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Kinetic sedimentation of *Rhizobium*-aggregates produced by leguminous lectins

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Summary

Lectins from *Canavalia brasiliensis* (CnBr), *Cratylia floribunda* (CFL), *Vatairea macrocarpa* (VML) and *Phaseolus vulgaris* (PHA) aggregate *Rhizobium* bacteria. The relationship between specific sedimentation rate, v' (based on bacterial dry biomass) of bacterial aggregates and lectin concentrations was hyperbolic and showed bacterial surface affinity by lectins. *R. tropici* (Rt), *R. leguminosarum bv. phaseoli* (Rlp) and *R. etli* (Re) surfaces showed predominantly receptors of galactosidic nature. The Rt surfaces showed very high affinities ($k_s = \pm 8.6 \times 10^{-8}$ ag lectin protein ml^{-1}) by Gal-specific lectins (PHA and VML), and very low affinities ($k_s = \pm 4.9 \times 10^{-6}$) by Glc-specific lectins (CnBr and CFL). The Rlp surface had intermediate affinities by lectins. The Re surface showed high affinities by PHA ($k_s = \pm 1.26 \times 10^{-8}$) and intermediate affinities by VML, CnBr and CFL. The relationship between sedimentation specific v'' (based on lectin weight) and bacterial density was a sigmoid and showed lectin affinity by Rt surfaces. The bacterial sedimentation showed positive cooperative binding of lectins. The V''_{max} induced by Glc-specific lectins was ±20% of that produced by Gal-specific lectins. The PHA affinity (k_s = 1.19 mg dry biomass ml^{-1}) was larger than VML (k_s = 1.23). The Glc-specific lectin affinities were smaller than those of Gal-specific. The apparent binding site number of lectins (n_{app}) was: 2.7-PHA; 2.2-VML; 3.2-CFL and 3.2-CnBr. The dissociation constant, k_s, of lectin-binding kinetics decreased with sugar-hapten treatment (10 μM). The n_{app} decreased in PHA and CFL, increasing in VML + sugar-hapten treatment. This study showed that there is a difference in *Rhizobium* surfaces for lectin binding.

Introduction

Lectins are proteins or glycoproteins that bind reversibly and specifically to mono and oligosaccharides, without enzymatic modifications. Lectins are used as probes to study the structural and functional role of cell surface carbohydrates. They require configurational and structural complementarity of carbohydrates for interaction to occur and, in general, these molecules have more than two binding sites, a property that allows cell agglutination or complex carbohydrate precipitation (Wu *et al.* 1997). The lectins often bind to natural polysaccharides with high affinity and their interaction with simple monosaccharides is far weaker (Weis & Drickamer 1996).

Rhizobium leguminosarum bv. *phaseoli, R. etli* and *R. tropici* are gram-negative bacteria which induce nodules and fix atmospheric nitrogen on *Phaseolus vulgaris* [L.] bean plants, in a process characterized by the molecular signal exchanges with specific determinants of the plant (Laeremans & Vanderleyden 1998). According to Kijne *et al.* (1997) and Hirsch (1999), lectins expressed by legume roots may have a role in this signaling process, which is currently under much study. Brelles-Mariño *et al.* (1996), recently reported that pre-incubation of *R. etli* with Phytohemagglutinin (PHA), significantly increased the number of infection threads in bean roots. In addition, Mestrallet *et al.* (1999) showed that pre-incubation of *R. leguminosarum* and bean roots with PHA significantly increased the weight of the nodule and the dry matter and nitrogen content of plants. Lodeiro *et al.* (2000) pre-incubated *Bradyrhizobium* with soybean lectins and found stimulation of bacterial absorption to the host's root surface. Two reviews (Kijne *et al.* 1997; Hirsch 1999) consider the '*lectin recognition hypothesis*'. This hypothesis considers the role of lectins in the process of nodulation in leguminous plants. Lectins may serve as a molecular

glue to hold the *Rhizobium* on the surface of the plant host cell or they may have a more active signalling role. Direct evidence has been adding importance to the role of lectins, for example: (1) *Rhizobium* aggregation in the rhizosphere; (2) *Rhizobium* binding to the root hair tip surface; (3) recognizing lipochitin oligosaccharide; and (4) recognizing lipopolysaccharide-LPS in *Rhizobium* (Kijne *et al*. 1997; Hirsch 1999).

The *Rhizobium* LPS consist of an outer-membrane-anchored hydrophobic lipid A portion, and of a polysaccharide moiety, containing a conserved core (inner and outer core) and an O-antigen variable fragment (Laeremans & Vanderleyden 1998; Price 1999). For successful infection (e.g. bean and soybean), the presence of LPS is strictly required (Dieblod & Noel 1989; Laeremans & Vanderleyden 1998). The studies with peanut lectin binding, with surface LPS isolated from *Bradyrhizobium* species, showed strongly that the lectin–LPS interaction has affinity for specific LPS (Jayaraman & Das 1998).

The activity of lectins is often measured by agglutination assay, and is expressed as a titre which is defined as the reciprocal of the highest lectin dilution that agglutinates bacteria and bacterial polysaccharide–erythrocyte complexes. In this case, the affinity of lectins for carbohydrates can be compared by their agglutination inhibition, however, this method is semiquantitative (Hatakeyama *et al*. 1996). To better approximate lectin–bacteria interaction, quantitative measures of the lectin-binding activity for strain-specific surface carbohydrates, are of fundamental importance. The present study utilized a method based on spectrophotometric measures and analysed the sedimentation kinetics of lectin-dependent *Rhizobium* aggregates, using four different leguminous lectins and three *Rhizobium* strains.

Materials and methods

Bacteria, lectins and reagents

Rhizobium tropici – CIAT899 (Colombia origin centre), *R. leguminosarum* bv. *phaseoli*-USDA 2671 (England origin centre) and *R. etli* – CFN42 (Mexico origin centre), were provided by USDA/ARS/Beltsville *Rhizobium* Germplasm Collection. The legume lectins: *Canavalia brasiliensis* – CnBr, *Cratylia floribunda* – CFL (Glc-specific) and *Vatairea macrocarpa*-VML (Gal-specific) were provided by *BioMol-Lab./Departamento de Bioquímica/UFC-Fortaleza-Brasil*. The PHA lectin (Gal-specific), bovine serum albumin (BSA), MES (2-morpholinoethanesulfonic acid sodium salt) buffer, glucose (Glc), galactose (Gal) and other reagents were acquired from Sigma-Aldrich Chemicals Inc. (St. Louis, MO, USA).

Growth conditions

Growth medium YM [3.4 mM NaCl; 2.9 mM K_2HPO_4; 6.6 mM $MgSO_4$; 10^{-2} mM $FeCl_3$; 55 mM mannitol; 1 g yeast extract l^{-1} – pH 6.8] was used in the culture of *Rhizobium*. The inocula ($\pm 10^6$ bacteria ml^{-1}) were established (20 ml YM medium; 150 rev min^{-1}; at 30 °C for 24 h) and stored at 4 °C. The *Rhizobium* biomass was obtained from cultures at the end of the exponential phase (24 h), from an inoculum of 2×10^3 bacteria ml^{-1}.

Bacterial suspensions and lectin solutions

Rhizobium cells (24 h culture) were centrifuged ($25{,}000 \times g$ for 15 min at 25 °C), re-suspended in MES buffer (10 mM MES, 1 mM $CaCl_2$, 1 mM $MnCl_2$ and 3 mM NaCl) and centrifuged again ($7000 \times g$ for 10 min at 4 °C). These steps were repeated twice. This bacterial suspension was homogenized in a screen nylon monofilament (Schweiz., Seidengazefabrik AG Thal) with 16 μm mesh-opening and used to obtain standardized bacterial suspensions in MES buffer. The $OD_{595} = 0.6$ for the final volume of 120 μl, in U-bottom wells of microtitre plate, was the reference to adjust the maximum bacterial density. The appropriate dilutions of bacterial suspensions were obtained using MES buffer and stored under refrigeration. The counting of total cells in bacterial suspension, were carried out with a Neubauer camera (Hirschmann Techcolor-0.10 mm; 0.0025 mm^2). The dry biomasses (mg ml^{-1}) were determined gravimetrically from standard bacterial suspensions and dried at 100 °C for 6 h. Lectin solutions (0.002 g ml^{-1}) in MES buffer, were incubated at 43 °C (ultrasonic cleaner) and at room temperature for 30 min each. At the end, the lectin solutions were centrifuged ($22{,}000 \times g$ for 5 min at room temperature) and the pellets discarded. Protein determinations in the supernatant were carried out according to the Bradford method, using BSA as reference. The monosaccharide solutions were prepared in MES buffer.

pH effects in sedimentation

The standardized lectin solutions (CnBr, CFL, PHA and VML) and bacterial suspensions (3.04 mg dry biomass ml^{-1}) in MES buffer, were used for pH 5.5 and 6.7. The lectin concentration tests ($\leq 10^{-1}$ ag ml^{-1}) were obtained by serial dilution, and samples of dilutions (60 μl) were transferred to microtiter plate wells. The refrigerated bacterial suspension was pre-incubated (30 °C to 5 min), homogenized (vortex) and samples (60 μl) simultaneously dispensed in the wells (with or without lectin). Therefore soon after, absorbance (A_{595}) was monitored (25 s of interval for 5 min) in a spectrophotometer microplate reader.

Lectin–Rhizobium interaction – affinity of bacterial surfaces by lectins

Three experiments were accomplished (one for bacterial strain), each with three replicates. The treatments were: (1) CnBr, (2) CFL, (3) VML and (4) PHA lectin

solutions (< 0.05 ag ml^{-1}) and, (5) control (without lectin) in MES buffer (pH 6.7). The lectin solutions (2× final concentration) were established in 2-fold serial dilution using a microtitre plate. These samples of standardized–homogenized bacterial suspensions were simultaneously dispensed in well columns, some with and some without lectin treatments and their absorbance monitored.

Lectin–Rhizobium interaction – affinity of lectins for bacterial surface

The experiment was carried out to evaluate the sedimentation of bacterial suspensions under lectin and lectin + sugar-hapten effects (Glc or Gal) with the *R. tropici* strain. This was repeated three times. The treatments were: lectin (1) with, and (2) without sugar-hapten specific (10 μM), and (3) control without lectin. The final lectin concentrations were: 3×10^{-3} ag CFL ml^{-1}; 4×10^{-3} ag CnBr ml^{-1}, 10^{-3} ag PHA ml^{-1} and 6×10^{-4} ag VML ml^{-1}. The lectin solutions with or without sugar-hapten (both with 2× final concentration) were obtained by serial dilution, respectively, in MES and MES + 20 μM sugar-hapten, in a microtiter plate. The standardized and refrigerated bacterial suspensions were pre-incubated, homogenized and diluted (for 2× final concentration), by 2-fold serial dilution in MES buffer (60 μl), in a microtitre plate. The bacterial suspensions were simultaneously added to the wells with lectin and lectin + sugar-hapten treatments, and absorbance was monitored.

Instrumentation

The automated reading at 595 nm (spectrophotometer, Bio-Rad Microplate Reader-Model 550) was used to obtain the absorbance variation of bacterial suspensions with lectin ($A_{595}^{\text{treatment}}$) and without lectin ($A_{595}^{\text{control}}$) effects (Figure 1). Well column readings of the microtitre plate were monitored. The average of the readings for lectin and control treatment were used to determine ΔA_{595}^{1}. The ΔA_{595} dynamic time-dependent (t) can be described by quadratic equation (1).

$$\Delta A_{595} = a + bt + ct^2 \quad (1)$$

The initial sedimentation rate (v), in ΔA_{595} s^{-1}, was calculated from the slope at the inflection point of the ΔA_{595} curve by the first derivate of equation (1). The specific initial sedimentation rates, based on bacterial biomass ($v' - \Delta A_{595}$ s^{-1} mg dry biomass^{-1}) and on lectin weight ($v'' - \Delta A_{595}$ s^{-1} ag lectin protein^{-1}) were used for the sedimentation kinetic analysis. Double-reciprocal plots, Scatchard plots and least square regression of data, were used to estimate kinetic parameters with the help of Statistic soft (Statsoft Inc., Tulsa).

[1] $\Delta A_{595} = A_{595}^{\text{Treatment}} - A_{595}^{\text{Control}}$.

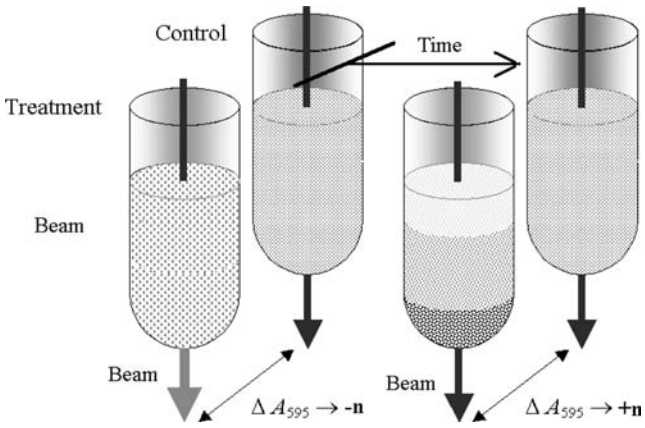

Figure 1. Scheme used to obtain a differential absorbance ($\Delta A_{595} = A_{595}^{\text{treatment}} - A_{595}^{\text{control}}$) variation in sedimentation of bacterial aggregates induced by lectins. The vertical beam attenuation in bacterial suspension (60 μl) contained in wells of microtitre plate.

Lectin level and bacterial density effects on total bacterial aggregates

The production of bacterial aggregates (diameter > 7 μm) by CnBr, were evaluated for *R. tropici* in two ways. In the first, intermediate bacterial density (2.02 mg dry biomass ml^{-1}) was tested for CnBr concentration of 10^{-2} and 10^{-7} ag ml^{-1}, with and without Glc (10 μM). In the second, concentration of 10^{-2} ag CnBr ml^{-1}, with and without Glc, was tested for bacterial densities of 1.01 and 3.03 mg dry biomass ml^{-1}. The bacterial suspensions and lectin treatments were built as before mentioned (pH 6.7). The direct countings of bacterial aggregates were done with Neubauer camera.

Results and discussion

The experimental ΔA_{595} data for pH and lectin effects are shown in Figure 2. The time-dependent ΔA_{595} dynamics are being hypothesized to be due to two events. The first event is bacterial aggregate formation by lectin or cell–cell contact effect. This induces a decrease in the $A_{595}^{\text{treatment}}$, as opposed to A_{595}^{control}. It is associated with a decrease in the light-interfering bacterial surface. The second event is the approach of bacterial aggregates in the course of its sedimentation. This last event induces an increase in the time-dependent $A_{595}^{\text{treatment}}$. This is related to an increase in the light-interfering surface in the sedimentation front. Both events happen simultaneously and drive the ΔA_{595} according to the quadratic model. In the first stage of the model we observed that ΔA_{595} increased to the maximum constant rate. The aggregates-approach and cell–cell contact events reached a stationary state. In the second stage, the ΔA_{595} increased in smaller rates at a time, and the aggregation reaction tended towards a new equilibrium, which decreased the cell–cell contact event (free lectin decrease) and, consequently, decreased the aggregates-approach event. The extension and intensity

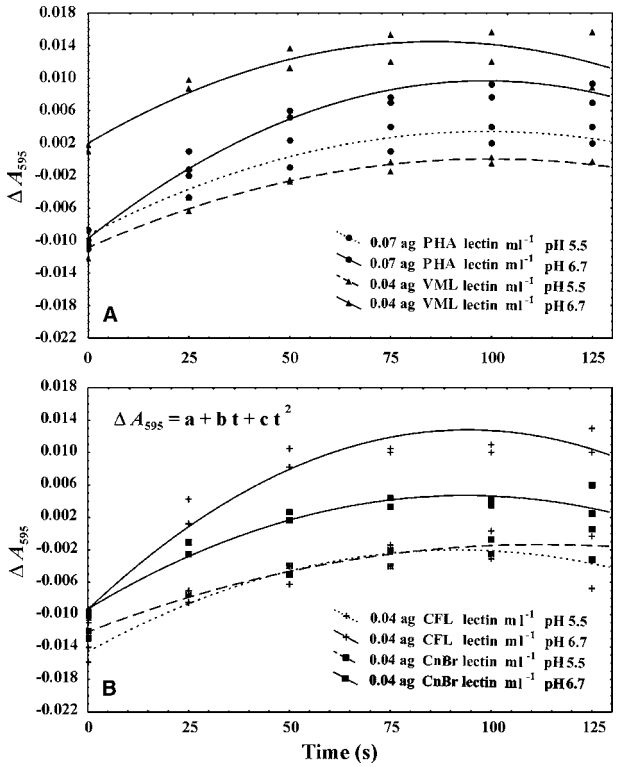

Figure 2. Time-dependent sedimentation of *Rhizobium tropici* aggregates under pH and lectin effects. The lectin used were: (A) *Phaseolus vulgaris* – PHA and *Vatairea macrocarpa* – VML; and (B) *Cratylia floribunda* – CFL and *Canavalian brasiliensis* – CnBr. The $\Delta A_{595} = A_{595}^{\text{treatment}} - A_{595}^{\text{control}}$. The bacterial density was 1.52 mg dry biomass ml^{-1}.

of these events showed the ΔA_{595} dynamic with pH and type of lectin dependence.

The pH increase from 5.5 to 6.7 showed an increase in the speed of *v*, in bacterial suspension for four tested lectins. The CnBr (2.19–4.00 ΔA_{595} s^{-1}) and CFL (3.26–5.82 ΔA_{595} s^{-1}), increased $\pm 80\%$, however, the PHA (3.23–4.94 ΔA_{595} s^{-1}) and VML (2.73–3.62 ΔA_{595} s^{-1}) had an increased of $\pm 42\%$. Among these, Gal-specific lectins showed the smallest *v* variations according to pH change. For Calvete *et al.* (1999), the CFL oligomerization was pH-dependent, showing that there are homogeneous tetramers in pH > 5.5. Grangeiro *et al.* (1997) reported that CnBr increased molecular mass when pH increased to 5.5, suggesting a mixture of dimer and tetramer forms. Cavada *et al.* (1998), observed that VML is predominantly a dimeric protein in neutral pH, and that tetramers and larger aggregates of VML were also present. PHA is known to be a non-pH-dependent tetrameric glycoprotein (Loris *et al.* 1998). The similarity of the behaviour of the tested lectins in neutral pH (oligomeric forms), can partially explain the high *v* and, consequently, the increase of lectin affinity by the polysaccharide-receptors (Weis & Drickamer 1996).

The sensibility observed in the detection of *v* for small concentration of lectins used can be attributed to additional effects: (1) high affinities of the lectin by natural polysaccharides (Weis & Drickamer 1996); (2) the displacement of bacterial bulk associated to high sensibility of spectrophotometric measures; (3) the high bacterial density used which accelerates the aggregation process (Bos *et al.* 1999); and (4) the U-bottom well which favours aggregate approximation in the course of sedimentation towards the reading zone.

Figure 3 shows the initial sedimentation specific v' for three *Rhizobium* species under lectin effect (log–log scale plots). The v' and lectin concentration relationship follows the Michaelis–Menten kinetic and shows affinity of bacterial surfaces for lectins. In general, the maximum specific initial sedimentation (V'_{max}) showed around $3.0 \pm 1.0 \times 10^{-4}$ ΔA_{595} (s^{-1}) mg dry $biomass^{-1}$, and the dissociation constant (k_s) varied between 10^{-8} and 10^{-6} ag lectin protein ml^{-1}.

The V'_{max} is an indirect measure of lectin receptor concentration on the bacterial surface and k_s is related to receptor affinity by lectin. These results suggest that there are *Rhizobium* surfaces which interact with the four lectins tested and that they differ in the quanti-qualitative forms. The *R. tropici* (Rt), *R. leguminosarum bv. phaseoli* (Rlp) and *R. etli* (Re) surfaces, present predominantly galactosidic receptors. The galactosidic receptors' concentration is high in Rt surface (3.7×10^{-4} and 4.0×10^{-4} ΔA_{595} s^{-1} mg dry $biomass^{-1}$ for VML and PHA). However, these receptors showed very high (2.03×10^{-8} ag lectin protein ml^{-1}) and high (1.51×10^{-7} ag lectin protein ml^{-1}) affinities by VML and PHA lectins. For the Re surface, galactosidic receptors showed high (7.50×10^{-7} ag lectin protein ml^{-1}) and very high (1.26×10^{-8} ag lectin protein ml^{-1}) affinities for VML and PHA lectins. However, the Re surface showed inferior concentrations of these receptors (3.3×10^{-4} ΔA_{595} s^{-1} mg dry $biomass^{-1}$). The Rlp surface was similar to the Re surface with regards to VML receptor concentration, although with less affinity (1.94×10^{-7} ag lectin protein ml^{-1}). The PHA receptors in the Rlp surface showed a very low concentration (2.4×10^{-4} ΔA_{595} s^{-1} mg dry $biomass^{-1}$), but similar affinity with those in the Rt surface. For glucose-specific lectins, high concentrations of CFL and CnBr receptors were observed on the Rt surface (3.4×10^{-4} and 3.9×10^{-4} ΔA_{595} s^{-1} mg dry $biomass^{-1}$) with very small affinities ($\pm 4.9 \times 10^{-6}$ ag lectin protein ml^{-1}). Re and Rlp surfaces showed high affinities with CFL (1.07 and 6.42×10^{-7} ag lectin protein ml^{-1}) and CnBr (6.49 and 5.96×10^{-7} ag lectin protein ml^{-1}) lectins. However, theses strains showed inferior concentrations of glucosidic receptors ($<2.6 \times 10^{-4}$ ΔA_{595} s^{-1} mg dry $biomass^{-1}$) on their surfaces. In general, when both V_{max} and k_s parameters of binding were considered, a ranking of bacterial surface affinity by lectins could be defined. For Gal-specific lectin we have: Rt > Rlp = Re. For Glc-specific lectin, we have the following binding preference: Re > Rlp > Rt. According to Carlson *et al.* (1995), the LPS core region is highly conserved between Rlp and Re, and these are generally referred to as the core trisaccharide unit →4)-α-D-GlcpA-(1→4)-[α-3-O-Me-6-deoxy-Talp-(1→3)]-α-L-Fucp-(1→ and core tetrasaccharide unit α-D-Gal-(1→6)-[α-D-GalA-(1→4)]-

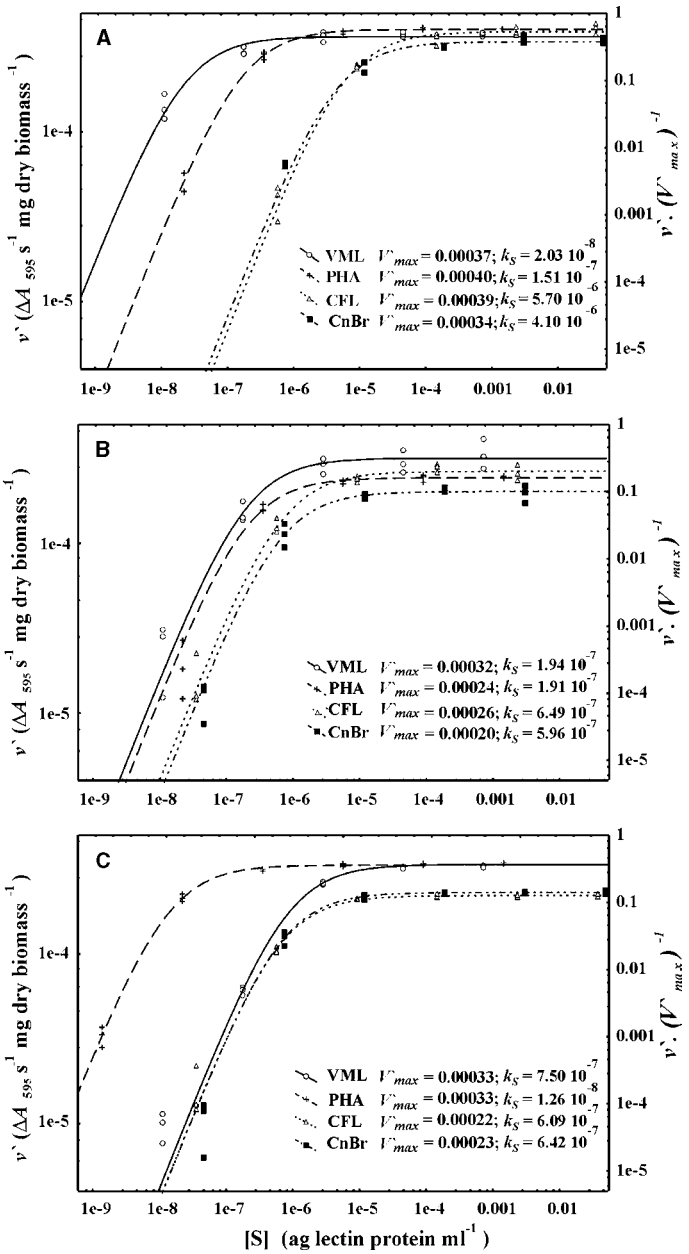

Figure 3. Initial specific sedimentation (v') of bacterial aggregates for three *Rhizobium* species under lectin effects. The *Rhizobium* were: (A) *R. tropici*; (B) *R. leguminosarum* bv. *phaseoli*; and (C) *R. etli* (all with ±2.5 mg dry biomass ml^{-1}). The tested lectins were: *Vatairea macrocarpa* – VML, *Phaseolus vulgaris* – PHA, *Cratylia floribunda* – CFL and *Canavalia brasiliensis* – CnBr. The V'_{max} and k_s are the Michaelis kinetic parameters.

α-D-Man-(1→5)-Kdo. The structure of the O-antigen in the Re CE3 strain is a polymer of a repeating unit of →4)-α-D-GlcpA-(1→4)-[α-3-O-Me-6-deoxy-Talp-(1→3)]-α-L-Fucp-(1→ (Forsberg *et al.* 2000). The Rt CIAT899 O-antigen, is a polymer of a repeating unit of →4)-β-D-Glcp-(1→3)-[α-2-O-Ac-6-deoxy-Talp-(1→3)]-α-L-Fucp-(1→ (Gil-Serrano *et al.* 1995). For the Tal/Gal interaction with lectins, the docking experiments with Gal showed that VML has a network of connected hydrogen bonds (residues Asp87, Gly105, Asn129, Leu213 and Ser214) to 3-OH, 4-OH and 6-OH, from the pyranose ring (Ramos *et al.* 1999). However, the epimeric 2-OH of Tal probably does not interact with the binding site of VML and PHA, and it does not participate in the discrimination of receptors (Weis & Drickamer 1995; Loris *et al.* 1998).

In addition to the LPS structural differences between *Rhizobium* species, there are differences in symbiotic plasmids, nodulation host ranges and origin centre of those bacteria. Rlp and Re (with genetic instability) possess multiple copies of *nif* genes and have a relative narrow host range, centering on beans; while Rt (with genetic stability) has a single copy of the *nifH* gene and nodulates bean plants, as well as many others tropical legumes (Eardly *et al.* 1995). In this way, we can infer that there is *Rhizobium* differentiation based on bacterial surface affinity ranking by lectins, showing an apparent degree of correlation with nodulated host ranges by

Rhizobium sp. This could be associated with the bacterial LPS configuration, which could provide specificity to the bean nodulation process.

Figure 4 shows specific initial sedimentation (v'') for Rt aggregates under bacterial density × lectin type interaction effects (liner-liner scale plots). This lectin effects on v'' were evaluated with or without sugar-hapten. For the lectins tested, this v'' vs. bacterial density relationship, was kinetic with sigmoidal behaviour, which shows affinity of lectins by bacterial surfaces. The Scatchard plots (not shown) of lectin-dependent specific sedimentation data, indicate that there is a positive cooperative binding behaviour of lectin tested by bacterial surface. It is important to emphasize that the v'' responds with more sensitivity to changes in bacterial density, if the binding mechanism is sigmoidal. Similar binding behaviours were obtained by Williams *et al.* (1992) with Con A lectin. They proposed positive cooperativity for binding of the trisaccharide, based on several points of a Scatchard plot (microcalorimetry data), which exhibited downward deviations at low fractional occupancy of the Con A. In this paper, different sigmoidicities of v'' were observed for lectins tested in *R. tropici*. For PHA and VML lectins, the V''_{max} estimates were 0.84 and 1.24 ΔA_{595} s^{-1} ag lectin protein^{-1} with bacterial density > 3 mg dry biomass ml^{-1}. The v'' variation associated to bacterial density in the PHA, showed a larger cooperativity degree ($n_{app} = 2.70$) and similar affinity[2] ($ks = 1.19$ mg dry biomass ml^{-1}) by bacterial surface of that demonstrated by VML ($n_{app} = 2.24$ and $k_s = 1.23$ mg dry biomass ml^{-1}). The Gal-hapten (10 μM) co-administration with specific lectins, affected differently the estimated kinetic parameters. The cooperative binding of PHA under Gal effect decreased to 2.03. The V''_{max} estimated for PHA was not affected by Gal, however, for VML, the V''_{max} induced decreased to 0.88 ΔA_{595} s^{-1} ag lectin protein^{-1}. The v'' cooperative degree under the VML binding effect, increased with Gal to 2.82. For these lectins, there was a decrease of affinity by bacterial surface (±1.21 to ±2.13 mg dry biomass ml^{-1}) under Gal effect. The Glc-specific lectins demonstrated different behaviour in the kinetic parameters of v'' (Figure 3). The V''_{max} induced by CFL and CnBr, resulted in ±20% (±0.219 ΔA_{595} - s^{-1} ag lectin protein^{-1}) of that obtained with Gal-specific lectins. The CFL showed larger V''_{max} (0.26 ΔA_{595} s^{-1} ag lectin protein^{-1}) than that of CnBr (0.21 ΔA_{595} s^{-1} ag lectin protein^{-1}). Similar CFL and CnBr

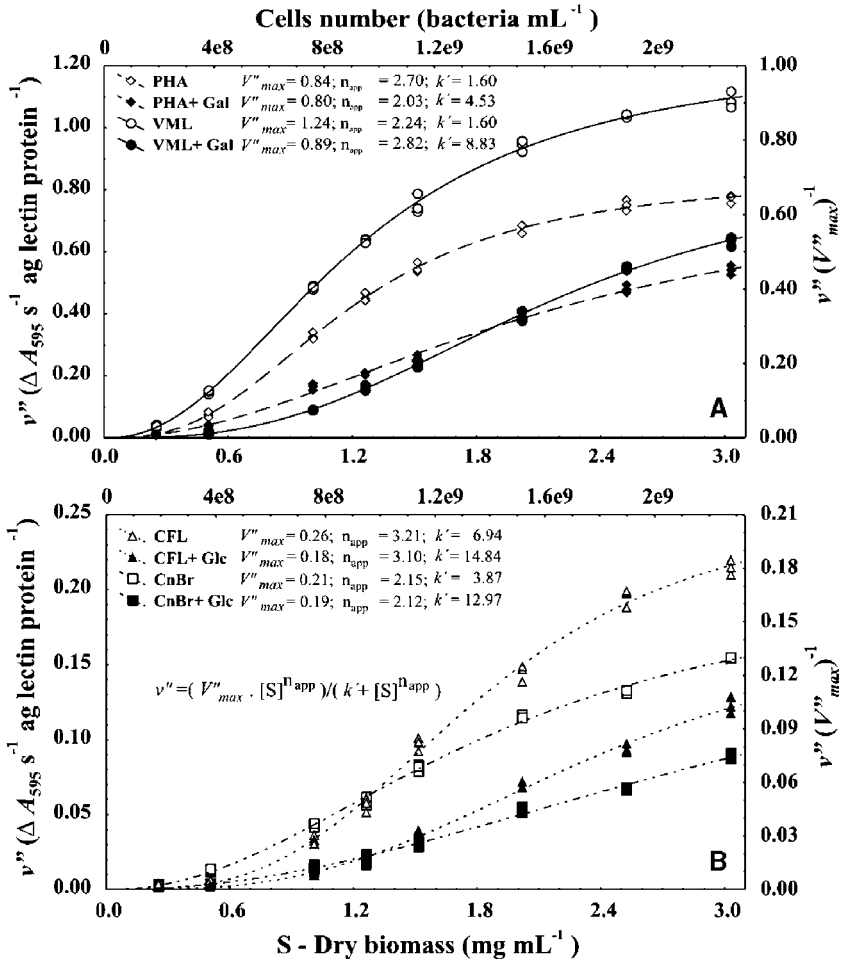

Figure 4. Initial specific sedimentation (v'') of *Rhizobium tropici* aggregates for bacterial density and lectin effects. The lectins were: (A) *Phaseolus vulgaris* – PHA and *Vatairea macrocarpa* – VML; and (B) *Cratylia floribunda* – CFL and *Canavalia brasiliensis* – CnBr (all from 10^{-4} to 10^{-3} ag ml^{-1}). The Galactose (Gal) and Glucose (Glc), were the sugar-haptens used. The V''_{max} n_{app}, and k', are Hill kinetic parameters.

Table 1. The total number of *Rhizobium tropici* CIAT899 aggregates induced by lectin: bacterial density and Canavalian brasiliensis (CnBr) concentration effects

Treatments	Lectin concentration[a] (ag lectin protein ml^{-1})		Bacterial density[b] (mg dry biomass ml^{-1})	
	7.4×10^{-7}	4.9×10^{-2}	1.01	3.03
	10^6 aggregates ml^{-1}			
CnBr	[c]3.14 ± 0.11	4.24 ± 0.11	2.00 ± 0.06	6.25 ± 0.09
CnBr + 10 μM Glucose	2.67 ± 0.16	3.79 ± 0.18	1.92 ± 0.08	5.81 ± 0.08
Control[d]	3.15 ± 0.11	3.70 ± 0.08	1.92 ± 0.05	4.94 ± 0.06

[a] used bacterial density: 2.02 mg dry biomass ml^{-1},
[b] used lectin concentration: 4.9×10^{-2} ag lectin protein ml^{-1},
[c] Average of 5 replicates,
[d] Without lectin.

affinity was observed. However, in CnBr there was a larger cooperative degree ($n_{app} = 3.21$) than in CFL ($n_{app} = 2.15$), in relation to sedimentation reaction. V''_{max} and the affinity to bacterial surface of CFL and CnBr, decreased with Glucose-hapten (10 μM). However, the reduction of V''_{max} in CnBr was very little. The cooperativity behaviour of v'' with CFL and CnBr did not show detectable Glc effects. These results indicate that: (1) VML and PHA showed greater affinities by Rt surface than CFL and CnBr; (2) lectin-dependent *Rhizobium* aggregation is affected by sugar-hapten; (3) the v''associated to bacterial density showed a positive cooperative binding mechanism of lectins; (4) the cooperative binding data, indicates that there are two and three binding sites, respectively, in CnBr and in PHA-VML-CFL, all with strong interaction by Rt surface receptors. The binding inhibition observed through the kinetic v'' by sugar-hapten, confirms that the process in study is induced by the lectinic interaction. The sugar-hapten does not affect the V''_{max} of PHA and CnBr treatments, indicating that there is a competitive inhibition mechanism or similarity between the sugar-hapten and the Rt surface receptors. However, the sugar-hapten affects the V''_{max} of VML and CFL treatments, indicating that there is a non-competitive inhibition, that is, that the sugar-hapten does not imitate the surface receptors. The biological significance of the cooperative binding of lectins to the bacterial surface, can be a strategy for strain selection and/or size increase of bacterial inoculum (by bacterial adhesion and/or aggregation increase in infection sites of plant roots) that can attribute competitive advantage to a particular strain. This can improve the efficiency of the pre-infection phase, to establish *Rhizobium*-legume symbiosis, as observed in Brelles-Mariño *et al.* (1996), Mestrallet *et al.* (1999) and Lodeiro *et al.* (2000).

Table 1 shows the results of the total number of bacterial-aggregates (NBA_T) dependent on lectin concentration and bacterial density. These control treatments (without lectin) in both assays, were the bacterial aggregation background. For intermediate bacterial density (2.02 mg dry biomass ml^{-1}), the concentration of 4.9×10^{-2} ag CnBr ml^{-1}, increased the NBA_T in relation to control treatment. The Glc, co-administered (10 μM) with 4.9×10^{-2} ag CnBr ml^{-1}, showed an NBA_T similar to the control treatment. However, when Glc was co-administered with 7.4×10^{-7} ag CnBr ml^{-1}, the NBA_T was smaller than that induced by the same concentration of the lectin and its control treatment. It is probable that bacterial lectins are contributing to the aggregation background. For bacterial density of 1.01 mg dry biomass ml^{-1}, the lectin effects in bacterial aggregation were not clearly observed. However, for high bacterial density (3.03 mg dry biomass ml^{-1}), the CnBr and CnBr + Glc treatments showed larger NBA_T than that of the control. The CnBr + Glc treatment induced a smaller NBA_T than that of the CnBr treatment. The bacterial aggregation induced by CnBr is consistent with the bacterial sedimentation study, and therefore it is a direct evidence of this process. The spectrophotometric measurements associated to sedimentation showed sensibility and closer approximation to particle mass change. The kinetic studies here reported will assist future characterization of the *Rhizobium* surface.

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