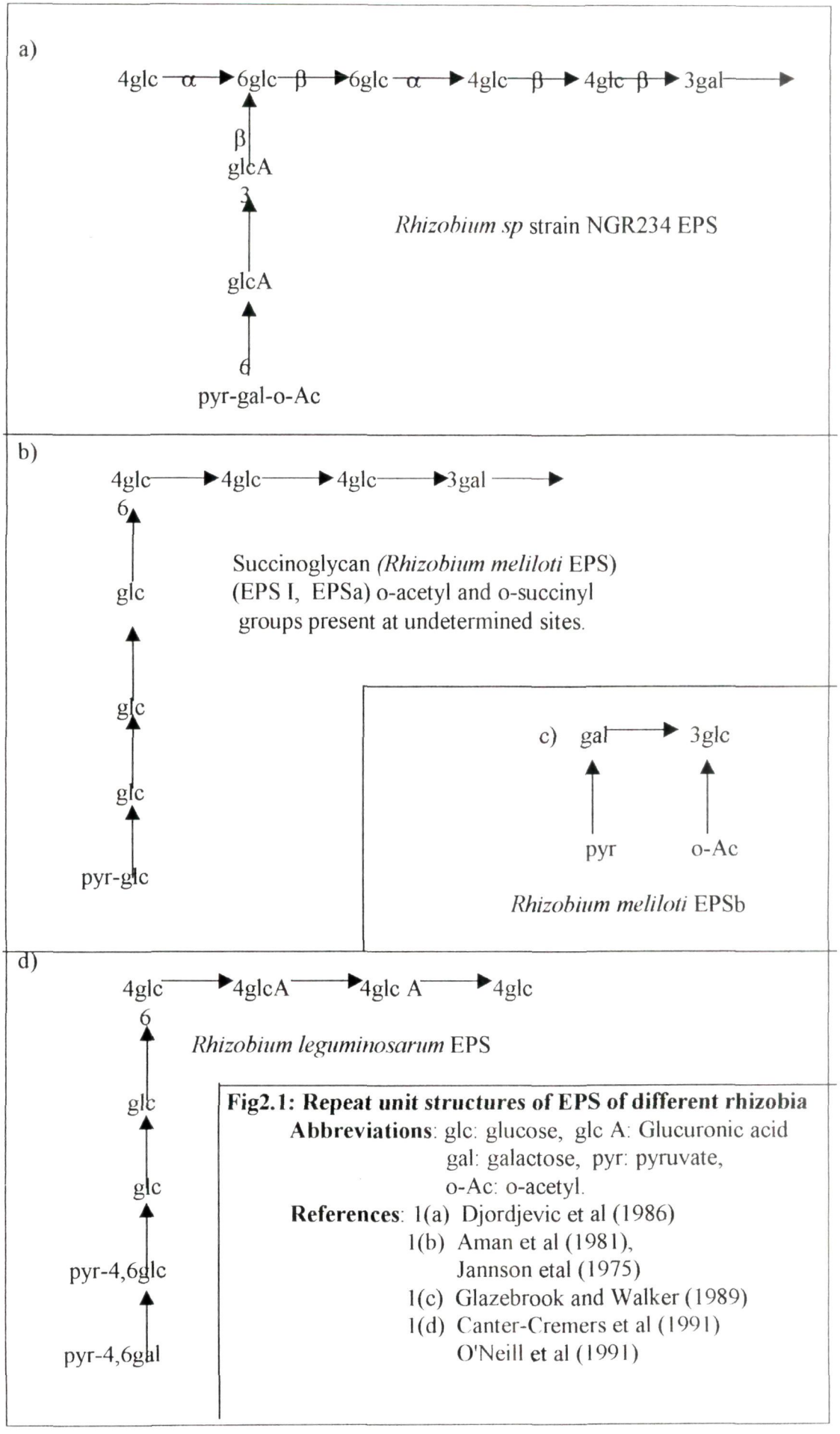
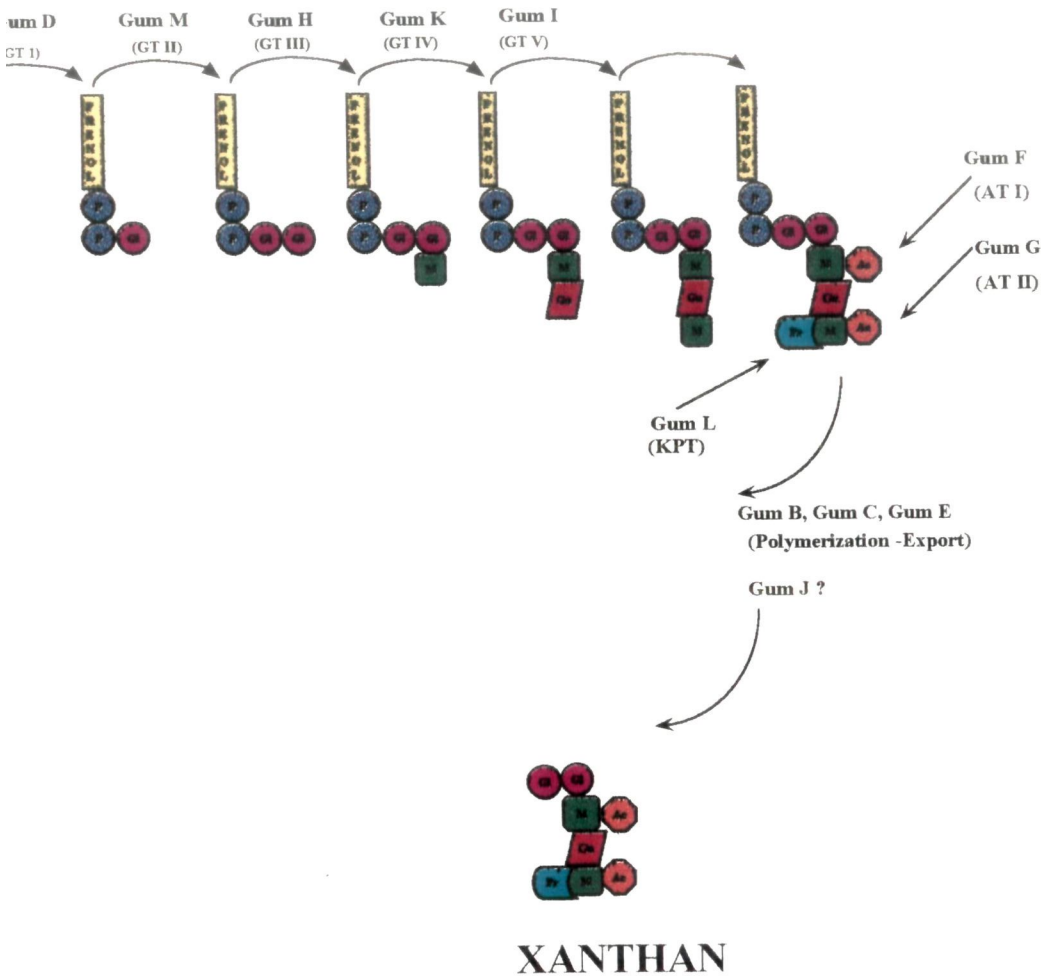


Fig2.1: Repeat unit structures of EPS of different rhizobia

Abbreviations: glc: glucose, glc A: Glucuronic acid
gal: galactose, pyr: pyruvate,
o-Ac: o-acetyl.

References: 1(a) Djordjevic et al (1986)
1(b) Aman et al (1981),
Jannson etal (1975)
1(c) Glazebrook and Walker (1989)
1(d) Canter-Cremers et al (1991)
O'Neill et al (1991)



XANTHAN

Biosynthesis of Xanthan gum(EPS) by *Xanthomonas campestris* as proposed by Katzen *et al*(1998)

ABBREVIATIONS:

P- phosphate, Gl-glucose,M-mannose ,Gu-glucuronic acid, Ac-acetyl group, Pr-pyruyl group
GT-glucosyl transferase, AT-acetyltransferase , KPT-ketal pyruvate transferase

The heteropolysaccharide synthesis is relatively complex. In the first step nucleotide diphosphate intermediates are synthesized. In the second step monosaccharide residues from the corresponding nucleotide are transferred to the carrier lipid(C-55 undecaprenyl phosphate) located on the cell membrane to form oligosaccharide repeating units. At this stage, pyruvate, acetate are selectively added. The transfer of the growing polysaccharide chain from its carrier lipid to the new subunit is the last step in the biosynthesis of the polymer. The EPS is ready to be secreted out. These steps were deduced based on the studies on *Klebsiella aerogenes* by Troy, (1979), on *Rhizobium meliloti* by Tolmasky *et al.*, (1982) and on *Xanthomonas campestris* by Ielpi *et al.*,(1981a 1981b, 1983, 1993).

The biosynthetic pathway of EPS of *Xanthomonas campestris* which is called xanthan gum has been characterized by Ielpi *et al.*,(1981). It involves the action of glycosyltransferases I, II, III, IV, V which add glucose, mannose, glucuronic acid and mannose, respectively to an isoprenoid lipid carrier. Pyruvate and acetate are added to the oligosaccharide by ketolase and acetylase respectively and then the oligosaccharide chain is lengthened. The carrier lipid is recycled by a dephosphorylation step and the polymer is released into the milieu as free slime. Figure 2 depicts the biosynthetic pathway of xanthan gum.

2.9 Genetics of EPS biosynthesis

In *Rhizobium meliloti* two megaplasmids, pRmeSu47a and pRmeSu47b which help in EPS biosynthesis have been reported(Finan *et al.*, 1986; Hynes *et al.*, 1986) .The *exo* gene cluster required for the biosynthesis of succinoglycan (EPS I) has been traced to the megaplasmid pRmeSu47a (Long *et al.*, 1988; Reuber *et al.*, 1991).Megaplasmid pRme Su47b contains gene cluster called as *exp* or *muc* for the biosynthesis of EPS b(EPS II)(Glazebrook and Walker, 1989; Zhan *et al.*, 1989).

The *exo* gene cluster for succinoglycan synthesis consists of at least 12 genetic complementation groups (likely transcription units) within a 22 kb region. Several genes outside the *exo* cluster were also found to be important for succinoglycan synthesis.(Buendia *et al.*, 1991; Long *et al.*, 1988; Puhler *et al.*, 1991; Reuber *et al.*, 1991). It was found that the mutations in eight complementation groups of the *exo* cluster(*exo P,M,A,L,T,Y,Q* AND *B*) and one chromosomal locus(*exo C*) completely abolished succinoglycan production.

Buendia *et al.* (1991) and Canter-Cremers *et al.* (1990) have reported that the *exo B* gene encodes the enzyme UGE, whereas PGM is encoded by *exo C* (Uttaro *et al.*, 1990). Both these are important for precursor synthesis. *exo A* is speculated to code for a glycosyl transferase, that adds the first glucose residue following galactose to the succinoglycan repeat unit. The *exo* gene cluster may not act as transcriptional regulator.

Many *exo* genes of both *Rhizobium meliloti* SU47 and *Rhizobium* sp NGR234 have been found to function similarly These similarities in gene function may reflect similarities in EPS structure of *Rhizobium meliloti* SU47 and *Rhizobium* sp NGR234, or similar mechanisms of polymerization and secretion. This similarity in gene organization may reflect a common evolutionary origin of the two *exo* regions. (Zhan and Leigh, 1990).

Mutations in the *exo* gene cluster of *Rhizobium meliloti* SU47 lead to altered EPS synthesis. Mutations in *exo H* gene lead to the secretion of (succino)glycan which lacks the succinyl group. Also it was found that such a glycan had reduced low molecular weight fractions(Leigh *et al.*, 1987).The *exo D* and *Z* genes were found to be outside the *exo* gene cluster and are thought to affect EPS synthesis indirectly by altering the membrane properties(Reed *et al.*, 1991, Charles *et al.*, 1991).

2.10 Regulation of EPS biosynthesis

The latest model for the regulation of succinoglycan biosynthesis in *Rhizobium meliloti* has been put forward by Cheng and Walker(1998).

Exo S is a protein which has two domains, one in the plasma membrane and another in the cytoplasm. According to this model Exo S is activated due to an unknown environmental signal. Then a minor change occurs in the sensing domain

and activates the cytoplasmic domain which has a kinase activity. Chv I, a regulator protein is then phosphorylated and becomes active. This activated protein activates the transcription of the *exo* genes leading to the production of succinoglycan.

Regulatory mechanisms mediated by *exo X* and *exo Y* genes and their homologues have been discovered by Gray *et al.*(1990) in several species of rhizobia. Both these proteins were found to act in a counterbalancing manner. *exo X* was found to inhibit EPS synthesis whereas *exo Y* was found to stimulate EPS synthesis. Both genes were found to act post translationally. *exo Y* was found to be homologous to the *gum D* gene of *Xanthomonas campestris* which is required for the addition of the first sugar residue to the lipid carrier.(Reed *et al.*, 1991).Another mechanism for the action of Exo X and Exo Y has been proposed by Gray and Rolfe(1990) in which Exo X binds to Exo Y to form a complex in which the action of Exo Y is inhibited thereby preventing the initiation of EPS biosynthesis.The *exo* X and *exo* Y genes or their homologues are reported to be present in *Rhizobium* sp NGR234 (Gray *et al.*, 1990), *Rhizobium meliloti* (Reed *et al.*, 1991). *Rhizobium leguminosarum* (Borthakur *et al.*, 1986; Borthakur *et al.*, 1988; Latchford *et al.*, 1991), *Xanthomonas campestris* (Borthakur *et al.*, 1986; Reed *et al.*, 1991).

2.11 Functions of EPS

2.11.1 EPS in plant pathogens and microorganisms of different habitats

EPS play an important role in the growth and development of many micro-organisms. Genetic studies have indicated that EPS could be the virulence factor of many plant pathogens. Mutants of *Erwinia. stewartii* , *Erwinia amylovora, Xanthomonas campestris, Pseudomonas solanacearum* which are defective in EPS production have been found to have reduced virulence.(Coplin and Majerczak, 1990; Bellman and Geider, 1992; Ramirez *et al.*, 1988; Cook and Sequeria, 1991; Denny and Baek, 1991).

Erwinia stewartii is a bacterium that causes stewart's wilt and necrosis in corn . It inhabits xylem vessels of the host plant and the EPS produced by it blocks the xylem vessel and prevents water transport. This leads to wilting of the plants. It can also grow in intercellular spaces of corn leaves and produce water soaked lesions (Leigh and Coplin, 1992).The water and nutrients held by EPS are released from the damaged cells. This promotes water soaking symptoms and maintains favourable conditions for the bacteria to multiply. EPS mutants of *Erwinia stewartii* were found to form smaller lesions and turn necrotic soon after infection(Coplin and Majerczak, 1990).In vascular and canker diseases the hydrostatic pressure created by EPS in the blocked xylem vessel can cause them to rupture and release bacteria into the adjacent vessels or the surrounding stem cortex and pith and thereby aiding in the systemic infection (Leigh and Coplin, 1992).

Bradshaw-Rouse *et al.*, (1981) have studied the effect of a corn agglutinin on 22 strains of *Erwinia stewartii* with varied virulence. They observed that EPS mutants that failed to produce EPS or produced very little EPS were agglutinated by the agglutinin where as the wild strains (those producing EPS normally) were not agglutinated. This proved that corn agglutinin agglutinates EPS mutants and prevents their systemic movement, but wild strains are not affected and they can cause systemic infections. A similar observation made by Romerio *et al.*,(1981) showed that a protein agglutinin in apple trees binds to EPS mutants of *Erwinia amylovora* and retards passage of the bacteria in the trees and thus prevents its systemic movement and infection.

EPS have also been reported to suppress host defenses. Symptoms appeared sooner and the bacteria grew much faster when EPS was infiltrated along with the EPS mutant of *Xanthomonas campestris* p.v. *malavacearum* into the plants. When the experiment was repeated with incompatible races of *Xanthomonas campestris* p.v. *malavacearum*, the purified EPS prevented a hypersensitive response. This may be due to the masking of bacterial epitopes by EPS from plant host defenses(Rudolph *et al.*, 1989).

EPS also help micro-organisms of other habitats. Hino *et al.* (1997) have reported that certain *Pseudomonads* present in oil rigs produce an EPS that protects the bacteria from the toxic effect of crude oil. Gordan,(1987) has proposed that hydration of EPS helps in the gliding movement of the diatoms . This observation is

supported by Lind *et al.*(1997) who have shown that monoclonal antibodies specific to diatom EPS bind to it and inhibit the movement of the diatoms. This finding suggests that EPS are important for the mobility of diatoms.

2.11.2 EPS in *Rhizobium* -legume symbiosis:

A number of roles for EPS in nodulation have been speculated(Yang *et al.*,1992). EPS might (i) mask determinants on the rhizobial surface, thus protecting it from host defenses. (ii) be a part of complex signalling process that occurs between plants and bacteria. (iii) be involved in positive recognition enabling directional growth and penetration of infection thread.

Niehaus *et al.*(1993) have reported that EPS may be involved in the suppression of plant defenses. EPSa deficient mutants *Rhizobium meliloti* 0540 induced pseudonodules of spheroid, irregular shape. Bacteria were not present in these nodules. The cells of alfalfa in the pseudonodules had thickened cell walls containing callose and phenolic compounds. Phenylalanine ammonia lyase (PAL), an enzyme thought to be an indicator of onset of plant defense was found in higher quantaties (upto 10 times higher) in pseudonodules. Peroxidases involved in lignification of plant cell walls displayed higher activity. The wild type strains induced normal nodules and plant defense responses were also very low.

Structure specific role for EPS in *Rhizobium*-legume symbiosis could be in nodule formation. Leigh *et al.* (1985) have observed that *Rhizobium meliloti* mutants that were deficient in the production of EPS formed ineffective (nonnitrogen fixing) nodules on alfalfa. The ineffective nodules did not have bacteroids(intercellular bacteria), shepherd's crook and infection threads. Leigh *et al.* (1987) have reported about *Rhizobium meliloti* mutants that produced EPS (succinoglycan) without the succinyl group. Such mutants that formed nodules did not contain bacteria. The root hair curling was delayed and infection threads aborted in the nodule cortex. Similar results were observed with *Rhizobium meliloti exo* mutants deficient in EPS production. It resulted in the formation of abnormal nodules. There was a delay in root hair curling. Further the infection threads aborted in the peripheral cells and did not penetrate the cortex. Also the nodules formed on the secondary roots rather than on the primary roots as is in the normal case(Yang *et al.*, 1992).

It has been demonstrated that the symbiotic function of *exo* mutants of *Rhizobium leguminosarum* bv *trifolii* could be restored by high molecular weight EPS. In contrast, symbiotic could not be restored in case of *Rhizobium meliloti exo* mutants(Djordjevic *et al.*, 1986; Hirsch, 1992). Battisti *et al.* (1992) have found that only low molecular weight EPS from *Rhizobium meliloti* is effective. EPS from nonspecific species do not promote invasion; thereby suggesting that rhizobial EPS could be important for recognition of the host legume plant at molecular level.

An important result was obtained by the studies of Philip-Hollingsworth *et al.*,(1989). They transferred *hsn* genes that encode for the production of EPS from *Rhizobium trifolii* ANU843 to *Rhizobium leguminosarum* 300. The recombinant *Rhizobium leguminosarum* was then found to produce an EPS similar to that of *Rhizobium trifolii* and also it bound to the clover lectin, Trifolin, to which *Rhizobium trifolii* bound normally. The recombinant *Rhizobium leguminosarum* also caused infection and nodulation on clover roots. This experiment demonstrated that the interaction of the *Rhizobium trifolii* with a clover lectin with its EPS may contribute to host specificity in the white clover- *Rhizobium trifolii* symbiosis.

Binding of cell surface polysaccharides of rhizobium; LPS, EPS, has been well documented(Wolpert and Albersheim, 1976; Dombrink-Kurtzman *et al.*, 1983). Although most workers agree that lectins are involved in recognizing carbohydrates on rhizobial cell surface, thereby aiding in the recognition process, they are only a part of the story.

MATERIAL AND METHODS

III MATERIAL AND METHODS

The experiments to isolate and characterize surface components of *Rhizobium* were conducted in the Department of Biochemistry at the University of Agricultural Sciences, G.K.V.K., Bangalore. The material used and the methods followed are described below.

MATERIAL

Chickpea (*Cicer arietinum .L.*) variety Annigeri-1 seeds were obtained from ICRISAT, Hyderabad. Rhizobial strains TAL1148, TAL1000, P132, were obtained from the Department of Agricultural Microbiology, University of Agricultural Sciences, G.K.V.K., Bangalore. The 0.45 μm nylon membrane filters were obtained from Millipore Corporation, Bedford, U.S.A. The chemicals, cetyl trimethyl ammonium bromide, 2-thiobarbituric acid, β-mercaptoethanol, 2-keto-3-deoxy-octulosonic acid, ortho-hydroxy-biphenyl were obtained from Sigma Chemical Co, St Louis, U.S.A. TEMED was obtained from Biorad Laboratories Inc, California, U.S.A. DEAE-Sephadex A-50, Sephadex G-50, Sepharose- 4B were products of Pharmacia, Sweden. All other chemicals used were of the reagent grade. Absorbances were read using Elico colorimeter model SL171 and Shimadzu UV 1201 spectrophotometer.

METHODS

3.1 Confirmation of parental strains

Three rhizobial cultures obtained were streaked separately on YEMA(Yeast Extract Mannitol Agar) containing Congo Red and incubated for 7 days at 28^{0}C.Well isolated white colonies were streaked on YEMA slants with appropriate labels and stored at 4^{0}C. Composition of YEM broth is listed in Appendix-I.

The three rhizobial strains were subjected to Gram's staining to validate them as belonging to the *Rhizobium* species.

3.1.1 Gram's staining

Gram's staining for the three strains was carried out according to the procedure described by Somasegaran and Hoben(1985). Reagents for Gram's staining are listed in Appendix-II.

3.2 Isolation and purification of EPS

EPS from the three rhizobial strains were isolated as per the method of Robertson *et al.*, (1981) with some modifications. The rhizobial cells were grown in

sterilized YEM broth as 1.5 litre cultures with rotary shaking, harvested in the late exponential phase by centrifugation at 9000 rpm for 20 min at 4^0C. The supernatant was used for the isolation of EPS and the cells were used for the isolation of LPS.

The supernatant obtained after harvesting the cells was filtered through a 0.45μ m nylon membrane filter under vacuum to remove any residual bacteria that were trapped in the viscous supernatant. The culture filtrate was concentrated by evaporation under reduced pressure to $1/5^{th}$ of the original volume, dialyzed against distilled water(1 liter x 6 changes) for 24 hours at 4^0C. The EPS were precipitated by the addition of 4.0% CTAB to the dialyzed supernatant at room temperature with constant stirring till the supernatant became clear. The precipitated EPS were dissolved in 100ml of 10% sodium chloride solution, reprecipitated by adding 2 volumes of acetone. This precipitate was dissolved in 100ml of 10% sodium chloride solution and dialyzed extensively against distilled water for 2 days (1 liter x 12 changes) at 4^0C. The salt free solution was then lyophilized. The total carbohydrate and glucuronic acid contents of each EPS sample were estimated as described in 3.3.1 and 3.3.2, respectively.

3.2.3. Purification of EPS by gel filtration on Sephadex G-50 column.

Ten mg of the EPS sample was dissolved in 1ml of 10mM Tris-HCl buffer (pH 7.2) and applied to a Sephadex G-50 column (30cm x 1.25cm) that had been preequilibriated with the same buffer. Samples were also eluted with the same buffer. A flow rate of 60ml per hour was maintained and 3ml fractions were collected. The

eluted fractions were assayed for total carbohydrates by the phenol-sulfuric acid method. Carbohydrate containing fractions were pooled together, dialyzed against distilled water (1 liter x 6 changes) at 4^0C and lyophilized.

3.3 Characterization of EPS

3.3.1 Estimation of total carbohydrates.

Total carbohydrate content was estimated by the phenol-sulfuric acid method (Dubois *et al.*, 1956) using glucose as standard. Reagents for the phenol-sulfuric acid method are listed in Appendix-III.

Aliquots of standard solution of glucose containing 10-100 μg of glucose were pipetted out into a series of test tubes and the volume was made up to 1ml with double distilled water. 0.5ml of phenol reagent was added to each test tube and mixed thoroughly. To each test tube 5ml of concentrated sulfuric acid was added and the contents were mixed thoroughly. After incubation at room temperature for 20min, the absorbances were read at 490nm against the reagent blank. The absorbances were plotted against the concentration levels to obtain the standard graph. Samples were appropriately diluted and treated as above to estimate the total carbohydrate content.

3.3.2 Estimation of glucuronic acid

Glucuronic acid in the EPS samples was estimated by the O-hydroxy-biphenyl method (Blumenkrantz and Asboe-Hansen 1973). Reagents for O-hydroxy-biphenyl method are listed in Appendix-IV.

Aliquots of 0.2 ml, containing 0.5 to 20µg of glucuronic acid were pipetted out into a series of test tubes. 1.2 ml of sulfuric acid-tetraborate reagent was added. The tubes were refrigerated in crushed ice. The mixture was shaken in a vortex mixer and the tubes were heated in a water bath at 100^{0}C for 5 minutes. They were immediately cooled in ice and 20µl of OHBP reagent was added to all the tubes. The tubes were shaken and within 5 minutes absorbances were read at 520 nm against a reagent blank. The absorbances were plotted against concentration levels to obtain a standard graph.

Samples of EPS were appropriately diluted and treated as above to estimate the glucuronic acid content.

3.3.3 Gel filtration chromatography of EPS on Sepharose 4B column

EPS sample(10mg) was dissolved in 1 ml of 10mM EDTA-TEA buffer (100mM EDTA solution was titrated to pH7.0 with TEA and the volume was made up to 1000 ml) and applied to a Sepharose 4B column (30cm x 1.25cm) that had been preequilibriated with the same buffer. The column was eluted with the same buffer

maintaining a flow rate of 25 ml per hour with 3 ml fractions being collected. The eluted fractions were assayed for total carbohydrates by the phenol-sulfuric acid method. The absorbances were plotted against the fraction numbers to get the elution profiles of the samples.

3.3.4 SDS-PAGE of EPS

Discontinuous slab gel electrophoresis was carried out to obtain EPS band patterns using 5 per cent stacking gel and 10 per cent separating gel as per the method of Laemmli (1970). Stock solutions for SDS-PAGE are listed in Appendix-V.

EPS samples were dissolved in the sample buffer in the ratio 2:1(w/v). The mixtures were heated in boiling water bath for 15 minutes. 120μg of TAL1000, P132 and 500μg of TAL1148 sample were loaded to the gel tracks and electrophoresed. A current of 8mA per track was maintained till the tracking dye entered the resolving gel and was increased to 10mA per track till the dye reached the bottom of the gel.

3.3.5 Silver staining the gel.

After the run, the gel was silver stained by the method of Tsai and Frasch (1982) with some major modifications. Reagents for staining and developing the gel are listed in Appendix-VI.

Immediately after electrophoresis, the gel was fixed in Fixer solution-I overnight. The gel was then treated in Fixer solution-II for five minutes and was washed thrice with double distilled water (500ml) at 10 minute intervals. It was then incubated in the modified silver stain for 15 minutes with gentle agitation which was followed by wash in double distilled water for 15 minutes with 2 changes as against 2 minutes recommended by Tsai and Frasch. The gel was then treated in the freshly prepared developer solution till the bands developed clearly. It was finally rinsed in double distilled water and stored in double distilled water.

3.4 Isolation of LPS

The LPS were isolated by the method of Johnson and Perry (1976).

The rhizobial cells were washed thrice with 500ml of 1% sodium chloride solution and centrifuged at 9500rpm for 15min at 4^0C followed by 3 washings with distilled water. Bacterial cells were then suspended in 15ml of 50mM sodium phosphate buffer(pH 7.0) with 5mM EDTA, 0.05%(w/v) sodium azide. The suspension was incubated at 70^0C for 10 minutes with stirring. An equal volume of 90% (v/v) phenol solution preheated to 70^0C was added and the mixture was stirred for 15 minutes. The suspension was rapidly cooled to 10^0C by placing in crushed ice. The mixture was centrifuged at 10000 rpm for 15 minutes. The resulting aqueous phase was removed by aspiration and the phenol phase was discarded. The aqueous phase was again

reextracted with an equal volume of 45%(v/v) phenol solution at 70°C. The LPS preparations were dialyzed against distilled water (1 liter x 6 changes) at 4°C. Precipitate formed, if any during dialysis was removed by centrifugation and the supernatant was lyophilized to get LPS.

3.5 Chemical analysis of LPS

3.5.1 Estimation of total carbohydrate content

The total carbohydrate content of LPS samples was determined by the method of Dubois *et al.*, (1956) as described in section 3.3.1.

3.5.2 Estimation of total Phosphorus.

Estimation of total phosphorus in LPS samples was done by the method of Fiske and Subbarow (1925). The reagents for Fiske and Subbarow method are listed in Appendix-VII.

Aliquots of standard solution containing 2-20 μg phosphorous were pipetted out into a series of test tubes and the volume was made upto 4.2ml using double distilled water. 0.6ml of molybdate reagent and 0.2ml of ANSA reagent were added to each test tube, vortexed and incubated at room temperature. Absorbances of the

solutions were read at 660nm against a reagent blank. The absorbances were plotted against concentration levels to obtain the standard graph.

Samples were appropriately diluted and treated as above to estimate the phosphorus content.

3.5.3 Estimation of 2-Keto-3-deoxy-octulosonic acid.

KDO in LPS was estimated by the periodate-thiobarbituric acid method of Karkhanis *et al.*, (1978). Reagents for KDO estimation are listed in Appendix-VIII.

3.5.3.1 Sample preparation

One mg LPS was mixed in 2 ml of 0.2N sulfuric acid and kept in a boiling water bath for 1 hour. The mixture was cooled and centrifuged at 5000 rpm to remove any undissolved material.

3.5.3.2 Assay procedure

To 0.5ml of the supernatant, 0.25ml of periodate solution was added, vortexed and the mixture was incubated at room temperature for 20 min. To this mixture, 0.25 ml of sodium arsenite solution was added and vortexed, till the brown colour that was formed, disappeared.

To this, 0.5ml of thiobarbituric reagent was added, vortexed and the mixture was heated to 100^{0}C in a water bath for 15 minutes. To this mixture, 1ml of DMSO was added while the reaction mixture was hot, and was allowed to cool at room temperature. Absorbances were read at 548nm against a reagent blank.

A standard graph was constructed along with the sample using 1-10µg of KDO. The amount of LPS sample was calculated from the standard graph.

3.6 Isolation and purification of chickpea seed lectin

Chick seed lectin was isolated and purified according to the procedure followed by Shivayogi (1996). All operations were carried out at 4^{0}C unless otherwise mentioned.

10g of decorticated chickpea seeds were soaked in 100ml of 0.01M PBS(pH 7.3) over night. The seeds were homogenized in a Remi mixer at top speed for 1min. The homogenate was filtered through cheese cloth and the filtrate was centrifuged at 10,000 rpm for 10minutes. To the clear supernatant (100ml), finely pulverized ammonium sulfate was added gradually with constant stirring at 0^0 C over a period of 45 minutes till 70% saturation was reached. After 1 hour of stirring, the mixture was centrifuged at 10000rpm for 15 minutes. The precipitate was dissolved in a minimum volume of double distilled water.

This solution was dialyzed against double distilled water (1liter x 12 changes) using 10kD cutoff dialysis tubing. The small amount of precipitate which was formed

was removed by centrifugation. The dialyzed solution was mixed with DEAE-Sephadex A-50 which had been preequilibriated with 0.01M Tris-HCl buffer (pH 8.0).

The slurry was washed with 0.01M Tris-HCl buffer(pH 8.0) through a Whatman No.1 filter paper till no protein was detected in the eluate by modified Bradford's method (Spector 1978). Bound protein was eluted with 0.01M Tris-HCl buffer (pH 8.0) containing 0.1M NaCl. The eluate was dialyzed against double distilled water and lyophilized.

3.7 Protein estimation.

Protein estimation was carried out as per the method of Lowry *et al.*, (1951) using BSA as the standard. Reagents for Lowry's method are listed in Appendix-IX.

Aliquots of standard solution containing 20 - 120µg BSA were pipetted out into a series of test tubes and the volume was made up to 1 ml using distilled water. 5 ml of solution C was added to each tube and mixed thoroughly. After incubation at room temperature for 10 minutes, 0.5 ml FCR was added and the contents were mixed thoroughly. The solutions were allowed to stand at room temperature for 30 minutes and the absorbances were read at 660 nm against a reagent blank. The absorbances were plotted against the concentration to obtain the standard graph.

Lectin samples were appropriately diluted and treated as above to estimate the protein content.

3.8 Bacterial agglutination and inhibition of agglutination tests

3.8.1 Preparation of rhizobial cells.

After growing for 5 days in sterilized YEM broths, the specific and non specific strains of rhizobia were centrifuged at 5000 rpm for 20 minutes at 4^0C. They were washed with saline three times and resuspended in 0.01M Phosphate Buffer Saline(pH 7.3).

3.8.2 Bacterial agglutination assay in Durham's tubes.

Chickpea seed lectin solution(100 μg in 500 μl PBS) was taken in 3 Durham's tubes. 28 μl of cell suspension of each of the 3 rhizobial strains were suspended in the 3 Durham's tubes containing the lectin solutions. Controls for each strain were prepared separately by mixing the cell suspensions(28 μl) with 500 μl of PBS taken in 3 other Durham's tubes. The tubes were attached to a glass plate with a black background. Visible agglutination was monitored after 16 and 48 hours.

3.8.3 Bacterial agglutination assay by spectrophotometer.

Cell suspensions (28 µl) were mixed with chickpea seed lectin (100µg in 500µl PBS) separately in 1 ml cuvettes to an initial absorbance of 0.3 at 540 nm against a PBS blank. Respective controls were prepared by suspending the cells (28µl) in PBS only. The absorbances were recorded after one hour.

3.8.4 Assay of inhibition of chickpea seed lectin dependent bacterial agglutination by LPS and EPS.

3.8.4.1 Durham's tube method

LPS and EPS solutions (25,50,100,200 µg in 100 µl PBS) were mixed separately in eppendrof tubes with lectin solution (100 µg in 400µl PBS) and incubated at room temperature for 1 hour. These solutions were transferred to Durham's tubes of equal dimensions. 28 µl of the specific cell suspension was added to the tubes containing the solutions of lectin and polysaccharide mixtures. The tubes were attached to a glass plate with a black background. Visible agglutination was monitored after 16 and 48 hours.

3.8.4.2 Spectrophotometric method

LPS and EPS solutions (25, 50, 100, 200 μg in 100 μl PBS) were mixed separately with lectin solutions (100 μg in 400μl PBS) in eppendrof tubes and incubated at room temperature for 1 hour. These solutions were transferred to 1ml cuvettes. Specific strain rhizobial cells (28 μl) were suspended to an initial absorbance of 0.3 at 540nm. The absorbances were read against a blank of PBS after 1 hour.

EXPERIMENTAL RESULTS

IV EXPERIMENTAL RESULTS.

4.1 Isolation and purification of EPS

The CTAB precipitated EPS were obtained as white powder after lyophilization. The EPS were subjected to Sephadex G-50 column chromatography to remove any residual sodium chloride and CTAB. The elution profiles of the EPS of three strains; TAL1148, TAL1000, P132 is as shown in Figure 4.1.

4.2 Chemical composition of EPS

The chemical composition of EPS preparation before and after Sephadex G-50 chromatography is presented in Table 4.1 and 4.2. The total carbohydrate content varied with the strains. P132 EPS contained the highest amount of total carbohydrates(4.7 μmol/mg) whereas the least amount was determined in TAL 1148 EPS (0.066 μmol/mg). TAL1000 EPS had a value of 0.12 μmol/mg which was intermediate to that of TAL1148 and P132.

Glucuronic acid content was found to be higher in EPS of both the non specific strains TAL1000 and P132(0.053 μmol/mg and 0.312 μmol/mg respectively) whereas the specific strain of TAL 1148 had 0.007 μmol/mg which is lower compared to the other two strains.

Fig 4.1 Purification of EPS by Sephadex G-50 column chromatography.

EPS sample(10 mg) was dissolved in 1ml of 10 mM Tris-Hcl buffer and loaded onto a Sephadex G-50 column(30x1.25cm). The column was preequilibriated with 10mM Tris-HCl buffer(pH 7.2). The samples were eluted with the same buffer by collecting 3ml fractions at the rate of 60ml per hour. Each fraction was assayed for total carbohydrates by the phenol-sulphuric acid method.

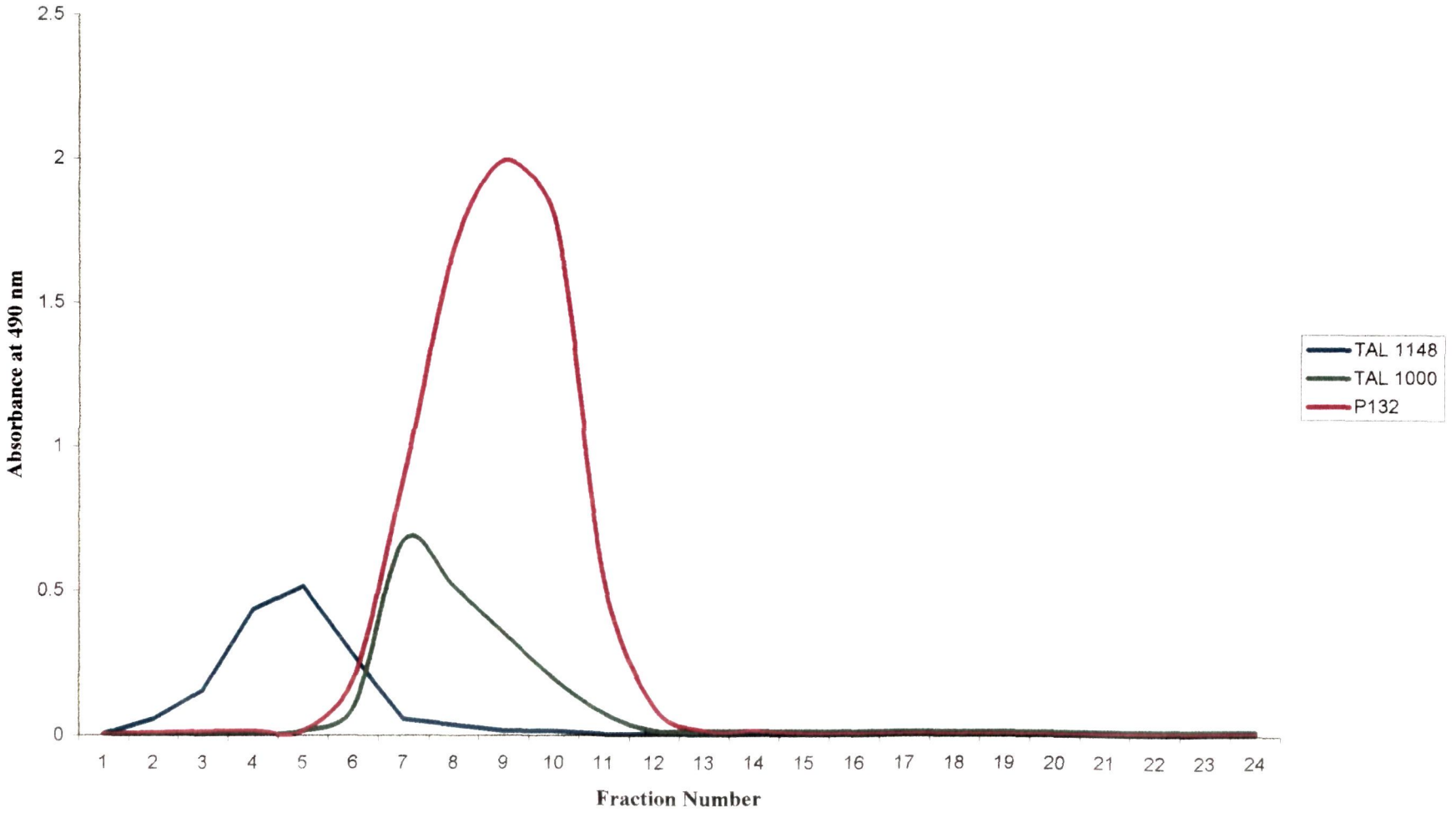

2.5
2
1.5
1
0.5
0
Absorbance at 490 nm
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Fraction Number
TAL 1148
TAL 1000
P132

Table 4.1 Chemical analysis of CTAB precipitated EPS

Strains	Total carbohydrates (μmol/mg)	Glucuronic acid (μmol/mg)	Total carbohydrates : Glucuronic acid
TAL 1148	0.023	0.004	5.75
TAL 1000	0.071	0.022	3.23
P132	4.700	0.302	15.56

Table 4.2 Chemical analysis of EPS after Sephadex G-50 chromatography

Strains	Total carbohydrates (μmol/mg)	Glucuronic acid (μmol/mg)	Total carbohydrates : Glucuronic acid
TAL 1148	0.066	0.007	9.43
TAL 1000	0.120	0.053	2.26
P132	4.790	0.312	15.35

The EPS samples after Sephadex G-50 chromatography were designated as pure based on the presence of higher amounts of total carbohydrates and glucuronic acid.

4.3 Gel filtration of EPS

The elution profile of EPS from Sepharose 4B column is shown in Figure 4.2. EPS samples of all the three strains revealed a single broad peak. This elution profile suggested that EPS of all the three strains studied have high molecular weight components.

4.4 SDS-PAGE analysis of EPS

SDS-PAGE band pattern of EPS of TAL1148, TAL 1000, P 132 is presented in Figure 4.3. TAL 1000 and P 132 had strikingly similar band pattern showing 3 clear bands. EPS from TAL 1148 presented a different band pattern with 8 distinct bands. 3 bands(band 3,5,6) of all the three strains had similar electrophoretic mobility.

4.5 Chemical analysis of LPS

The total carbohydrate and KDO contents of LPS isolated from the 3 strains is presented in Table 4.3. TAL 1148 contained lesser amount of total carbohydrates compared to the LPS of TAL1000 and P 132. KDO content in TAL 1148 LPS was found to be higher than that of the LPS of TAL 1000 and P 132. Phosphate content in

Fig 4.2 Gel filtration of EPS on Sepharose 4B column

EPS sample (10 mg) was was dissolvedin 1ml of 10mM EDTA-TEA buffer (pH 7.0) and loaded on to a Sepharose 4B column(30x1.25cm) preequilibriated with the same buffer and samples were eluted with the same buffer. 3ml fractions were collected at a rate of 25ml per hour. Each fraction was assayed for total carbohydrates by the phenol-sulphuric acid method.

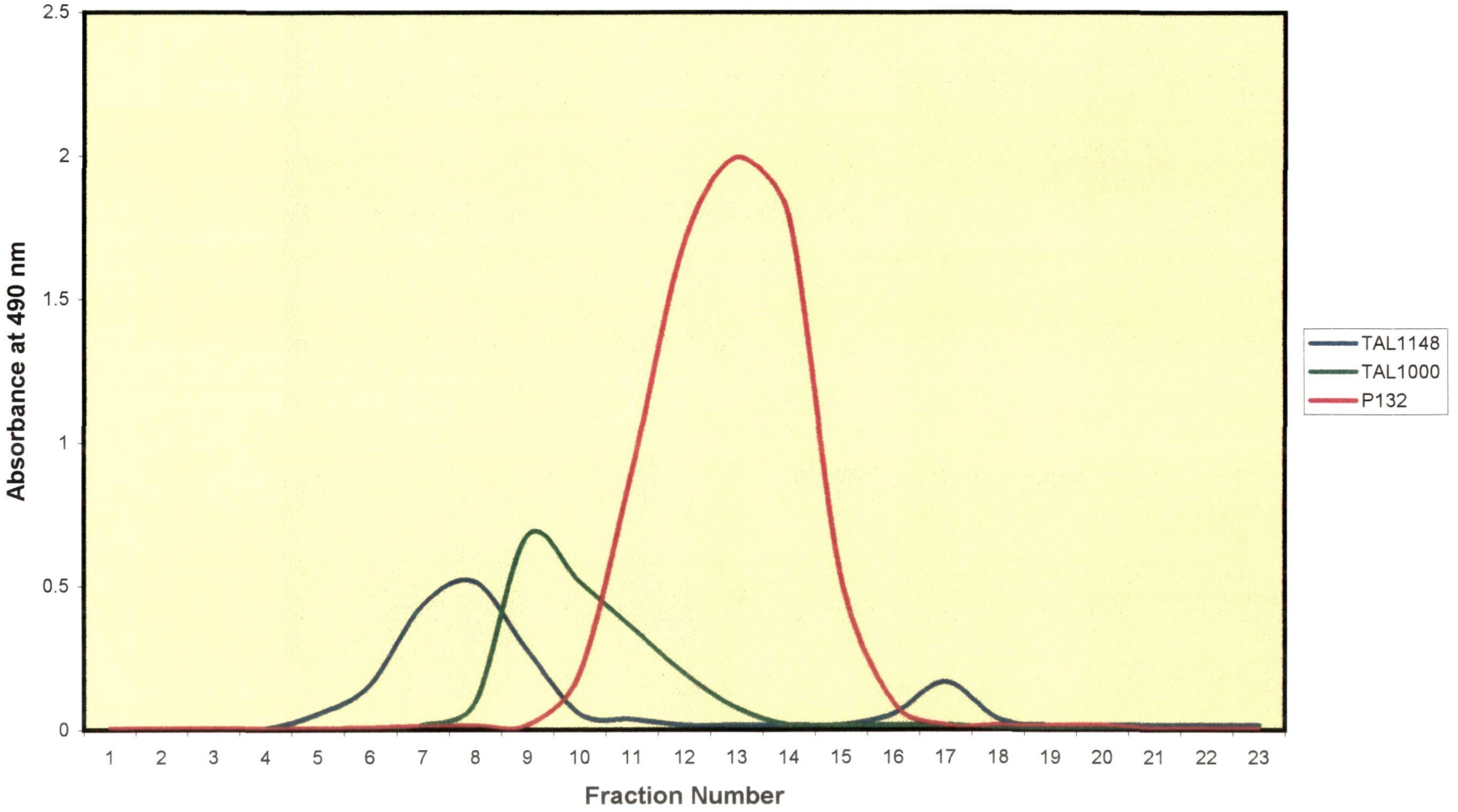

2.5
2
1.5
1
0.5
0
Absorbance at 490 nm
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Fraction Number
TAL1148
TAL1000
P132

Plate 1.: SDS-PAGE band patterns of chromatographed EPS isolated from different rhizobial strains.

Lane 1: TAL1148
Lane 2: TAL1000
Lane 3: P132

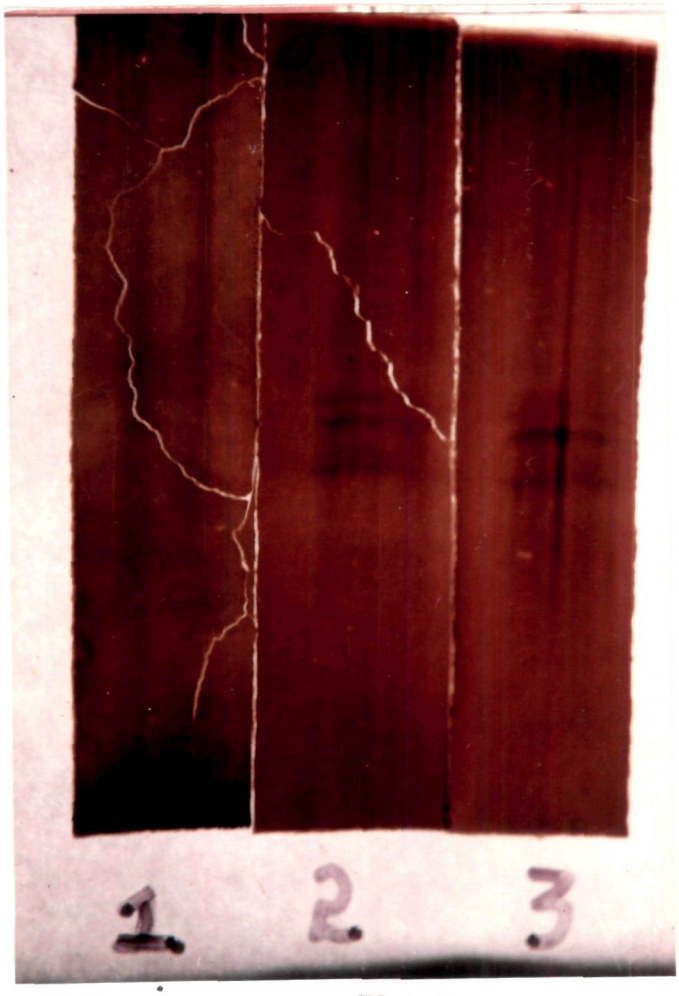


Plate 1

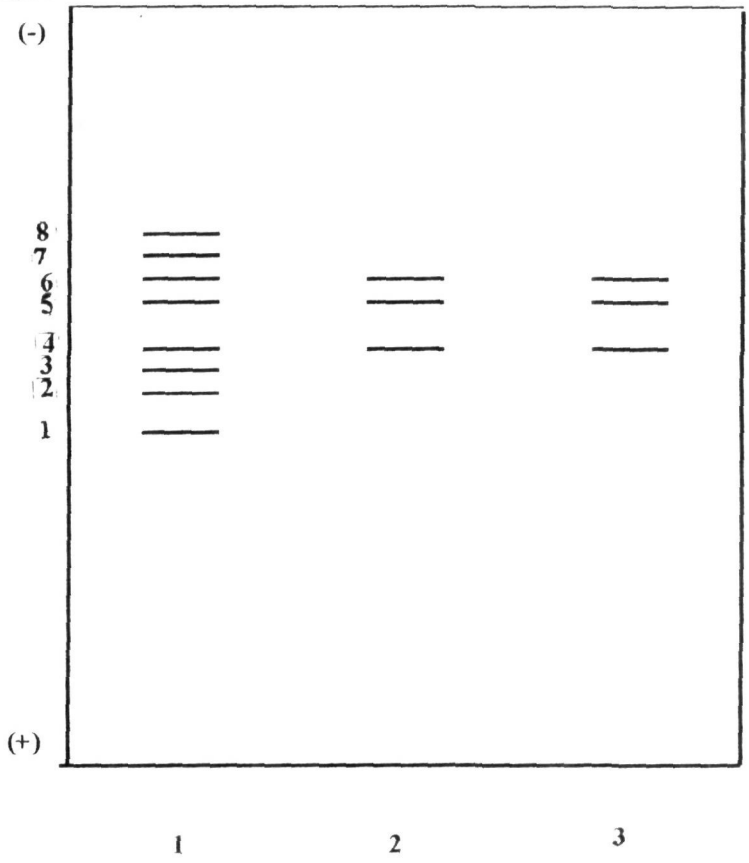


Fig 4.3 Schematic representation of SDS-PAGE bands of chromatographed EPS of different rhizobial strains. Lane 1: TAL1148, Lane 2:TAL1000 Lane 3: P132.

Table 4.3 Chemical analysis of LPS

Strains	Total Carbohydrates(T.C) (μmol/mg)	K.D.O. (μmol/mg)	T.C. : K.D.O.	Phosphate (μmol/mg)
TAL 1148	1.69	0.27	6.3	0.002
TAL 1000	2.34	0.12	19.5	0.079
P132	3.10	0.20	15.5	0.039

all the three LPS preparations was found to be low indicating negligible nucleic acid contamination.

4.6 Inhibition of chickpea seed lectin dependent agglutination of rhizobial cells by their purified LPS and EPS

The agglutination experiment of rhizobial cells was carried out in Durham's tubes. The agglutination was monitored after 16 hours and 48 hours. The tube C(plate 5 and 7) shows the settling down(agglutination) of TAL1148 (chickpea specific strain) in the presence of chickpea seed lectin, whereas the control tube(tube B of plates 4and 6) is turbid due to the presence of suspended cells. The tubes G and I (plates 2 and 3) containing TAL1000 and P132 strain cells,respectively along with chickpea seed lectin do not show any agglutination as compared to their respective controls(tubes F,H of plates 2 and 3) indicating that chickpea seed lectin differentially agglutinates specific strain *viz* TAL1148.

The results of the inhibition of chickpea seed lectin dependent agglutination of specific rhizobial strain TAL1148 by it's LPS and EPS are presented in the plates 5 and 7.At 16 hours, tube D which contained preincubated lectin+LPS and rhizobial cells showed turbidity whereas tube E which contained preincubated lectin+EPS showed partial agglutination. Tube C which contained lectin and rhizobial cells showed partial settling of cells. At 48 hours, in tube C rhizobial cells were completely agglutinated, whereas tube D which contained preincubated lectin+LPS and rhizobial cells showed turbidity. Tube E which contained preincubated lectin+EPS showed considerable

Assay of chickpea seed lectin dependent agglutination of rhizobial cells by EPS, LPS.

PLATES 2, 3, 4, 5, 6, 7.

A: Blank[PBS only].

B: Control 1: TAL 1148 cells + PBS.

C: Chickpea seed lectin + TAL 1148 cells.

D: [Chickpea seed lectin + TAL 1148 LPS] + TAL 1148 cells.
preincubated for one hour at room temperature

E: [Chickpea seed lectin + TAL 1148 EPS] + TAL 1148 cells.
preincubated for one hour at room temperature

F: Control 2: TAL 1000 cells + PBS.

G: Chickpea seed lectin + TAL 1000 cells.

H: Control 3: P 132 cells + PBS.

I: Chickpea seed lectin + P 132.

TAL 1148: Chickpea specific strain.

TAL 1000: Groundnut specific strain.

P 132: Cowpea specific strain.

Assay mixture contained PBS (0.01M pH 7.3), 28µl of rhizobial cells, 100 µg of chickpea seed lectin in 400µl PBS, and 200µg of EPS, LPS in 100µl PBS. Total volume of the blank was 500 µl. Total volume of the controls and assay mixtures was was 528 µl (inclusive of cells)

Plate 2: Effect of interaction of chickpea seed lectin with non specific strains of chickpea, TAL 1000 and P 132 after 16 hours.

F: Control 2: TAL 1000 cells + PBS.
G: Chickpea seed lectin + TAL 1000 cells.
H: Control 3: P 132 cells + PBS.
I: Chickpea seed lectin + P 132

Plate 3: Effect of interaction of chickpea seed lectin with non specific strains of chickpea, TAL 1000 and P 132 after 48 hours.

F: Control 2: TAL 1000 cells + PBS.
G: Chickpea seed lectin + TAL 1000 cells.
H: Control 3: P 132 cells + PBS.
I: Chickpea seed lectin + P 132

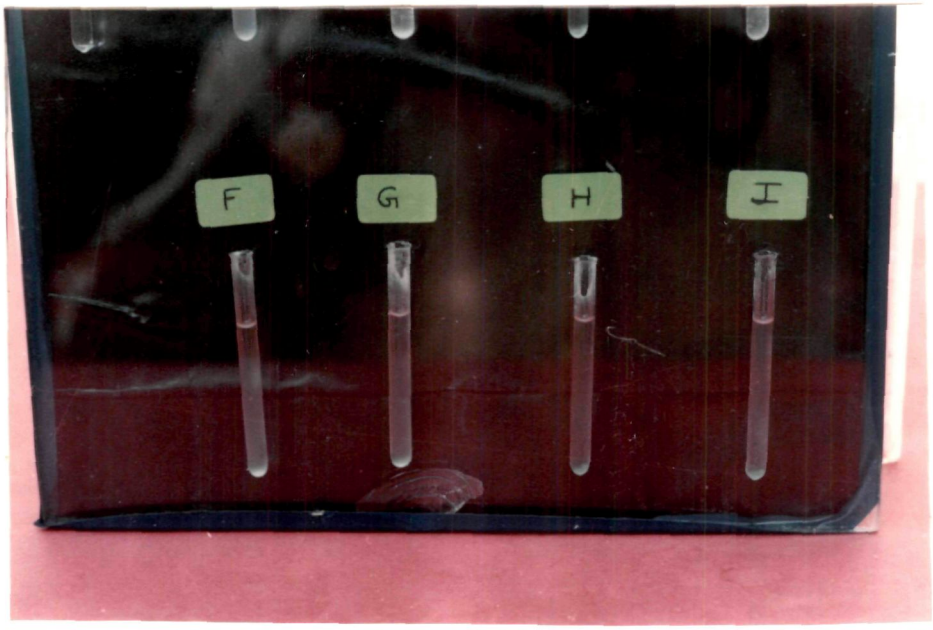


Plate 2

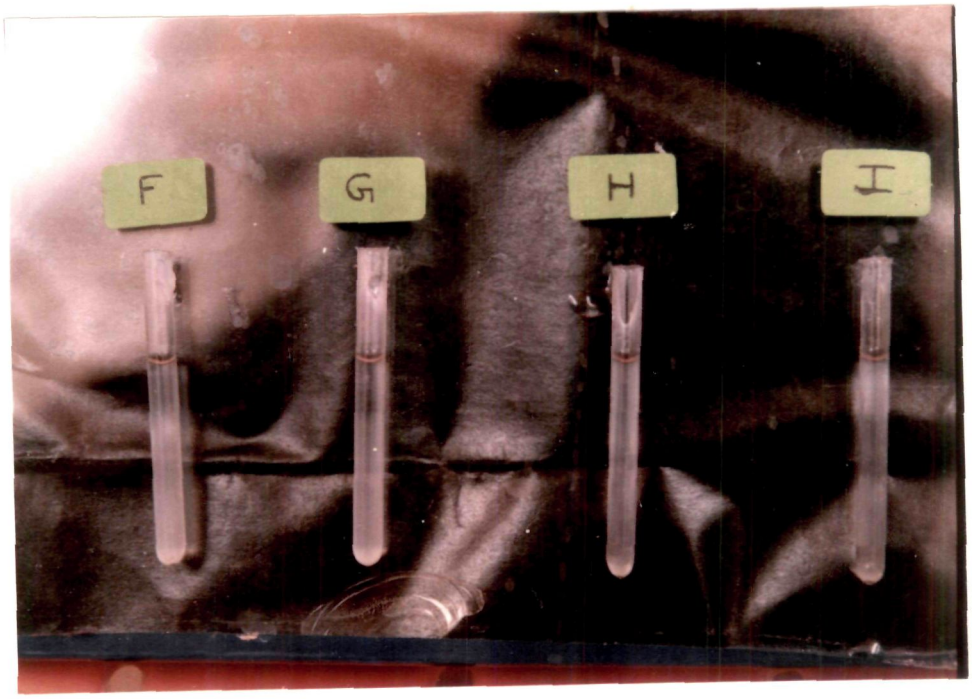


Plate 3

Plate 4: Effect of interaction of chickpea seed lectin with specific strain of chickpea TAL1148 and it's inhibition of chickpea seed lectin dependent agglutination by EPS, LPS shown along with blank and control(after 16 hours).

A: Blank[PBS only].
B: Control 1: TAL 1148 cells + PBS
C: Chickpea seed lectin + TAL 1148 cells.
D: [Chickpea seed lectin+TAL1148 LPS] + TAL1148 cells. preincubated for one hour at room temperature
E: [Chickpea seed lectin+TAL1148 EPS] + TAL1148 cells. preincubated for one hour at room temperature

Plate 5: Near focus of the effect of interaction of chickpea seed lectin with specific strain of chickpea, TAL1148 and it's inhibition of chickpea seed lectin dependent agglutination by EPS, LPS shown along with control(after 16 hours).

A: Blank[PBS only].
B: Control 1: TAL 1148 cells + PBS
C: Chickpea seed lectin + TAL 1148 cells.
D: [Chickpea seed lectin+TAL1148 LPS] + TAL1148 cells. preincubated for one hour at room temperature
E: [Chickpea seed lectin+TAL1148 EPS] + TAL1148 cells. preincubated for one hour at room temperature

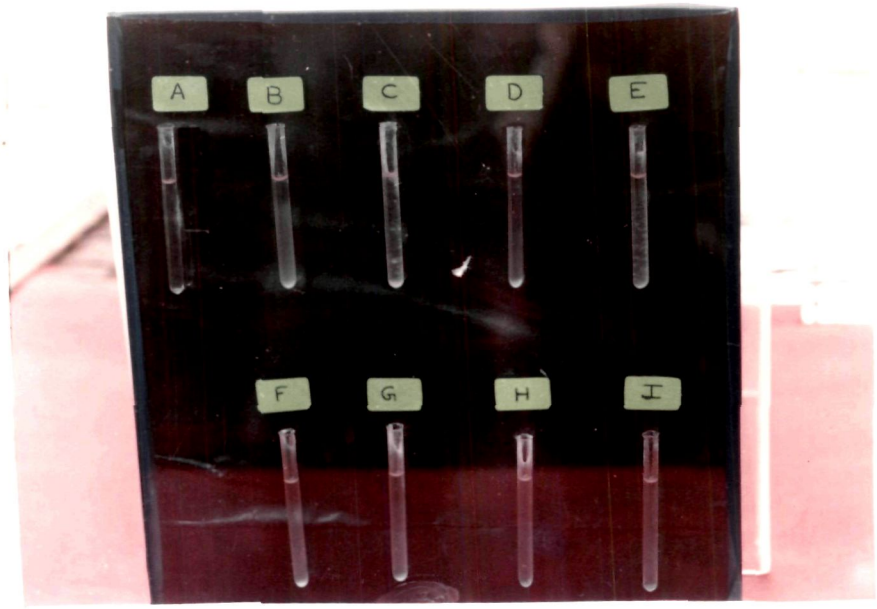


Plate 4

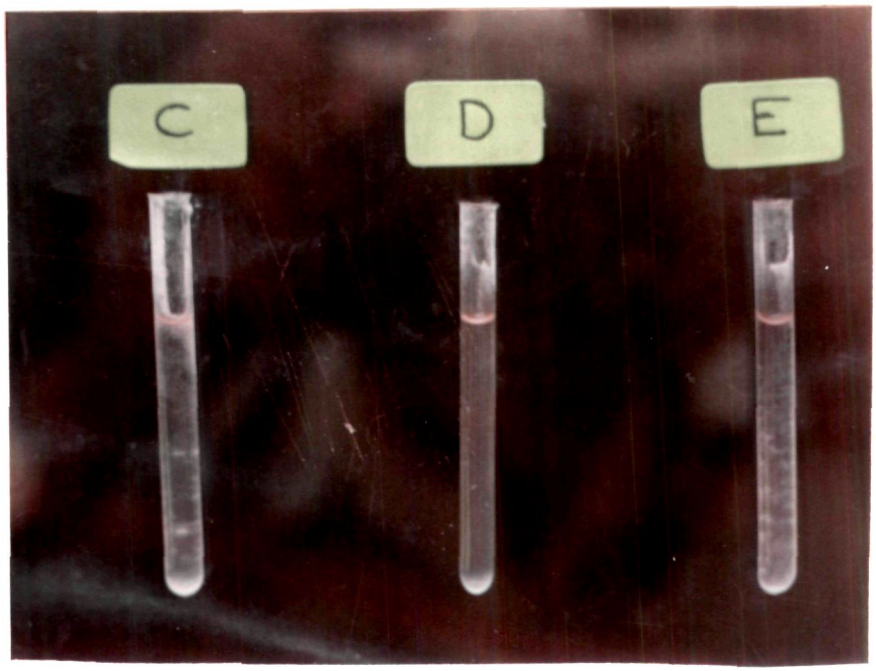


Plate 5

Plate 6: Effect of interaction of chickpea seed lectin with specific strain of chickpea TAL1148 and it's inhibition of chickpea seed lectin dependent agglutination by EPS, LPS shown along with blank and control(after 48 hours).

A: Blank[PBS only].
B: Control 1: TAL 1148 cells + PBS
C: Chickpea seed lectin + TAL 1148 cells.
D: [Chickpea seed lectin+TAL1148 LPS] + TAL1148 cells. preincubated for one hour at room temperature
E: [Chickpea seed lectin+TAL1148 EPS] + TAL1148 cells. preincubated for one hour at room temperature

Plate 7: Near focus of the effect of interaction of chickpea seed lectin with specific strain of chickpea, TAL1148 and it's inhibition of chickpea seed lectin dependent agglutination by EPS, LPS shown along with control(after 48 hours).

A: Blank[PBS only].
B: Control 1: TAL 1148 cells + PBS
C: Chickpea seed lectin + TAL 1148 cells.
D: [Chickpea seed lectin+TAL1148 LPS] + TAL1148 cells. preincubated for one hour at room temperature
E: [Chickpea seed lectin+TAL1148 EPS] + TAL1148 cells. preincubated for one hour at room temperature

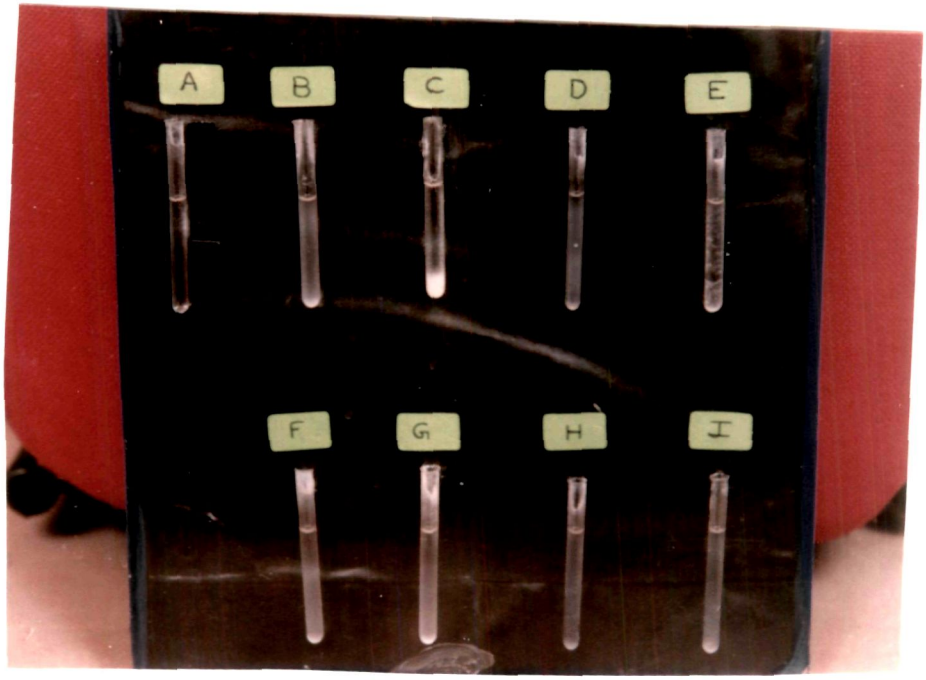


Plate 6

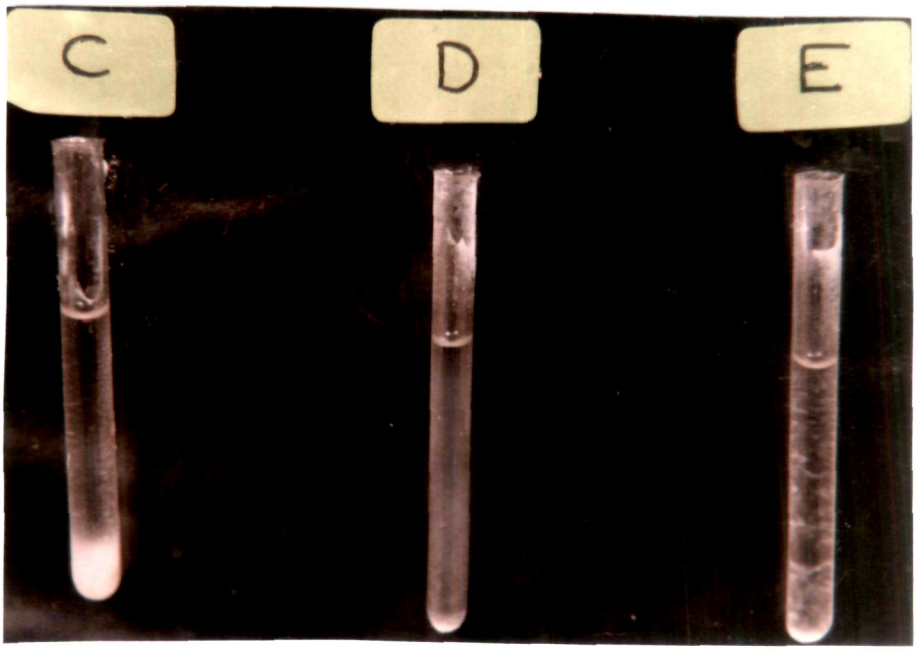


Plate 7

agglutination.These results suggested that LPS bound to the chickpea seed lectin to a greater extent and inhibited the lectin dependent agglutination to a greater extent than EPS which bound the lectin to a lesser extent.

The results of the agglutination of bacterial cells of specific and non specific strains of chickpea by chickpea seed lectin as monitored spectrophotometrically is presented in Table 4.4. The ΔA_{540} is very pronounced in case of TAL1148 whereas it was negligible in case of nonspecific strains, TAL1000 and P132.

Table 4.5 shows the inhibition of agglutination of specific bacterial cells by different concentrations of LPS and EPS as monitored spectrophotometrically. Inhibition of agglutination of specific strain cells by chickpea seed lectin was observed with as little as 25µg of LPS and at 200µg of LPS, the agglutination was inhibited to almost 96%. The inhibition of agglutination of specific strains cells by EPS was lower compared to that by LPS. Maximum inhibition of agglutination by EPS was 59% as against 96% observed with LPS under similar conditions.

Table 4.4 Agglutination of specific and non specific strains of rhizobia by chickpea seed lectin.

Strain	Change in absorbance Δ 540 nm on reaction with chickpea seed lectin		
	Absorbance		Δ540nm
	Initial	Final	
TAL 1148	0.300	0.210	0.090
TAL 1000	0.300	0.296	0.004
P132	0.300	0.294	0.006

Table 4.5 Inhibition of chickpea seed dependent agglutination of chickpea specific rhizobial strain (TAL1148) cells by their purified LPS and EPS

Assay mixture	Absorbance (540nm)		Δ_{540nm}	% inhibition
	Initial	Final		
rhizobial cells+ 100µg chickpea seed lectin	0.300	0.210	0.09	-
100µg chickpea seed lectin + different concentrations of LPS (pre-incubated) + rhizobial cells				
LPS concentrations				
25 µg	0.300	0.231	0.069	24
50 µg	0.300	0.263	0.037	59
100 µg	0.300	0.283	0.017	82
200 µg	0.300	0.296	0.004	96
100µg chickpea seed lectin + different concentrations of EPS (pre-incubated) + rhizobial cells				
EPS concentrations				
25 µg	0.300	0.220	0.080	12
50 µg	0.300	0.232	0.068	23
100 µg	0.300	0.244	0.056	38
200 µg	0.300	0.263	0.037	59

DISCUSSION

V DISCUSSION

In the present study, isolation and characterization of EPS from rhizobia, specific and non specific to chickpea has been carried out with a view to understand their role in *Rhizobium* -legume symbiosis.

EPS were precipitated from the culture supernatant with CTAB according to the method of Robertson *et al.*,(1981). Ethanol also has been used for the precipitation of EPS from culture supernatant.The disadvantage of this method is that sometimes other polysaccharides like β-1,2 glucans also get precipitated with EPS. CTAB is used for reprecipitation to obtain pure EPS(Hino *et al* .,1997).The CTAB precipitated EPS were purified by gel filtration chromatography on Sephadex G-50 to remove residual sodium chloride and CTAB. Table 4.2 shows that there has been an increase in the total carbohydrate content of EPS isolated from all the three rhizobial strains, indicating that salt contamination has greatly reduced.The total carbohydrate content of EPS from TAL1148 and TAL1000 increased from 0.023 µmol/mg to 0.066 µmol/mg and 0.07µmol/mg to 0.12 µmol/mg respectively while that of P132 changed to negligible amounts.

The glucuronic acid content was also found to be higher in the purified EPS of all the three strains. In respect of TAL1148 and TAL1000, the glucuronic acid content increased almost two fold, from 0.004 µmol/mg to 0.007µmol/mg and 0.022 µmol/mg to 0.053 µmol/mg respectively, while that of EPS of P132 showed a slight increase from 0.302 µmol/mg to 0.312 µmol/mg. The ratio of total carbohydrate to

glucuronic acid may be used for the characterization of EPS. The ratio was highest in the EPS of P132, and least in the EPS of TAL1000(15.35 and 2.26 respectively). Tal1148 EPS showed a ratio of 9.43. The ratio is an indicator of the acidic nature of EPS. Accordingly, EPS of P132 is the least acidic of all the three EPS, whereas TAL1000 is the most acidic. Djordjevic *et al* .,(1986) have analysed the composition of EPS from *Rhizobium* sp NGR234. They have reported a value of 5160 nmol/mg of total carbohydrate, 1120 nmol/mg of glucuronic acid, 5.2 nmol/mg of pyruvate and 5.6 nmol/mg of acetate. The ratio of total carbohydrate to glucuronic acid was 4.61. This value is not similar to the values determined for the three rhizobial strains studied.

The significance of varied ratio of total carbohydrate to glucuronic acid for *Rhizobium*-legume symbiosis is not clear.The presence of glucuronic acid in all the three EPS demonstrates that these are heteropolysaccharides. *Rhizobium meliloti* SU47 produces a succinoglycan called EPS I or EPSa which contains glucose, galactose, pyruvate, acetyl and succinyl groups(Aman *et al* ., 1981; Jannson *et al* ., 1975). Under conditions of nutritional stress, another EPS called EPS II or EPSb is secreted. This contains galactose, glucose, pyruvate and acetate.(Her *et al.*, 1990; Levery *et al* ., 1991). Both of these do not contain glucuronic acid. However almost all other rhizobial EPS contain glucuronic acid. For example *Rhizobium leguminosarum* b.v *viciae, trifolii, phaseoli* (Canter-Cremers *et al.*, 1991; Hollingsworth *et al* ., 1988; McNeil *et al* ., 1986; Robertson *et al* ., 1981) *Rhizobium* sp NGR 234 (Drodjevic *et al* .,1986).

The anionic nature of the EPS is due to the presence of uronic acids, pyruvate, acetate and phosphoric diester groups (Kenne and Lindberg, 1983). Because of this anionic nature it is possible to run EPS on an SDS-PAGE. Due to the difference in uronic acid content among the three EPS studied, the amount of sample loaded on to the gel also varied. The glucuronic acid content of TAL1148 is comparatively very low, therefore higher amount of sample was required to confer sufficient anionic charges on EPS, so that it can be moved in an electric field. 120 g of TAL1000 and P132 EPS was sufficient to detect the bands. To detect TAL1148 EPS bands, 500 g sample was needed. Resolving gels of lower percentage (10%) had to be used for SDS-PAGE of EPS, presumably due to higher molecular weight they possessed. EPS of TAL1000 and P132 showed three distinct bands on SDS-PAGE whereas TAL1148 showed eight bands. Three electrophoretic bands(bands 3,5,6) were found to have similar mobility in all the three strains. It would be interesting to find out the chemical nature and functions of these three common bands found in different strains of rhizobia with different specifities. Though the carbohydrate composition and structure of EPS of many bacteria have been established(Aman *et al* .,1981; Poveda *et al* ., 1997a; Poveda *et al* ., 1997b) PAGE patterns of any EPS are yet to be reported.

The elution profile of the EPS from Sepharose 4B has revealed a single peak in all the three strains. This is in agreement with SDS-PAGE pattern which indicates the presence of a major high molecular weight component. As the amount of total sugars varied with the samples the area of the peak also varied. P132 EPS showed the largest peak area, whereas TAL1148 showed the least peak area. The elution profile

of Xanthan gum on Bio-gel A 5m column showed a single peak as reported by Ielpi *et al* ., (1981). It was shown by Leigh and Lee, (1988) that a low molecular weight fraction was eluted when EPS of the *exo* mutants of *Rhizobium meliloti* were fractionated on Bio-gel A 5m column, whereas both high molecular weight and low molecular weight fraction were eluted from wild strains. Elution profile of EPS on the gel filtration column may therefore be used for their characterization.

Chickpea specific strain of *Rhizobium,* TAL1148 was shown to be specifically agglutinated by chickpea seed lectin. However, the protein induced bacterial agglutination could not be inhibited by any of the simple sugars tested(Shivyogi, 1996). This is in agreement with the observation of Kolberg *et al.*,(1983) who described isolation and properties of a chickpea seed lectin for which only some glycoproteins like Ig M and fetuin were effective inhibitors of hemagglutination whereas simple sugars were ineffective. In addition, the present study demonstrated the inhibition of agglutination by chickpea seed lectin by LPS isolated from specific rhizobial species. It is most probable that a complex structure is needed for efficient interaction. Therefore, the chickpea seed lectin used in these studies is indeed a lectin and it conforms to the definition of lectin which according to Goldstein *et al* ., (1980) is "sugar binding protein or glycoprotein of non immune origin which agglutinates cells and or glycoconjugates".

Several researchers have shown the ability of host lectins to interact with various surface polysaccharides of *Rhizobium* (Wolpert and Albersheim, 1976; Kamberger 1979; Kato *et al* ., 1979; Bhagwat and Thomas, 1980; Hrabak ,1981).

In the present work, it has been shown that the chickpea seed lectin dependent agglutination of the specific strain TAL1148 was inhibited to a greater extent by it's LPS (96%) than with it's EPS (56%). These observations are supported by earlier observations of Maiti and Podder (1989) that peanut agglutinin interacts more strongly with LPS of *Bradyrhizobium arachis* than with it's EPS or CPS. These results were based on the quantitative precipitin reactions. LPS concentration necessary for 50 % inhibition of precipitin reaction was 10 times less than that of EPS, CPS and there was no difference between EPS and CPS concentration for 50 % inhibition.

Putnoky *et al* . (1990), after genetic analysis have shown that *Rhizobium meliloti* strain 41 mutants with mutations in either the *exo* or *lps* genes, formed effective nodules on alfalfa but double mutants with mutations in both *exo* and *lps* genes were not able to form effective nodules. This demonstrated that *exo* and *lps* genes determine similar functions in the course of nodule development, suggesting that EPS and LPS may provide equivalent information for the host plant.

Williams *et al* . (1990) have demonstrated that when a gene *lps* Z^+ is introduced from *Rhizobium meliloti* strain 41 into the *exo* mutant of *Rhizobium meliloti* SU 47(that produces non nitrogen fixing nodules on alfalfa due to EPS deficiency) the EPS production is not restored but LPS composition, structure produced by it is altered and nitrogen fixing nodules are produced. This suggests that *Rhizobium meliloti* EPS can perform the same function in nodule development.

EPS has been found to be essential for effective nitrogen fixing in indeterminate nodules whereas LPS has been found to be necessary for the determinate nodule formation. Chickpea is an indeterminate nodule forming legume.

In the present study LPS have been found to be more effective inhibitors of lectin dependent agglutination of specific strain rhizobial cells than EPS. But EPS binding to lectin(upto 60%) cannot be discounted in the wake of reports that LPS and EPS can substitute each other's functions(Putnoky *et al* ., 1990; Williams *et al* ., 1990). The binding of host lectins to specific rhizobia cannot be a mere coincidence. Final word is yet to be written on the roles of all these three molecules namely lectins, LPS, EPS in the *Rhizobium*-legume symbiosis.

SUMMARY

VI SUMMARY

Surface polysaccharides (EPS, LPS) of different rhizobial strains, specific and non specific to chickpea were isolated and purified to study their interaction with chickpea seed lectin.

1. EPS free from salts were prepared from three strains of rhizobia, namely TAL 1148, TAL 1000, and P 132.

2. EPS of all the rhizobial strains were found to be heteropolysaccharides.

3. EPS of all the strains varied in their chemical composition.

4. Elution profiles of EPS from Sepharose 4B column indicated that all the three EPS contain a high molecular weight component

5. The two non specific strains, TAL 1000, and P 132 showed three distinct bands of similar electrophoretic mobility, whereas TAL 1148 displayed eight bands with three of them having similar electrophoretic mobility as that of the non specific strains.

6. LPS of the specific strain, TAL 1148 inhibited the lectin dependent agglutination of the specific strain to a larger extent than the EPS

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***Originals not seen**

APPENDIX

APPENDIX- I

Reagents for yeast extract mannitol agar (YEMA) with congo red.

Mannitol	10.00 g
Yeast extract	0.50 g
K_2HPO_4	0.50 g
$MgSO_4.\ 7H_2O$	0.20 g
NaCl	0.10 g
Agar	20.00 g
Congo Red	3 ml
Distilled water	1000 ml
PH	6.95 to 7.05

Note : For YEM broth, do not add agar and congo red.

APPENDIX-II

Reagents for Gram's staining

1. Gram's stain

Solution A :

Crystal violet (85% dye)	.....2.0 g
Ethyl alcohol (95%)	.....20.0 ml

Solution B :

Ammonium oxalate	.....0.8 g
Double distilled water	.....80.0 ml

Dilute solution A five times with distilled water and mix with an equal volume of solution B.

2. Lugol's Iodine

Iodine	.....1g
Potassium Iodide	.....2-5 g
Double distilled water	... .300 ml

Allow for 24 hours for the iodine to dissolve. It may be necessary to add a few crystals of potassium iodide.

3. Counter stain

Saffranine (2.5 %solution)	.....25 ml
Double distilled water	.....75 ml

APPENDIX - III

Reagents for phenol - sulfuric acid method

1. Phenol reagent : 5% phenol (v/v) in distilled water.

4. Concentrated sulfuric acid AR grade

3. Standard glucose solution : Dissolve 100 mg of glucose in 100 ml of distilled water in a volumetric flask. Dilute 1 ml of this solution to 10 ml. This diluted solution contains 100µg of glucose in 1 ml.

APPENDIX IV

Reagents for glucuronic acid estimation.

1. Ortho-hydroxy-biphenyl solution.

   A 0.15% solution of ortho-hydroxy-biphenyl is prepared in 0.5% sodium hydroxide and the reagent solution is stored in an amber bottle for 1 month in refrigerator for 4 hours.

2. Sulfuric acid/sodium tetraborate solution.

   0.0125M solution of sodium tetraborate is prepared in concentrated sulfuric acid.

APPENDIX-V

Stock solution for SDS-PAGE

1. Acrylamide solution (30% : 0.8%) ; Dissolve 30g of acrylamide and 0.8g of bis.acrylamide in some volume of distilled water and made up to 100ml.
2. Resolving gel buffer : 1.5M Tris-HCl. pH 8.8 .
3. Stacking gel buffer : 1M Tris-HCl . pH 6.8.
4. Electrode reservoir buffer pH 8.3 ; Dissolve 14.4g glycine, 3.0g Tris and 1g of SDS in some volume of distilled water and make up the volume to 1000ml .
5. SDS solution : 10 per cent W/V, in distilled water.
6. Ammonium per sulphate solution : 10% W/V in distilled water.
7. Sample solubilisation buffer : Mix 1g of SDS, 2 ml of glycerol, 2ml of Bromophenol Blue Tracking dye (0.1 % W/V solution in distilled water), 1.25 ml of 1 M Tris Hcl pH: 6.8, 2ml of β - mercaptoethanol and make upto 10ml with distilled water. When this solution is diluted to single strength , sample buffer will contain 5% W/V SDS, 10 per cent V/V glycerol, 5% V/V β -mercaptoethanol and 0.0625 M Tris - Hcl pH 6.8.

APPENDIX VI

Reagents for silver staining the gel.

1. Fixer solution I: A solution containing 40% ethanol, 5% acetic acid in double distilled water.

2. Fixer solution II: A solution containing 40% ethanol, 5% acetic acid and 0.85% periodic acid in double distilled water.

3. Staining reagent: 29 ml of 0.1N sodium hydroxide, 2.1 ml of ammonium hydroxide are mixed in a beaker. 20% silver nitrate solution is mixed to this solution with constant stirring till a light brown precipitate is formed. 115 ml of double distilled water is added. It is filtered through a filter paper to get a colourless solution. This is used for staining.

4. Developer solution: 50 µl of 37% formaldehyde and 5 mg of anhydrous citric acid in 100 ml double distilled water.

APPENDIX-VII

Reagent for total phosphorous estimation

1. Molybdate reagent : 2.3 g of ammonium molybdate is dissolved in 20 ml of water and transferred to a 100 ml of volumetric flask containing 30 ml of 10 N H_2SO_4. Dilute the solution to the mark.
2. 1,2,4- amino napthol sulphonic acid reagent : place 195 ml of 15 % sodium bisilphite solution in a glass stoppered cylinder. Add ANSA and 5 ml of 20 % sodium sulphite and shake till the powder dissolves. Filter through Whatmann 1 filter paper and store in brown bottle .
3. Standard phosphate solution : Dissolve 0.1 g of K_2HPO_4 in some water and transfer it to

   a 100 ml volumetric flask containing 1 ml of 10 N H_2So_4, dilute to the mark. Dilute 5.6 ml

   of the solution to 50 ml. 1 ml of this contains 20 µg of phosphate.

APPENDIX - VIII

Reagents for KDO estimation

1. 0.2 N H_2SO_4

2. 0.5 M HCl

3. Periodate solution : Dissolve 0.1116g of periodic acid in 10 ml of 60 mM H_2SO_4

4. 0.2 M sodium arsenite in 0.5 M HCl : Dissolve 2.598 g of sodium arsenite in 100 ml of
   0.5M HCl.

5. 0.6% aqueous thiobarbituric acid solution

6. Dimethyl sulfoxide (DMSO)

APPENDIX-IX

Reagents for Lowry's method

1. Alkaline copper reagent

Solution A : 4 gm sodium hydroxide and 20 g sodium carbonate (anhydrous) dissolved in 1000 ml of water.

Solution B :1 ml of 1.35 percent sodium potassium tartrate and 0.1 ml of 5.5 % Copper sulfate.

Solution C : Solution A (50 ml) mixed with 1 ml of solution B just before use.

2. Folin -Ciocalteu reagent

To sodium tungstate (100g) and sodium molybdate (25g) about 700ml of distilled water , 50 ml of 85% phosphoric acid and 100 ml of conc. HCl are added and gently refluxed for 10 hours.Then add 150 g of Lithium sulfate, 50 ml of distilled water & few drops of Bromine.The solution is boiled to remove excess Bromine ,cooled and the volume made upto 1000 ml and filtered.

3. Standard albumin solution

A stock solution of BSA containing 2 mg per ml and diluted to 1:10 to obtain a working standard solution of 200 µg BSA per ml.