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A Lectin from *Sesbania aculeata* (*Dhaincha*) Roots and Its Possible Function

S. Biswas[1], A. Saroha[1,2], and H. R. Das[1]*

[1]*Institute of Genomics and Integrative Biology, Delhi University Campus, Mall Road, Delhi 110007, India; fax: 91-11-27667471; E-mail: hdas@igib.res.in; hasidas@yahoo.com*
[2]*Faculty of Natural Sciences, West Bengal University of Technology, Kolkata 700 064, India*

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Abstract—A lectin was isolated from the roots of *Sesbania aculeata*. This is a glucose specific lectin having 39 kDa subunit molecular weight. The expression of this lectin was found to be developmentally regulated and observed to be the highest in the second week. The lectin was purified by affinity chromatography using Sephadex G-50 and found to have 28% homology with *Arabidopsis thaliana* lectin-like protein (accession No. CAA62665). The lectin binds with lipopolysaccharide isolated from different rhizobial strains indicating the plants interaction with multiple rhizobial species.

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Key words: *Sesbania*, root lectin, lipopolysaccharides

Lectins are carbohydrate-binding proteins of non-immune origin that agglutinate cells or precipitate polysaccharides or glycoconjugates [1]. They are most abundant in seeds of various legumes and are reported to account for 1-10% of the total seed protein [2]. The lectins in the vegetative tissues of legumes may or may not be identical to their seed counterparts. The qualitative and quantitative differences in lectins localized among different tissues and organs within a single genotype of legume suggested that lectins are developmentally regulated [3, 4]. They may be involved in the growth and differentiation of plants. The root lectin has been reported to be an important determinant for host specificity [5, 6]. The interactions between lectins and carbohydrates of exogenous origin are considered as the determining factor in the establishment of the plant–microbe interaction [6, 7]. Therefore, the symbiotic rhizobial strains elicit the formation of root or stem nodules on host legumes where they colonize following a complex infection process [8, 9]. It has been reported that *Sesbania* roots establish symbiotic interactions with diverse rhizobial taxa (*Sinorhizobium saheli*, *S. meliloti*, *Rhizobium huautlense*) whereas stem showed interaction strictly with *Azorhizobium caulinodans* [10]. *Sesbania aculeata* has been widely used as green manure to improve soil fertility [11-13] and is a native to northern India and neighboring parts of Asia. This has been cultivated for various purposes. It was also reported to be a cross-infective plant [14] and hence it has been hypothesized that *S. aculeata* might contain novel lectins to facilitate the cross infection which subsequently might be important for increasing the fertility of the soil. The lectins from the seeds of *S. arabica* and *S. bispinosa* have already been reported [15, 16]. However, the lectins or lectin-like molecules from other vegetative parts of *Sesbania* species including *S. aculeata* have not yet been reported. It is, therefore, pertinent to investigate the lectins from the roots of *S. aculeata* for application in agrobiotechnology and biotechnology.

MATERIALS AND METHODS

Preparation of *S. aculeata* root extract. Seeds of *S. aculeata* cultivar H-1(DG-1) were first washed with ADW (autoclaved distilled water) and placed on moist autoclaved filter paper in a Petri dish for germination. The germinated seeds were sown in the soil in the IGIB garden (Delhi, India). The plants were uprooted after one,

Abbreviations: ADW, autoclaved distilled water; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; ELLBA, enzyme linked lectin binding assay; HA, hemagglutination; LPS, lipopolysaccharide; PMF, peptide mass fingerprinting; RRBC, rabbit erythrocytes; SRA, *Sesbania* root agglutinin; TBS, Tris-buffer solution.

* To whom correspondence should be addressed.

two, three, and four weeks of growth and washed thoroughly with tap water followed by ADW. The roots were excised, crushed, and homogenized in TBS (10 mM Tris-HCl, 0.15 M NaCl), pH 7.2, containing 0.5 μg/ml of PMSF. The homogenate was centrifuged at 23,700*g* for 1 h at 4°C, and the supernatant was dialyzed overnight against 1 mM TBS (pH 7.2). The protein concentration of root extract (supernatant) was determined by the Lowry method [17].

Hemagglutination (HA) of *Sesbania* extracts. The lectin activity of the root extract (supernatant) was tested using rabbit erythrocytes (RRBC) as described previously [18]. A 10-μl sample was mixed with 10 μl of 4% RRBC suspension, incubated at room temperature for 30 min, and observed for HA activity under a microscope. The reciprocal of the highest dilution of the lectin or the extract giving complete agglutination of RRBC at 30°C for 30 min is taken as HA titer. Unless otherwise mentioned, one HA unit is defined as the minimum amount of lectin required for 100% agglutination of RRBC under the above assay conditions. Specific activity of the lectin is defined as the number of HA units per mg protein.

Sugar specificity of lectin. The carbohydrate specificity of *S. aculeata* root lectin was determined by incubating 1 HA unit of lectin (10 μl) initially with an equal volume of serially diluted sugars (arabinose, D-glucose, N-acetyl-D-glucosamine, D-galactose, N-acetyl-D-galactosamine, cellobiose, fructose, D-mannose, L-fucose, raffinose, ribose, maltose, xylose, α-methyl-D-mannopyranoside, mannose, lactose, methyl-1,6-deoxy-α-D-glucopyranoside from Sigma (USA)) in TBS at 30°C for 15 min. This was further incubated for 30 min at 30°C after adding 20 μl of 4% RRBC suspension and examined for agglutination.

Purification of *S. aculeata* lectin. Affinity column chromatography was performed to purify the lectin of *S. aculeata* root extract using Sephadex G-50 [18]. The matrix was soaked in ADW for 2 h, loaded in the column (15 × 2.5 cm), equilibrated with TBS (10 mM, pH 7.2), and in each batch the root extract (15 mg) was added onto the column. The unbound material was washed and the bound protein was eluted with TBS containing 100 mM glucose. Protein contents in the fractions were monitored at 280 nm spectrophotometrically. The protein positive fractions were pooled, dialyzed against TBS (1 mM), lyophilized, and stored at 4°C. The root lectin thus obtained was designated as *Sesbania* root agglutinin (SRA). Protein concentration and HA activity of SRA was determined as described earlier [17, 18].

Gel electrophoresis. Native polyacrylamide slab gel electrophoresis was performed by running purified lectin along with native marker in 8% gel at 10 mA in electrode buffer containing 250 mM glycine and 25 mM Tris [19]. The SDS-polyacrylamide slab gel electrophoresis was performed with protein molecular weight markers in a 12% SDS gel following the method of Laemmli [20]. Electrophoresis was carried out at 10 mA in the above electrode buffer containing 0.1% SDS (Sigma). The gels were stained by Coomassie Brilliant Blue (0.02% Coomassie Brilliant Blue R-250 in 35% methanol, 5% acetic acid).

Effect of metal ions on hemagglutination activity of SRA. The SRA was dialyzed extensively against TBS (1 mM, pH 7.2). An aliquot of 3 μg of SRA was incubated with different concentrations (from 0.06 to 2 mM) of chloride salts of Mg^{2+}, Ca^{2+}, and Mn^{2+} at 30°C for 30 min and then supplemented with 25 μl of 4% (v/v) RRBC and assayed for HA activity [18].

Two-dimensional gel electrophoresis (2D-PAGE). A Protein IEF cell (Bio-Rad, USA) was used to perform the first dimension isoelectrofocusing [21]. The lyophilized SRA purified by the affinity columns was dissolved in 125 μl of rehydration buffer containing 8 M urea, 2% Triton X-100, 65 mM dithiothreitol (Sigma), and 0.2% ampholytes (Bio-Rad). The sample was loaded on ready-made IPG strips (7 cm, pH 4-7) and run according to the manufacturer's instructions. The second dimension SDS-PAGE was performed by using a Mini-Protean II slab cell vertical system from Bio-Rad. The strip was equilibrated for 20 min in SDS equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 4% (w/v) SDS, 20% (w/v) glycerol) containing 2% (w/v) dithiothreitol and then for 20 min in the same buffer containing 2.5% (w/v) iodoacetamide. The equilibrated strip was then run on 12% polyacrylamide SDS gel and proteins were separated [21].

The gel was then fixed in 5% methanol and 1% acetic acid in ADW for 15 min followed by 10 min washing (thrice) and sensitized with sodium thiosulfate (90 sec) followed by 30 sec washing (thrice) with ADW. Gel was stained (30 min) in silver nitrate (200 mg/100 ml), washed (60 sec) with ADW (thrice), and developed with sodium carbonate (6 g/100 ml ADW) containing 50 μl formaldehyde and 2 ml sodium thiosulfate. The approximate p*I* value of SRA was estimated by analyzing the silver stained gel visually. The gel spot was cut out with a sterile blade and stored at −20°C until being analyzed by MALDI-TOF.

Matrix-assisted laser desorption ionization (MALDI) analysis. The protein spot of purified SRA from 2D gel was subjected to in-gel digestion with trypsin, and the tryptic peptides were analyzed by MALDI-TOF to check for their masses [22]. The digest mixture and 10 mg/ml HCCA matrix (α-cyanohydroxycinnamic acid; Bruker Daltonics, Germany) prepared in 1 : 1 acetonitrile and 0.1% TFA was mixed in 1 : 1 ratio. The above mixture (0.8 μl) was loaded on the MALDI ground steel target plate and the sample was allowed to dry at room temperature for 10 min. The PMF (peptide mass fingerprinting) spectrum was acquired on a MALDI-TOF/TOF mass spectrometer (Autoflex II; Bruker Daltonics) equipped with a pulsed nitrogen laser (337 nm) in positive reflectron mode. Operating conditions were as follows: ion source 1 voltage – 19 kV, ion source 2 voltage – 16.5 kV, lens volt-

age – 20 kV, optimized pulsed ion extraction time – 120 nsec, matrix suppression – 400 Da. Spectra were acquired in 800-3500 m/z range and 200 shots were averaged. The spectra were externally calibrated using Bruker's peptide mix containing angiotensin I_$(M+H)^+$mono = 1296.68480, bombesin$(M+H)^+$mono = 1347.73450, ACTH_Clip_(1-17)$(M+H)^+$mono = 2093.08620, somatostatin(28)$(M+H)^+$mono = 3147.47100. The PMF spectrum was searched against the MASCOT database (Matrix Sciences, UK) using carbamidomethylation of cysteine and oxidation of methionine as modifications of peptides (100 ppm mass tolerance and 1 missed cleavage). The intact mass of the lectin was confirmed by acquiring the mass spectrum in a linear positive mode and detector bias gating below mass of 4000 Da using sinapinic acid as a matrix prepared in 1 : 1 acetonitrile and 0.1% TFA.

Biotinylation of lectins. The purified lectin (1 mg) was biotinylated according to Sigma manufacture's guidelines for protein labeling by carbodiimide coupling. Biotinylation of SRA was confirmed by dot blot experiment for which the biotinylated lectin was loaded and air dried on the nitrocellulose membrane. The nonspecific binding sites were blocked with 4% BSA for 2 h at 37°C, washed with PBS (phosphate-buffered saline), incubated with streptavidin–peroxidase (0.1 mg/ml), and developed in 4-chloronaphthol (3 mg/ml) solution in PBS (pH 7.0) containing 5 µl/ml H_2O_2.

Enzyme linked lectin binding assay (ELLBA) for lipopolysaccharides (LPS). The LPS from rhizobial strains such as SB2 and SB2M3 (*Sesbania* specific), GN17, IGR 92 (peanut specific), SB16 (soybean specific), P14-93 (pea specific), and Rcd301 (cicer specific) were isolated by the hot water–phenol method [23]. The suspension of above LPSs (10 µg/100 µl) was coated in triplicate in 96-well microtiter plate (Nunc, Denmark), incubated overnight at 37°C, and washed with washing buffer (10 mM PBS, pH 7.2, 0.02% Tween 20) followed by blocking with 1% BSA (100 µl) in PBS and incubating at 28°C (2 h). The blocking buffer was removed, and the plate was washed (five times) followed by addition of biotinylated lectin (1 µg) and incubation at 28°C (3 h). The wells were again washed, 100 µl of streptavidin–horseradish peroxidase conjugate (1 : 700 dilution in ADW) was added, and plates were incubated at 28°C for 45 min and finally developed with *o*-phenylenediamine (1 mg/ml in 0.05 M citrate buffer and 5 µl/ml H_2O_2) for 30 min. The reaction was stopped by adding 2 N H_2SO_4 to each well and binding efficiency was checked by reading absorbance at 492 nm in an ELISA reader (Spectramax Plus; Molecular Devices, USA).

RESULTS AND DISCUSSION

Lectins have been detected most frequently in Leguminosae and Euphorbiaceae plants. There are reports which showed that the legume lectins are not only restricted to seeds but also present in other vegetative tissues at different stages of plant development [4, 24, 25]. However, the lectins from vegetative tissues are less characterized. Lectins present in the roots of leguminous plants have been reported to play a role in the recognition of specific *Rhizobium* sp. [5, 6, 26], which is still a subject of controversy [27]. The plant lectins are mostly considered to bind exogenous carbohydrates ligands to play symbiotic or defensive roles, which contrasts with the numerous animal lectins that exhibit functions in connection with their endogenous ligands. However, recent observation from our laboratory on a peanut stem lectin SL-1 indicates that this vegetative tissue lectin may act as physiological buffer/regulator in maintaining the effective cytokinin concentration in the plant [28].

Purification and characterization of *S. aculeata* root lectin. Root extract of *S. aculeata* was found to agglutinate rabbit erythrocytes. This agglutination was inhibited most effectively by 50 mM glucose. Therefore, the lectin was purified using a Sephadex G-50 column. The elution profile is illustrated in Fig. 1a. Binding of the lectin with Sephadex G-50 and its subsequent elution with glucose led to 5-fold purification. This affinity purification showed 13% recovery of the total protein. In each batch, about 20-25 plant roots were considered and the data presented in Table 1 represent purification from one batch.

SDS-PAGE of purified SRA showed a single band of molecular weight ~39 kDa (Fig. 1b), while a band of ~80 kDa was seen in native PAGE (Fig. 1c), indicating that the SRA might be a dimer composed of two identical subunits of molecular mass ~39 kDa each. MALDI-TOF analysis also confirmed the molecular mass with a single peak at m/z 39 kDa (Fig. 2). The 2D-PAGE of purified SRA was carried out to check the presence of isoforms. The 2D gel pattern showed only one protein spot of approximate p*I* 6 indicating that SRA does not have isoforms (Fig. 3). The protein spot of purified SRA was then excised and subjected to in-gel digestion with trypsin, and the tryptic peptides were analyzed by MALDI-TOF to check for their masses [29]. Since the genomic and proteomic database of *S. aculeata* is not available, the PMF spectrum of the purified SRA was searched for any homology with the available proteins in the NCBI database. We observed that the digestion of purified lectin with trypsin and PMF spectrum of obtained peptides in the 800-3500 mass range (m/z) showed 28% homology with *Arabidopsis thaliana* lectin-like protein (accession No. gi|995619|) (Fig. 4 and Table 2).

It was observed that lectin SRA was developmentally regulated and activity was highest during 2-3 weeks, which is the prime time for rhizobial infection. Further, the addition of metal ion does not alter hemagglutination activity of SRA, indicating that the lectin activity of SRA was independent of metal ions. In this respect, SRA is akin to peanut agglutinin (PNA) isolated from peanut seeds; how-

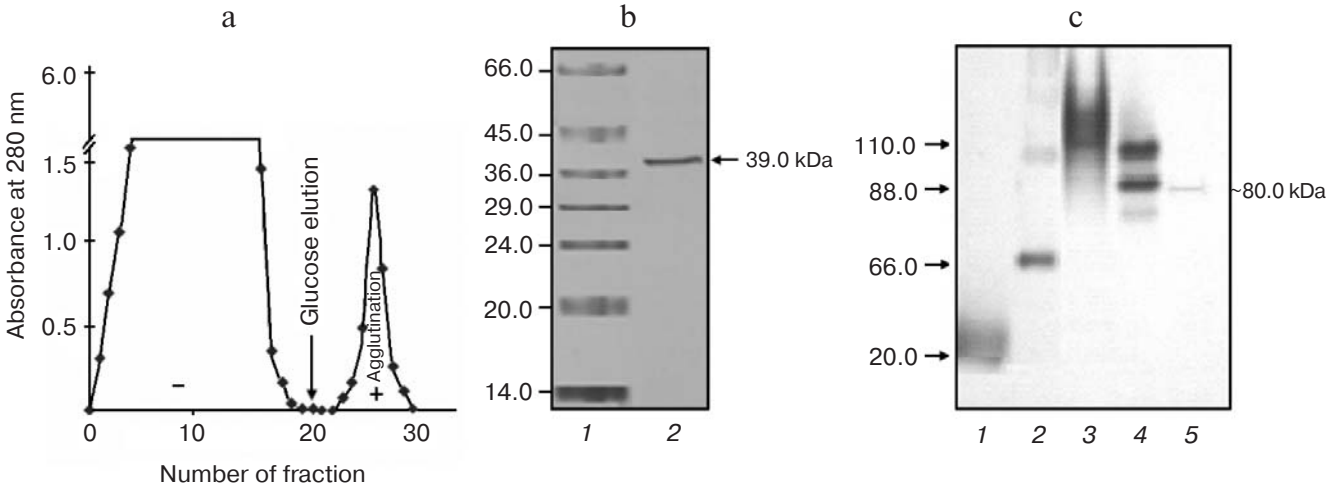

Fig. 1. a) Elution profile of glucose-specific *S. aculeata* lectin from Sephadex G-50 column. b) SDS-PAGE of SRA stained by Coomassie blue: *1*) molecular weight marker protein; *2*) SRA (*Sesbania* root lectin). c) Native PAGE of SRA stained by Coomassie blue: *1*) trypsin inhibitor (20 kDa); *2*) BSA (66 kDa); *3*) pea seed lectin (110 kDa); *4*) cicer seed lectin (88 kDa); *5*) SRA (~80 kDa).

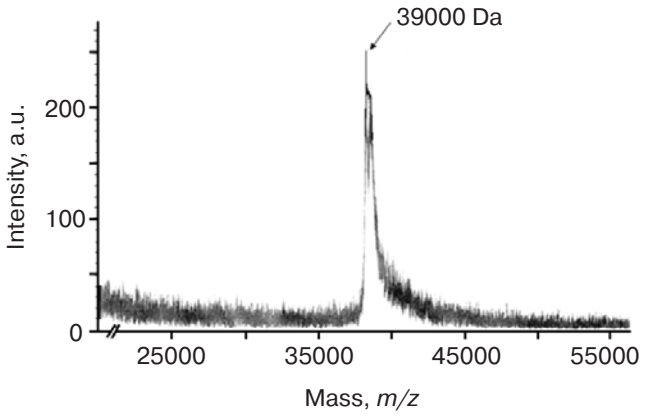

Fig. 2. Intact molecular mass determination of SRA using MALDI-TOF/TOF in linear positive mode.

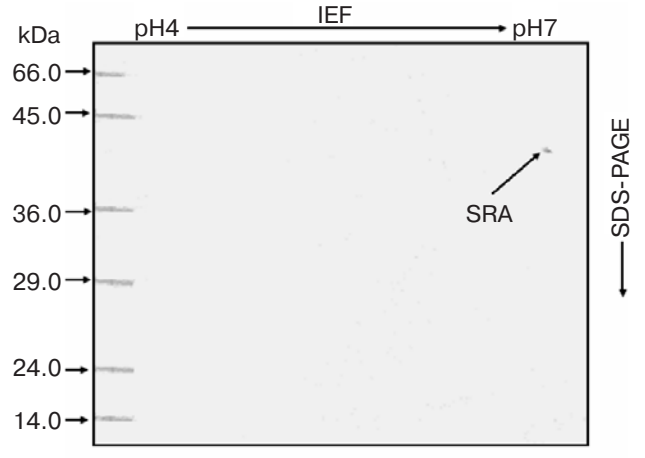

Fig. 3. 2D gel electrophoresis of purified SRA.

ever, they differed from peanut root lectin PRA II, which required metal ion Mn^{2+} for its optimal activity [18]. There are other lectins that require metal ions such as Ca^{2+} and Mn^{2+} for the maintenance and lectin activity [27, 30].

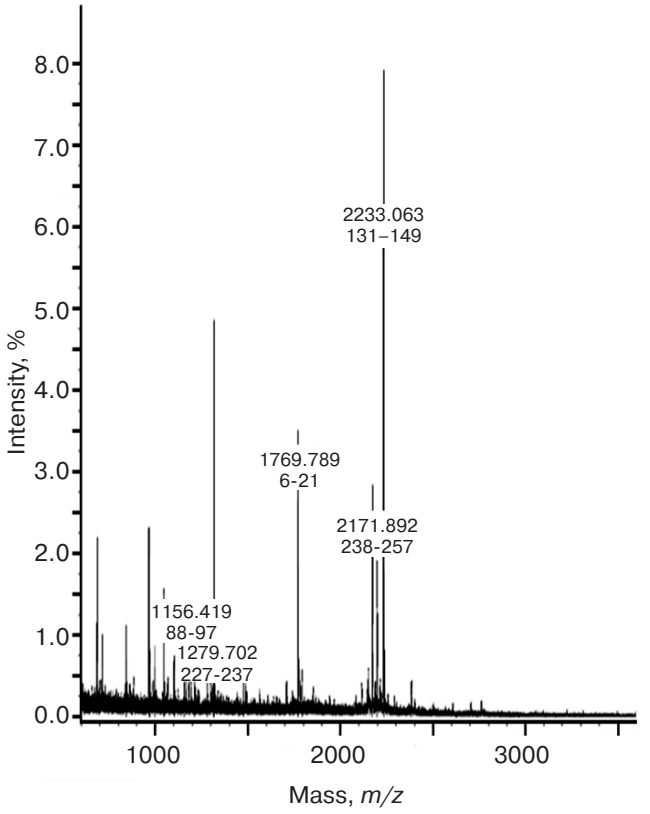

Fig. 4. Peptide mass fingerprinting spectrum of purified SRA from 2D gel using MALDI-TOF/TOF in reflectron positive mode. The mono-isotopic m/z values are annotated at the top of each peak.

Table 1. Purification of *S. aculeata* root lectins by affinity chromatography

Preparation	Root extract, ml	Protein concentration, mg/ml	Total protein, mg	Specific activity, HA units/ mg protein	Purification, fold	Yield, %
Crude root extract	15	1	15	4	1	100
Fraction after affinity purification	30	0.66	2	20	5	13

Table 2. M/Z ratios of peptides and their sequences showing homology with *Arabidopsis thaliana*

Serial No.	m/z	Sequence
1	1155.412	TSFTFSITPR
2	1278.694	KPKRPLIQGSR
3	1768.782	LCFLALFLANAAFAVK
4	2170.885	VLNDVLLQNMYAGFAGSMGR 247: Oxidation (M)
5	2232.056	TNDGKSENNLIFIEFDTFK

Note: Complete sequence of lectin like protein [*Arabidopsis thaliana*]: MQIHKLCFLALFLANAAFAVKFNFDSFDGSNLLFLGDAELGPSSDGVSRSGALSMTRDETPFSHGQGLYINPIQFKSSNTSSPFDFKTSFTFSITPRTKPNSGQGLAFVIVPAADNSGASGGGYLGILNKTNDGKSENNLIFIEFDTFKNNESNDISGNHVGININSMTSLVAEKAGYWVQTLVGKRKVWSFKDVNLSSGERFKAWIEFRSKDSRNTITIAPENVKKPKRPLIQGSRVLNDVLLQNMYAGFAGSMGRAGDVTMFGTGRLKTK.

Table 3. Inhibition of hemagglutinating activity of the purified *S. aculeata* root lectin

Inhibitor	Concentration, mM	Relative inhibitory potency
Glucose	3.12	1.0
Mannose	6.25	0.5
Me-α-D-mannopyranoside	6.25	0.5
Me-1,6-deoxy-α-D-glucopyranoside	6.25	0.5
N-Acetylglucosamine	12.5	0.25
Cellobiose	3.12	1.0
Maltose	6.25	0.5
Galactose	–	n.i.
N-Acetylgalactosamine	–	n.i.
Lactose	–	n.i.
L-Fucose	–	n.i.

Note: n.i., non-inhibitory. Fructose, ribose, xylose, raffinose, and arabinose were found to be non-inhibitory at a final concentration of 100 mM.

Carbohydrate specificity of *S. aculeata* root lectin. Monosaccharides, some of their derivatives, and disaccharides were screened as hapten inhibitors of hemagglutinating activity of the purified lectin (Table 3). The inhibitory potency of glucose was found to be the highest. Other inhibitor sugars are cellobiose, maltose, mannose, α-methyl-D-mannopyranoside, N-acetylglucosamine, and methyl-1,6-deoxy-α-D-glucopyranoside. However, galactose, N-acetylgalactosamine, fructose, L-fucose, ribose, xylose, raffinose, arabinose, and lactose are not inhibiting at 100 mM concentration. The hydroxyl groups at C-6, C-4, and C-3 positions of the pyranosyl ring are critical for binding of the monosaccharides to the lectin [31]. As methyl-1,6-deoxy-α-D-glucopyranoside is an inhibitor of this lectin, the C-6 hydroxyl group of monosaccharides is therefore not critical for sugar specificity of this lectin. A variation in orientation of hydroxyl group at C-4 is very crucial as in galactose or substitution by glycosidic linkage as in lactose abolishes the inhibitory potency. This glucose-specific lectin also accepts mini-

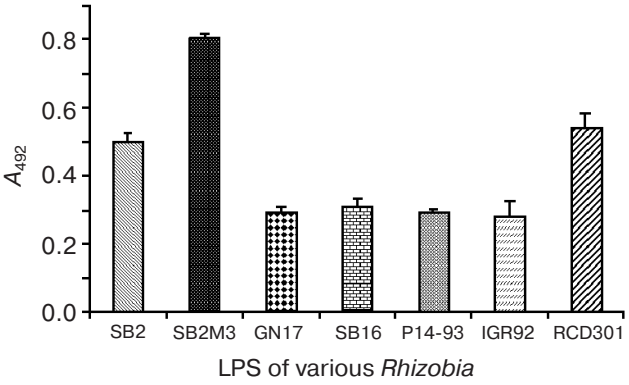

Fig. 5. Enzyme linked lectin binding assay of SRA with the LPS isolated from various rhizobial strains.

mum variation at C-3 position, as L-fucose is not an inhibitor of this lectin. This root lectin shows some similarity towards SL-1 lectin isolated from peanut stem lectin [32], but they differ with respect to α,β-linkage of glucose. SL-1 is inhibited by maltose but not by cellobiose, whereas this lectin is inhibited by both these saccharides indicating its lack of preference towards α- or β-linkage.

Binding to LPS. It has been reported that the lectin present on the roots of legumes might have a role in establishing nitrogen-fixing symbiosis [6, 33]. The root lectins probably recognize the LPS molecule present on the surface of rhizobia, and thus the interaction between rhizobia and plant takes place. This interaction is highly specific [5, 6]. Hence the binding efficiency of SRA (1 μg) with the LPS (10 μg) isolated from *Sinorhizobium saheli* strain SB2 and its mutant SB2M3, was examined using biotinylated SRA by ELLBA [6]. It was observed that the LPS of mutant strain (SB2M3) was binding more efficiently with SRA as compared to LPS isolated from wild strain (SB2). The difference was found to be highly significant ($p \leqslant 0.0001$) (Fig. 5), suggesting that the SB2M3 is a better mutant strain than the wild strain for the interaction. SB2M3 was reported to be the best mutant with respect to nodulation and cross infectivity with nonspecific leguminous plant [34]. Thus, the binding efficiency of SRA with the LPS of various other rhizobia such as GN17, IGR 92 (specific for peanut), SB16 (specific for soybean), P14-93 (specific for pea), and Rcd301 (specific for cicer) were tested by ELLBA. The binding efficiency of Rcd301 LPS was found to be comparable with specific LPSs of SB2 and SB2M3 (Fig. 5). The LPS from other nonspecific rhizobial strains showed significantly less cross reactivity with SRA. It was also observed that LPS of wild strain of cicer (Rcd301) has the highest binding among the other nonspecific LPS with SRA, but the binding efficiency of SB2M3 LPS with SRA was the most significant.

Binding study of SRA with the LPS of various nonspecific rhizobia showed that SRA present in the roots of *S. aculeata* can have binding efficiency with various rhizobia indicating that the nodulation of *Sesbania* may be possible by various rhizobia. The specific mutant SB2M3 was found to be the best rhizobial mutant strain for cross nodulation of nonspecific legumes and hence can be used to increase the fertility of soil for other nonspecific crops also.

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