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Ficus cunia agglutinin for recognition of bacteria

M Adhya, B Singha and B P Chatterjee*

Department of Biological Chemistry, Indian Association for the Cultivation of Science, Jadavpur, Kolkata 700 032, India

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Interaction of bacteria with lectin using anti-lectin antibody by ELISA is an established method. In the present study, we have devised a simple ELISA using a biotinylated lectin and antibiotin-HRP. *Ficus cunia* agglutinin (FCA), which has shown the specificity towards α/β anomers of GlcNAc and other –NAc containing sugars like LacNAc and GlcNAcβ(1-4/6)GlcNAc, was used as a model lectin for the study of interaction with immobilized microorganisms on ELISA plate. The bacterial cells of *E. coli*, *Pseudomonas aeruginosa, Klebsiella pneumoniae*, *Bacillus subtilis* and *Staphylococcus aureus* showed binding with FCA and the degree of binding was dependent on the bacterial surface antigen. This method is considered a simple technique to study the lectin-bacteria interaction.

Keywords: *Ficus cunia*, agglutinin, bacteria, ELISA

The usefulness of lectins in clinical microbiology[1] is attributed to their diverse carbohydrate specificity, nanogram sensitivity, commercial availability, ability to probe subtle differences between various clinical isolates and ease of conjugation with different chemical and biochemical reagents. Owing to their carbohydrate-binding specificity, they may replace immune sera for detecting various carbohydrate residues on microbial cells, which are composed of teichoic acids, peptidoglycans, lipopolysaccharides (LPSs) and capsular polysaccharides[2]. Lectins are versatile reagents for definitive identification and function as epidemiological markers. Though serotyping is a well-established method, yet it encounters problem in epidemiological study, particularly when all types of anti-sera are not available in pathological laboratories.

Helix pomatia agglutinin (HPA) could distinguish between the strains of *B. anthracis* and *B. mycoids* and was positive only for the latter[3]. HPA-coated magnetic microspheres were reported to bind *Listeria monocytogenes*[4], a frequent contaminant in food and dairy industry, but not with other members of *Salmonella*, *Bacillus* and *Streptococcus*. Concanavalin A (ConA) agglutinated *Staphylococcus aureus* and most strains of Streptococcus[5]. It agglutinated a variety of Gram-negative bacteria, by binding with their cell wall LPS[6] and also precipitated LPS from *Shigella flexneri*[7]. A sialic acid-binding lectin carcinoscorpin from Indian horseshoe crab *Carcinoscorpius rotunda cauda* agglutinated *E.Coli* K12 and *Salmonella minnesota* R 595 cells, but not *Vibrio cholerae*[8]. A LPS-specific lectin immulectin-2 from an insect *Manduca sexta* agglutinated FITC-labeled *E. coli* in a dose-dependent manner[9].

Lectins also find application as diagnostic reagents to *Neisseria* sp. The wheat germ agglutinin (WGA) agglutinated *N. gonorrhoeae*[10,11] and non-encapsulated *N. meningitides*, but not the encapsulated *N. meningitides*[12]. In clinical diagnosis, different techniques have been used for studying the lectin-microorganism interaction. Earlier, by direct agglutination of suspended bacterial cells, sixteen Habs and three Fisher types of *P. aeruginosa*[13], ten different serogroups of *Shigella dysenteriae*[14] and six different serotypes of *K. pneumoniae*[15] were grouped intra-specifically, with lectins of varied sugar specificity in our laboratory. Bacterial binding study with lectins TPL-1 and TPL-2, isolated from the hemolymph of a crab *Tachypleus tridentatus* was

*Author for correspondence
Phone: +91-33-2473-4971/5904; Fax: +91-33-2473-2805.
E-mail: bcbpc@mahendra.iacs.res.in

Abbreviations: FCA, *Ficus cunia* agglutinin; HPA, *Helix pomatia* agglutinin; WGA, wheat germ agglutinin; ConA, concanavalin A; SBA, soybean agglutinin; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PBS-T, PBS containing 0.05 % (v/v) Tween-20; ELISA, enzyme-linked immuno-sorbent assay; BSA, bovine serum albumin; antibiotin-HRP, antibiotin conjugated horseradish peroxidase; OPD, *o*-phenylenediamine; LPS, lipopolysaccharide; GlcNAc, N-acetylglucosamine; MurNAc, N- acetylmuramic acid; LacNAc, N-acetyllactosamine

reported by ELISA, where rabbit anti-TPL-1 and TPL-2 were used, followed by anti rabbit IgG-peroxidase[16].

In the present study, a simple, sensitive and reproducible method is reported for the detection of Gram-negative and Gram-positive bacteria *viz*., *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *B. subtilis* and *S. aureus* using biotinylated *Ficus cunia* agglutinin (FCA)[17] by ELISA.

Materials and Methods

Materials

Ficus cunia seeds were obtained from the receptacle of the fruits collected from the garden of our institute. Different strains of Gram-negative bacteria *viz*., *E. coli* (O26, O125, O127, O142, O158), *Pseudomonas aeruginosa* Habs-type (H1, H3, H6, H7, H11, H13) and Fisher-type F3, *Klebsiella pneumoniae* (K10, K15, K40, K53, K55) and Gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus* used in this study were kindly obtained from Prof. A K Guha of this department.

Fetuin-agarose, biotin (biotinamidocaproate-N-hydroxysuccinimide ester), antibiotin-HRP, *o*-phenylenediamine (OPD) and Bradford reagent were purchased from Sigma, USA. All other reagents used were of highest analytical grade.

Purification of *F. cunia* agglutinin (FCA)

The agglutinin was purified from the seeds essentially by the earlier described method[17], except the technique of affinity repulsion chromatography[18] which is based on the micro-electrostatic charges that can either repel or attract each other. Such electrostatic interactions are not substantial in solution containing salts, as charges are solvated, but becomes considerable in absence of salts. By exploitation of this induced electrostatic charge on affinity matrix, purification of FCA was carried by electrostatic repulsion using deionized water as eluent.

Briefly, the seeds were homogenized in the presence of saline (150 m*M*), centrifuged at 10,000 rpm for 30 min and the supernatant was loaded on to fetuin-agarose affinity column (5 cm × 1 cm) equilibrated with 20 m*M* PBS, *p*H 7.0. The unbound protein was washed with the equilibrating buffer. The bound protein was desorbed by deionized water and the fractions (1 ml) were collected in 1 *M* NaCl (150 µl). The fractions showing hemagglutinating activity were concentrated by ultrafiltraton and dialyzed in PBS. Protein content was measured by the method of Bradford[19] using BSA as the standard. The purity of FCA was judged by SDS-PAGE[20] and its properties were found to be the same as reported earlier[17].

Culture of bacterial cells

B. subtilis and different strains of *E. coli*, *Klebsiella* and *Pseudomonas* were cultured in nutrient agar medium (Difco) and *S. aureus* was cultured in brain heart infusion agar medium. The cultures were harvested in saline and transferred to 0.1 *M* Na_2CO_3/$NaHCO_3$ buffer (*p*H 9.6). The concentration of the bacteria was determined by measuring absorbance at 600 nm. The number of cells per ml was estimated[16], assuming 0.1 optical density unit was roughly equivalent 10^8 cells/ml.

Biotinylation of FCA

FCA was biotinylated using biotin as described previously[21]. Briefly, to 1.0 mg FCA in 1.25 ml PBS (*p*H 7.0), 2 ml of 0.025% (w/v) biotin solution was added. The mixture was left for 30 min at room temperature and dialyzed for 2 h against water, followed by PBS overnight. Biotinylated FCA was adjusted to 100 µg/ml with PBS containing 0.02% (w/v) NaN_3 and stored at –20 °C.

Bacterial cells recognition by FCA-biotin

All bacteria were heat-killed prior to be adsorbed in ELISA plates. The 100 µl of bacterial cell suspension (10^8 cells/ml) in 0.1 *M* Na_2CO_3-$NaHCO_3$ buffer (*p*H 9.6) was added to each well and incubated at 37°C for 2 h. The wells were washed with PBS-T and incubated with 0.1% (w/v) BSA in PBS-T at 37°C for 1 h. After washing, 100 µl of biotinylated FCA (0.1 ng to 1.0 µg) was added in PBS-T and incubated at 25°C for 1 h. Thereafter, after washing, the wells were incubated with 100 µl of antibiotin-HRP diluted 1:2000 (v/v) with PBS-T at room temperature for 1 h. The colour developed by addition of OPD (2 mg/ml) and 0.01% H_2O_2 in 0.05 *M* citrate-phosphate buffer (*p*H 5.0) was measured at 492 nm, after incubation at room temperature for 30 min in the presence of 50 µl 3 *N* H_2SO_4 in the ELISA reader. The wells containing bacterial cells without FCA-biotin and antibiotin-HRP were considered as control (absorbance value below 0.1). All experiments were carried out in triplicate and data presented were mean value of the results.

Results and Discussion

Different serotypes of Gram-negative (*E. coli*, *K. pneumoniae* and *P. aeruginosa*) and Gram-positive

(*B. subtilis* and *S. aureus*) bacteria were adsorbed in the wells of ELISA plate (10^7 cells/well). Biotinylated FCA, added with increasing doses from 0.1 ng to 1.0 µg per well, gave the extent of binding in dose-dependent manner, as evidenced by antibiotin-HRP assay at 492 nm and was found to be saturated at absorbance value of 3.0. The amount of FCA that corresponded to 50% binding to bacterial cells (Table 1) showed binding absorbance of 1.5 and varied from one serotype to other in the same bacterial species (Fig. 1). The present study demonstrated binding ability of FCA with different bacteria. Among Gram-negative bacteria, FCA showed strong binding with *E. coli* O158, as it

Table 1—Affinity of different bacterial cells to biotinylated FCA

Bacterial strains	FCA (ng)*	Binding intensity†	Bacterial strains	FCA (ng)*	Binding intensity†
E. coli			*P. aeruginosa*		
O26	63.74	+++	H1	95.17	+++
O125	47.01	++++	H3	120.08	++
O127	49.56	++++	H6	75.87	+++
O142	37..35	++++	H7	65.31	+++
O158	8.63	+++++	H11	63.17	+++
K. pneumoniae			H13	102.85	++
K10	114.07	++	F3	87.13	+++
K15	60.23	+++	*B. subtilis*	281.98	+
K40	47.99	++++	*S. aureus*	52.93	+++
K53	221.18	++			
K55	97.15	+++			

*Amount of FCA required for the absorbance 1.5 unit at 492 nm, corresponding to 50% binding
†The results were interpreted, according to the amount of lectin required for 50% binding to the bacteria as follows: +++++ (1-10 ng), ++++ (10-50 ng), +++ (50-100 ng), ++ (100-250 ng) and + (> 250 ng)

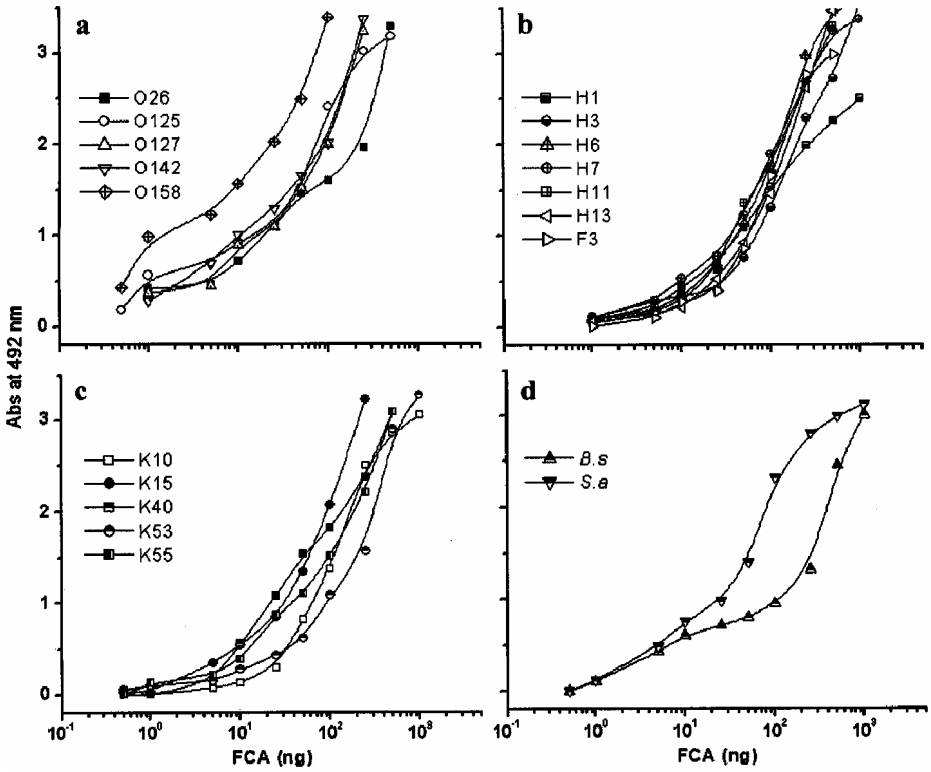

Fig. 1—Binding of FCA to different strains of (a) *E. coli* (b) *P. aeruginosa* (c) *K. pneumoniae* (d) *B. subtilis* (B.s), *S. aureus* (S.a) bacteria [Each well containing 10^7 bacteria cells was incubated with biotinylated FCA in a dose-dependent manner (0.1 ng to 1 µg/well). The detailed procedure is given in 'Materials and Methods']

required least amount (8.63 ng) for binding with the above strain, whereas among Gram-positive bacteria, binding was stronger with *S. aureus* (Table 1).

The observed effect was probably due to the binding of lectin with the polysaccharides, exposed on the bacterium surface. LPS, the major component of outer cell membrane of the Gram-negative bacteria[22] is essentially composed of a membrane bound lipid A moiety, attached to a polysaccharide chain of variable length and its composition is dependent on the bacterial strain. FCA has sugar specificity towards Meα/βGlcNAc and Galβ1-4GlcNAc as well as GlcNAcβ1-6GlcNAc, moiety present in lipid A backbone of the bacteria[17]. Further, a common antigen composed of repeating units of GlcNAc and N-acetyl-mannosaminuronic acid, cross-linked to palmitic acid residues[22] is found on the cell surface of all members of enterobacteriaceae like *E. coli* and *Klebsiella*. *B. subtilis* and *S. aureus* have a common structural motif of peptidoglycan layer covering their whole surface[22]. Peptidoglycan is composed of repeating units of GlcNAc and MurNAc and the polysaccharide parts are cross-linked by an inter-peptide bridge[23].

In conclusion, it could be stated that like other lectins used in clinical bacteriology[24], biotinylated FCA may be considered an important lectin for its usefulness in detecting Gram-positive and Gram-negative bacteria.

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