APPENDIX

Published Paper

(1) Rhizobial Lipopolysaccharide as the receptor in lectin-\textit{Rhizobium} Interaction. Indranil Bhattacharya, Sagarika Biswas, Rakha H Das and Hasi R. Das. Indian Journal of Biochemistry and Biophysics, Vol 41, April-June 2004, pp 89-95


Communicated Paper

(1) Isolation and Characterization of an acid tolerant mutant of \textit{Mesorhizobium ciceri} mutant Red 301AT capable of inducing effective nodules on \textit{Sesbania aculeata}. Canadian J. of Microbiology, M. Pathak, P. Agarwal, A. Saroha, S.Biswas, R.H.Das and H.R. Das

Oral presentation

(1) Isolation and Characterization of Lipopolysaccharide Mutants of Peanut Specific \textit{Bradyrhizobium} (GN17). Indranil Bhattacharya, Sagarika Biswas and Hasi R.Das. At XVIIIth Carbohydrate Conference, Indian Association for the Cultivation of Science, Kolkata, November 5-7, 2003

(2) Isolation of lectins from \textit{Sesbania aculeata} and their interaction with lipopolysaccharides of host infecting rhizobia. Sagarika Biswas, Sonu Gandhi, G.L.Sharma and Hasi Das 2nd International Conference on Recent Advances in Biomedical and therapeutic Sciences, Institute of Biomedical Sciences, Bundelkhand University, Jhansi, 6-8 January 2005

Poster Presentation

(1) Isolation and Characterization of Mutants \textit{Mesorhizobium} sp. obtained by Tn5 Mutagenesis. Praveen Agrawal, Monika Pathak, Ashish Saroha, Sagarika Biswas and H.R.Das. BIOTECH 2004, IGIB, Mall Road, Delhi.
Antibacterial Activity of *Citrus reticulata* Peel Extracts

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*Citrus reticulata*, Polymethoxylated Flavones. Antimicrobial Activity

Citrus peels were successively extracted with hexane, chloroform and acetone using a soxhlet extractor. The hexane and chloroform extracts were fractionated into alcohol-soluble and alcohol-insoluble fractions. These fractions were tested against different gram positive and gram negative bacteria. The EtOH-soluble fraction was found to be most effective. Fractionation of EtOH-soluble fraction on silica gel column yielded three polymethoxylated flavones, namely desmethylnobiletin, nobiletin and tangeretin. Their structures were confirmed by UV, \(^1\)H, \(^13\)C NMR and mass spectral studies. The findings indicated a potential of these natural compounds as bio-preservatives in food applications.

Introduction

The annual world production of citrus fruits is 98.4 million metric tons (FAO, 1997) and approximately 34% of the fruits are processed into juices. The juice yield is about half of the fruit weight. Processing of citrus into juices result in large amount of by-products (Bovill, 1996). The volatile oil and non-volatile oil of orange peel are widely used in food, drug and cosmetic industries (Shaw, 1979). Polymethoxylated flavones (PMF) are an interesting group of bioactive compounds present in citrus fruits. PMF are more active than the flavonoid glycosides in their antiadhesive effects in red blood cells and platelets (Robbins, 1974). PMF have also been shown to have anti-inflammatory properties and they inhibit histamine release thereby reducing allergic reactions (Middleton and Drezwicki, 1982). PMF such as nobiletin and tangeretin are more potent inhibitors of tumour cell growth than hydroxylated flavonoids. This difference in activity may be due to better membrane uptake of the PMF since methoxylation of the phenolic groups decreases the hydrophilicity of the flavonoids (Kandaswami et al., 1991). Nobiletin and sinensetin are effective in decreasing the erythrocyte aggregation and sedimentation in human blood (Robbins, 1976; Bracke et al., 1994). PMF have also been shown to have a cytotoxic effect toward cancerous cell invasion (Kupchan et al., 1963) and to act as antimutagenic agent (Francis et al., 1989). The objective of the present study was to isolate and identify the PMF present in the active fraction determined by evaluating the antibacterial activity of the citrus peel extracts. To our knowledge, this is the first report on the isolation of compounds 1 and 2 from *Citrus reticulata* [Blanco Coorg mandarin] peels and antimicrobial activity of their extracts.

Materials and Methods

Materials

*Citrus reticulata* [Blanco Coorg mandarin] oranges are cultivated in South India on a large scale in the Coorg district (The Wealth of India, 1992). Dried peels of Blanco Coorg mandarins were procured from a local fruit-processing factory during January–February 1998. All solvents and chemicals used were of AR and HPLC grades. MP: uncorr. UV spectra were measured using a Genesys-5 UV-visible spectrophotometer (Milton Roy, NY, USA). \(^1\)H and \(^13\)C NMR spectra were recorded at 400 and 100 MHz, respectively, on a Bruker AMX-400 FT instrument (Bruker, Rheinstetten, Germany). \(^13\)C NMR spectral assignments were given on the basis of SEFT spectra. TMS was used as the internal standard. Mass spectra were recorded using Shimadzu QP-5000 Quadrupole Mass Spectrometer (Shimadzu, Tokyo, Japan).

Extraction

Dried peels (100 g) of *Citrus reticulata* were powdered and successively extracted in a soxhlet extractor with hexane, chloroform and acetone for 8 h each. The extracts were filtered, concentrated under vacuum and the yields of hexane, chloroform and acetone extracts were 5.0, 1.6 and 3.6 g, respectively. TLC of hexane and chloroform extracts (hexane:EtOAc, 5:15 v/v) showed three...
spots with different concentrations. Hence, both the extracts were mixed for fractionation.

**Fractionation of PMF**

One ml each of hexane and chloroform extracts were mixed with 20 ml of EtOH, the precipitate formed was filtered. The supernatant was concentrated under vacuum and used for antimicrobial activity along with the precipitate. Ethanol soluble fraction (1.0 g) was impregnated with 2 g of silica gel and loaded on to the silica gel column. The compounds were eluted with hexane: EtOAc solvent mixtures of increasing polarity. Compound 1 eluted with hexane; EtOAc (78:22 v/v), whereas compounds 2 and 3 were eluted with hexane: EtOAc (70:30 v/v) and (60:40 v/v) respectively. The solvents from the eluates were evaporated under vacuum and recrystallized. Compounds 1, 2 and 3 were obtained with yields of 50, 110 and 500 mg, respectively. The compounds were dissolved in chloroform, spotted on TLC and developed using hexane: EtOAc (85:15 v/v). TLC plates were sprayed with 10% sulfuric acid in methanol (v/v) and heated at 110°C for 10 min. The Rf values of the compounds were calculated and compared with reported values. Finally, compounds 1, 2 and 3 were identified as desmethylnobiletin (6,7,8,3',4' pentamethoxy-5-hydroxyflavone), nobelitin (5,6,7,8,3',4' hexamethoxy-flavone) and tangeretin (5,6,7,8,4'-pentamethoxyflavone) by 1H, 13C NMR and mass spectra, respectively.

**Inoculum preparation**

Strains of *Bacillus cereus*, *B. subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* were obtained from the stock culture collection of Food Microbiology Department of the Institute. The bacterial cultures were maintained at 4°C on nutrient agar slants and subcultured at 15-day intervals. Prior to use, the cultures were grown in nutrient broth at 37°C for 24 h. A preculture was prepared by transferring 1 ml of this culture to 9 ml nutrient broth and incubated for 48 h at 37°C. The cells were harvested by centrifugation (1200 x g, 5 min), washed and suspended in sterilized saline.

**Antibacterial activity**

The fractions were tested against different micro-organisms by the method of Chen *et al.* (1998). To flasks containing 20 ml melted nutrient agar, different concentration (200, 400 and 800 µg/ml) of test material in propylene glycol were added. In case of control, equivalent amount of propylene glycol was added. One hundred µl (about 10^5 cfu/ml) of each bacterium to be tested was inoculated into the flasks under aseptic conditions. The contents were mixed thoroughly and media was then poured into sterilized petri dishes in quadruplet and incubated at 37°C for 20–24 h. The colonies developed after incubation were counted and the inhibitory effect was calculated using the following formula (Rico-Muñoz and Davidson, 1983).

\[
\% \text{ Inhibition} = \left(1 - \frac{T}{C}\right) \times 100, \text{ where } T \text{ is cfu/ml of test sample and } C \text{ is cfu/ml of control.}
\]

The minimum inhibitory concentration (MIC) was reported as the lowest concentration of the compound capable of inhibiting the complete growth of the bacterium being tested (Naganawa *et al.*, 1996).

**Results and Discussion**

The antimicrobial activity of different fractions from citrus peel is shown in Table 1. All fractions suppressed the growth of gram-positive bacteria at concentrations lower than that required for gram-negative bacteria. EtOH soluble fraction was most active against all the bacterial strains. The acetone extract was found least effective of all the tested fractions. In case of EtOH-soluble fraction MIC for *Bacillus cereus* and *Staphylococcus aureus* was observed at 300 µg/ml level, while for *B. subtilis* and *B. subtilis*, 500 µg/ml were required to bring about complete inhibition of growth. In case of gram negative bacteria, *Escherichia coli* and *Pseudomonas aeruginosa* MIC of EtOH soluble fraction was found to be 1200 and 600 µg/ml respectively. MIC levels of EtOH soluble fraction were lower than other fractions probably due to higher polymethoxylated flavone content. Results of the present study are in consistent with the antifungal activity of citrus polymethoxylated flavones reported by earlier workers (Del Rio *et al.*, 1998). They reported antifungal activity of PMF from essential oils of Citrus against *Phytophthora cit-
Table 1. Minimum inhibitory concentration (MIC) of citrus peel fractions*.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>MIC (µg/ml)</th>
<th>Hexane extract</th>
<th>Chloroform extract</th>
<th>Acetone extract</th>
<th>Ethanol soluble fraction</th>
<th>Ethanol in soluble fraction</th>
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<tr>
<td>Grampositive</td>
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<tr>
<td>Bacillus cereus</td>
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<td>Bacillus coagulans</td>
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<tr>
<td>Bacillus subtilis</td>
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<td>700</td>
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<tr>
<td>Staphylococcus aureus</td>
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<tr>
<td>Gramnegative</td>
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</tr>
<tr>
<td>Escherichia coli</td>
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<td>1600</td>
<td>2200</td>
<td>1200</td>
<td>1800</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>1000</td>
<td>1200</td>
<td>1600</td>
<td>600</td>
<td>1100</td>
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* Results of four replications.

Table II. ^1H NMR chemical shifts (δH in CDCl₃) of compounds 1–3.

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<td>5'</td>
<td>5.05 (d) (J=1.8 Hz)</td>
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Chemical shift values are in ppm and J values in parentheses (Hz).

Table III. ^13C NMR chemical shifts (δC in CDCl₃) of compounds 1–3.

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<td>5.05 (d) (J=1.8 Hz)</td>
<td>5.05 (d) (J=1.8 Hz)</td>
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</table>

Spectral and chemical characteristics

**Compound 1**

Pale yellow needles (MeOH): mp 144–145 °C; Rf 0.65; UV (MeOH) λ_max cm⁻¹ 245, 284, 341; AlCl₃ 263, 363, 368; AlCl₃ + HCl 290, 356; NaOMe 291, 314, 398; NaOAc 244, 284, 344; NaOAc + H₂BO₃ 244, 284, 341. MS, m/z (%): 388 (M⁺, 100%), 373 (M⁺ -CH₃, 18%), 358 (M⁺ - OCH₃, 100%). 327 (6%), 259 (4%), 194 (10%), 186 (20%), 165 (5%), 156 (5%), 147 (5%), 127 (14%), 91 (14%), 60 (60%). Compound 1 was identified as desmethylmobeutin (6,7,8,3',4' pentamethoxy-5-hydroxy-flavone) from these spectral data, chemical and physical properties (Kinoshita and Firman, 1996).

**Compound 2**

Pale yellow needles (MeOH): mp 138–139 °C; Rf 0.30; UV (MeOH) λ_max cm⁻¹ 247, 271, 324:
Fractionation of hexane and chloroform extracts by EtOH precipitation provides the enrichment of the antimicrobial activity. This enriched fraction was subjected to silica gel column chromatography using hexane and EtOAc with increasing polarity to obtain three compounds 1–3 in crystalline form. Based on the spectral data compounds 1, 2 and 3 (Fig. 1) are identified as desmethylnobiletin, nobiletin and tangeretin, respectively (Roitman and James, 1985; Horie et al., 1998; Sugiyama et al., 1993).

Conclusions

Hexane, chloroform and acetone extracts of peels of *Citrus reticulata* were found to possess antibacterial activity. Active principles were enriched in to the EtOH soluble fraction of hexane and chloroform extracts. EtOH soluble fraction was found to exhibit a high degree of antibacterial activity and has the potential to be used as a biopreservative.

![Compound 1](image1)

![Compound 2](image2)

![Compound 3](image3)

Fig. 1. Isolated polymethoxylated flavones from citrus peel.


Rhizobial lipopolysaccharide as the receptor in lectin-Rhizobium interaction

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Rhizobial specificity was examined on the basis of interaction between legume lectins (peanut, pea and soybean) and different rhizobial species (various bradyrhizobia specific for peanut, P 14-93 and SB16). Legume lectins showed higher affinity towards host-specific Rhizobium and lipopolysaccharides (LPS) isolated from those particular rhizobia. Two LPS mutants of peanut-specific Bradyrhizobium sp. (Arachis) strain GN17 were isolated by Tn5 mutagenesis. These mutants (GN17M1 and GN17M2) were characterized by their higher hydrophobicity with respect to the parent cells. The hexose content in exopolysaccharides (EPS) and LPS of the mutants was found reduced significantly, whereas 2-keto-3-deoxyoctulosonic acid (Kdo) and uronic acid in LPS were less by 20-times and thrice, respectively in the mutants. Glucose was the major sugar in LPS from all the strains. However, glucosamine appeared only in the mutants. Spectrofluorimetric analysis showed that LPS from GN17M1 mutant interacted most significantly with peanut root agglutinin or lectin (PRA II). The results indicate that LPS on the surface of rhizobial cells is the possible receptor for lectin.

Keywords: Lipopolysaccharide, peanut root lectin, exopolysaccharide, receptor, lectin-Rhizobium interaction

Plant lectins are proteins or glycoproteins of non-immune origin, possessing at least one non-catalytic domain, which binds reversibly to a specific mono- or oligosaccharide. Lectins present in the roots of leguminous plants have been reported to play a role in the recognition of specific Rhizobium sp. The symbiosis between Sinorhizobium meliloti and alfalfa, Rhizobium leguminosarum bv. viciae and pea and Bradyrhizobium japonicum and soybean has shown that the interactions are highly specific. Root lectins which bind to rhizobial surface polysaccharides play a crucial role in binding to only specific rhizobia, paving the way for rhizobial entry and proceed towards nodulation.

Earlier, we reported interaction between Rhizobium cell surface lipopolysaccharide (LPS) and the corresponding host lectin, which was also species-specific. Here, we have studied the binding efficiency between legume lectins from peanut (PRA II), pea (PSL) and soybean (SBA) with the host-specific Rhizobium or with their surface LPS. Further, to verify the role of LPS, the peanut-specific Bradyrhizobium strain GN17 was mutagenized. Parent as well as LPS mutants were characterized with respect to their cell-surface property and their binding efficiency with peanut root agglutinin/lectin (PRA II).

Materials and Methods

Biotinylation reagents, including biotin (long-arm) hydrazide (EDC), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide and hydroxysulphosuccinimide were purchased from Vector Laboratories, USA. Streptavidin peroxidase, o-phenylenediamine dihydrochloride (OPD), polyoxyethyleneborbiton monolaurate (Tween-20), 2-keto-3-deoxyoctulosonic acid (Kdo) and agarose were procured from Sigma Chemical Co., USA. PCR reagents were from Bangalore Gene and all other reagents were of local analytical grade.

Bacterial strains and media

Rhizobial strains were obtained mostly from the Department of Microbiology, Indian Agricultural Research Institute, N. Delhi. IGR 40 was procured from National Research Centre for Groundnuts, Junagarh, Gujrut. Escherichia coli WA803 and Sinorhizobium meliloti Rm101 were provided by Prof. Randhawa, Department of Biotechnology, University of Roorkee. Roorkee. Rhizobia were grown in TY medium and YEM medium at 28°C. Whereas Escherichia coli was grown in TY medium at
37°C. Antibiotics were supplemented as required at following concentrations: streptomycin 100 µg/ml for *Bradyrhizobium* strain GN17 and neomycin 40 µg/ml for *E. coli*. The strain GN17 was selected for Tn5 mutagenesis, as it was found to be neomycin-sensitive, which was the requirement in parent strain for this experiment.

**Purification and biotinylation of lectins**

Peanut root agglutinin (PRA II) was purified according to the method described earlier. Soybean agglutinin (SBA) and pea seed lectin (PSL) were purified by affinity chromatography, using standard procedures. All the three purified lectins were biotinylated by carbodiimide coupling, in accordance with the manufacturer’s guidelines (Vector Laboratories, USA).

**Binding of intact rhizobial cells with biotinylated PRA II**

Each well of polystyrene microtitre plate (NUNC) was coated with 100 µl cell suspension of rhizobial strains (~1 x 10^5 cells/100 µl) grown up to log phase. The plate was centrifuged at 5000 x g for 10 min at 28°C. The supernatant was removed and the plate was kept at 37°C for 2 hr for adsorption. The wells were washed thrice with the washing buffer PT (PBS, pH 7.2 plus 0.02% Tween 20) and 100 µl of blocking buffer consisting of 3% gelatin in PT buffer was added to each well and the plate was kept at room temperature for 1 hr. The blocking buffer was removed and the wells were washed thrice with 100 µl washing buffer. To each well 100 µl (1%) of biotinylated lectin PRA II was added and the plate was incubated for 3 hr at 28°C. The wells were washed with PT buffer, followed by addition of 100 µl streptavidin-HRP conjugate solution in distilled water (1:700) and incubated for 45 min at room temperature. The final colour was developed with o-phenylenediamine dihydrochloride (1 mg ml^-1 in 0.05 M citrate buffer with 5 µl H_2O_2) for 30 min. The reaction was stopped by adding 100 µl of 2 N H_2SO_4 to each well and the absorbance was read at 492 nm in an ELISA reader (Anthos, Austria).

**Isolation of lipopolysaccharide (LPS) and exopolysaccharide**

Rhizobial cultures were grown in YEM broth in an incubator shaker at 28°C for overnight. The cells were harvested (at OD_600 = 1) by centrifugation at 12,000 x g for 30 min. The cell pellet was washed with 0.01 M Tris-HCl (pH 7.2) and LPS was extracted by hot water-phenol method. The supernatant was precipitated by adding 2 vols of acetone. The precipitate obtained was then dissolved in 1% (v/v) H_2SO_4. Exopolysaccharide was quantified by orcinol sulphuric acid method for hexaside estimation.

**Interaction of rhizobial LPS with biotinylated lectins**

Each well of a microtitre plate was coated with 100 µl of rhizobial LPS suspension (1% LPS). The remaining procedure was similar to the one described for binding of rhizobial cells with biotinylated PRA II. Here, besides PRA II, the lectins SBA and PSL were also used.

**Tn5 mutagenesis**

Tn5 mutagenesis of *Bradyrhizobium* strain GN17 was carried out by the method described earlier, using *E. coli* WA803 (pGUS::Tn5 Cm' Neo') grown in TY broth to the stationary phase. The recipient rhizobial cells were also grown to stationary phase in TY medium. Mating was performed by mixing 1x10^7 cells of donor and 1x10^8 cells of recipient on TY plates, followed by incubation at 30°C for 24 hr. Two LPS mutants (GN17M1 and GN17M2) were selected on TY agar plate supplemented with neomycin 400 µg ml^-1 and streptomycin 100 µg ml^-1 after 5 days of incubation at 30°C. Insertion of Tn5 was verified by PCR amplification of an 818 bp product, encoded by a region 1533-2350 bp in Tn5. The genomic DNA of *Bradyrhizobium* strains was isolated following the method of Wilson et al. Amplification was performed by a minicycler (MJ Research, USA) for 25 cycles (94°C - 30 sec, 56°C - 45 sec, 72°C - 1 min 30 sec), using genomic DNA as template and a pair of oligonucleotide primers 5'-GGATGAGGATCGT-TTCGCGAT-3' and 5'-CCCCTCAGAGAAGACT-CGTC-3'. The PCR products from the parent strain and both the mutants were then analyzed on 0.8% (w/v) agarose gels.

**Assay for cell surface hydrophobicity**

The bacterial surface hydrophobicity was tested by two-phase partition method using n-hexadecane as the hydrocarbon phase. Briefly, bacteria grown in YEM broth up to log phase were harvested by centrifugation at 5,000xg for 10 min and resuspended in PBS buffer (pH 7.2) to obtain OD_540 = 1. For qualitative monitoring of hydrophobicity, equal volumes of cell suspension (3 ml) and hexadecane were mixed for each type of cells and were monitored for cell partitioning. For quantitative measurements,
bacterial suspensions (2 ml) were mixed with various volumes of hexadecane in a glass tube with a vortex mixer for 120 sec. Absorbance at 400 nm was recorded of the aqueous phase after allowing 15 min for hexadecane to rise completely. The hydrophobicity was expressed as percentage of initial absorbance of aqueous suspension.

**Analysis of LPS composition**

Neutral hexoses were determined by orcinol-sulphuric acid method. 2-Keto-3-deoxyoctulosonic acid (Kdo) was assayed by thiobarbituric acid method\(^{20}\). Uronic acids were estimated by carbozole method\(^{21}\).

**Carbohydrate analysis**

Native LPS samples (50 μg of hexose) were subjected to hydrolysis with 1% acetic acid at 100°C for 1 hr and centrifuged at 10,000 x g to remove the precipitates. Oligosaccharides in the supernatant were hydrolyzed with 2 M trifluoroacetic acid (TFA) for 16 hr as described earlier\(^{22}\). High performance anion exchange chromatography was performed on a Dionex DX 500 (Dionex, Sunnyvale, CA) with a CarboPac PA 1 column (4 x 250 mm), using pulsed amperometric detector with a gold working electrode. The waveform used for analysis was as follows: 0.05 V for 0.4 sec, 0.065 V for 0.2 sec and -0.15 V for 0.4 sec. Prior to injection of each sample the column was washed with 200 mM NaOH and pre-equilibrated with 18 mM NaOH. The flow rate was 1 ml min\(^{-1}\). Samples were eluted isocratically with 18 mM NaOH and 6-O-methyl-D-galactopyranose (25 μM) was used as an internal standard, as it was not found in any of the samples. The samples were injected at equal time interval since change in time intervals resulted in the shift of retention time of monosaccharides.

**Fluorescence emission spectroscopy**

Fluorescence measurements were taken on a Fluoromax-3 spectrofluorometer (Horiba, USA) at room temperature. An excitation wavelength of 280 nm was used and emission spectra in the range 300-400 nm were recorded. Excitation and emission slit width were 2 nm. The scans were made with an increment of 1 nm and with an integration time of 0.5 sec. The spectra were collected using Data Max Version 2. All the experiments were done, using a protein concentration of 27 μg ml\(^{-1}\) and the LPS concentration of 33 μg ml\(^{-1}\). Peanut root lectin (PRA II) was incubated with LPS for 30 min.

**Results and Discussion**

**Binding of intact cells and LPS with biotinylated lectin**

Cellular enzyme-linked lectin binding assay often permits detection of specific glycans in different cell samples and helps in monitoring alterations of cell-surface glycan expression\(^{23}\). Three different lectins (root lectin PRA II or seed lectins PSL and SBA) and their specific rhizobia species were considered to verify their cross-reactivity. It would be appropriate to use root lectins, as rhizobia come in contact only to legume root lectins. However, seed lectins PSL and SBA are known to have similar sugar specificity as their root lectins and are abundantly available, hence used for the study. Although PSL is having similar monosaccharide-specificity (glucose/mannose) as PRA II, yet, it shows no cross-reactivity with peanut-specific Bradyrhizobium strains as PRA II, but shows maximum binding to the peanut-specific Bradyrhizobium strains only (Table 1). Among peanut-specific strains, maximum binding was with the mutant GN17M1. All the biotinylated lectins (PRA II, PSL and SBA) showed high affinity

<table>
<thead>
<tr>
<th>Strain</th>
<th>Whole cell+PRA II</th>
<th>LPS+PRA II</th>
<th>LPS + PSL</th>
<th>LPS + SBA</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC 92</td>
<td>0.17±0.01</td>
<td>1.63±0.16</td>
<td>0.06±0.00</td>
<td>0.09±0.02</td>
</tr>
<tr>
<td>IGR 40</td>
<td>0.12±0.01</td>
<td>1.41±0.13</td>
<td>0.05±0.00</td>
<td>0.54±0.05</td>
</tr>
<tr>
<td>IGR 92</td>
<td>0.20±0.03</td>
<td>N.D</td>
<td>N.D</td>
<td>N.D</td>
</tr>
<tr>
<td>GN17</td>
<td>0.10±0.01</td>
<td>1.2±0.20</td>
<td>N.D</td>
<td>0.08±0.05</td>
</tr>
<tr>
<td>GN17M1</td>
<td>0.23±0.02</td>
<td>1.70±0.30</td>
<td>N.D</td>
<td>0.11±0.07</td>
</tr>
<tr>
<td>GN17M2</td>
<td>0.08±0.03</td>
<td>1.31±0.30</td>
<td>N.D</td>
<td>0.23±0.05</td>
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<tr>
<td>P 14-93</td>
<td>0.08±0.01</td>
<td>0.08±0.02</td>
<td>1.0±0.04</td>
<td>0.03±0.02</td>
</tr>
<tr>
<td>SB 16</td>
<td>N.D</td>
<td>0.10±0.02</td>
<td>0.07±0.01</td>
<td>2.80±0.03</td>
</tr>
</tbody>
</table>

ND, Not determined

Table 1 — Binding assay of lectins with rhizobial cells and lipopolysaccharide (LPS) from rhizobia
[Values are in O.D. mean of triplicate estimation ± S. D. from three experiments]
towards specific rhizobial cells or its LPSs only. Biotinylated ConA was also tested, but no significant binding was observed (data not presented) towards any of the rhizobial cells or their LPSs. The experiment indicated that the lectins interacted in a species-specific manner and the true receptors for the lectins appeared to be the rhizobial surface LPS.

**Hydrophobicity assays**

Two mutants GN17M1 and GN17M2 appeared to be altered in LPS synthesis from their non-mucoid colony and were thus selected for the study. Tn5 insertion was found only in GN17M1 (Fig. 1, lane 2). No product was amplified with the sample from GN17M2 (data not shown) and considered as spontaneous neomycin-resistant mutant. Usually, less mucoid colonies obtained by Tn5 mutagenesis were found to have defect in LPS molecules. In liquid cultures, mutants showed tendency to auto-agglutinate. Auto-agglutination generally results when bacteria with hydrophobic cell surface tend to adhere to one another resulting in the formation of clumps. The results indicated that the parent strain GN17 was hydrophilic, as it remained in the lower aqueous phase, whereas among the mutants, GN17M2 was seen to be more hydrophobic than GN17M1 (Fig. 2a). The results were validated when quantitative assay for hydrophobicity was carried out, which showed that the mutant strain GN17M2 was the most hydrophobic as 65% of cells were partitioned to n-hexadecane phase. The parent strain GN17 and the mutant GN17M1 partitioned to the hexadecane phase with
2% and 15%, respectively (Fig. 2b). LPS mutants of several bacterial species have been shown to be highly hydrophobic. Alteration in cell surface hydrophobicity of *B. japonicum* is correlated with the loss of O-antigenic part of LPS molecules. The hydrophobic behaviour of the cells is often attributed to the cell surface polysaccharides, EPS and LPS.

Exopolysaccharide contents

Hexose content was maximum in the parent strain GN17 and was 2-3 times higher than that of mutants (Table 2). Among the mutants, the yield of EPS from GN17M1 was more than that from GN17M2. The decrease in yield of EPS from mutants indicated alteration in the cell surface polysaccharides.

Colorimetric analysis of LPS

Results indicated that the ratio of the major LPS constituents varied considerably (Table 2). Hexose, Kdo and uronic acids were found to be maximum in the LPS from parent strain. Hexoses were the major carbohydrate constituents in all the strains. Kdo was much reduced in LPS from the mutants, while uronic acid level in mutants was 3-times less than the parent strain. No significant difference was found in the constituents of LPS between the mutants.

Carbohydrate analysis

The monosaccharide composition of TFA hydrolysate of native LPS for all the three strains is shown in Fig. 3. The monosaccharide constituents and their proportions varied in all the strains. Glucose was the major constituent in LPS from the strain GN17 and its mutants. Mannose was maximum in the mutant GN17M2, followed by GN17M1. Glucosamine was present only in the mutants LPS (Table 3). The ratio of glucose to mannose also varied in all the three strains; this ratio was important, since PRA II is a glucose/mannose-specific lectin. However, oligosaccharides are the true receptors of lectins and effective lectin binding depends on their structure and arrangement on the cell surface.

Binding of PRA II with LPS

PRA II-LPS interaction as examined by fluorescence spectroscopy (Fig. 4) showed that the purified PRA II spectrum \( \lambda_{	ext{max}} \) was around 330 nm, indicating the presence of tryptophan residues. The quenching of spectrum by the lectin was observed, when it was pre-incubated with GN17M1 LPS.

### Table 2 — Colorimetric analysis of *Bradyrhizobium* sp. (*Arachis*) exopolysaccharides (EPS) and lipopolysaccharides (LPS)

<table>
<thead>
<tr>
<th>Strain</th>
<th>EPS (µg/10^8 cells)</th>
<th>LPS (µg/10^8 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hexose</td>
<td>Kdo</td>
</tr>
<tr>
<td>GN17</td>
<td>188.3±13.8</td>
<td>466.6±40.3</td>
</tr>
<tr>
<td>GN17M1</td>
<td>83.9±3.3</td>
<td>333.0±40.0</td>
</tr>
<tr>
<td>GN17M2</td>
<td>57.0±5.7</td>
<td>276.0±20.5</td>
</tr>
</tbody>
</table>

*Fig. 3 — HPAE chromatograms for hydrolysates (2 M TFA, 16 hr, 100°C) of polysaccharides of LPS from peanut specific *Bradyrhizobium* strains GN17 (a): GN17M1 (b) and GN17M2 (c). The identity of the monosaccharides is as follows: (2), 6-O-methyl-galactose (internal standard); (4), galactosamine; (5), glucosamine; (6), galactose; (7), glucose; (8), mannose; and (9), ribose.*
Table 3 — Monosaccharide composition of lipopolysaccharides from Bradyrhizobium sp. (Arachis) strain GN17 and its mutants

<table>
<thead>
<tr>
<th>Monosaccharide</th>
<th>Retention time (min)</th>
<th>Molar ratio of monosaccharides&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>GN17</td>
</tr>
<tr>
<td>Unknown</td>
<td>3.7</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>7.2</td>
<td>—</td>
</tr>
<tr>
<td>Galactosamine</td>
<td>7.7</td>
<td>1.2</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>9.1</td>
<td>—</td>
</tr>
<tr>
<td>Galactose</td>
<td>9.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Glucose</td>
<td>10.4</td>
<td>2.1</td>
</tr>
<tr>
<td>Mannose</td>
<td>11.5</td>
<td>0.4</td>
</tr>
<tr>
<td>Ribose</td>
<td>14.9</td>
<td>1.9</td>
</tr>
</tbody>
</table>

<sup>a</sup>Molar ratio was calculated based on the internal standard (6-O-methyl-D-galactopyranose) = 4.0; ND, Not determined

suggesting a clear binding between the mutant LPS and the lectin. No quenching was observed with lectin samples pre-incubated with other LPS, suggesting the binding of GN17M1 LPS to PRA II to a region with tryptophan residues.

Similar fluorimetric studies indicating the binding of root lectin from Cajanus cajan and rhizobial LPS has been reported earlier. Also, hemagglutination inhibition assay of PRA II with rhizobial LPS has shown that the LPS from parent GN17 was a weak receptor for lectin interaction. Experimental results indicated that the mutagenesis of parent strain rendered the mutant to synthesize LPS molecules suitable for lectin binding. The findings in this study showed that the legume lectins were specific for rhizobia and the receptor for the lectin binding was
the cell surface LPS. However, further studies on LPS structure and linkage analysis are required for understanding the LPS receptor function.

References