Effects of *Euphorbia tortilis* (Shar-zaung) Latex Lectin on Human Erythrocyte Agglutination

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Lectin containing plants have been found in many botanical groups including mono and dicotyledons, molds and lichens. The lectins can be detected mostly in seeds, but they can also be found in other vegetative parts of the plants such as roots, leaves, barks, rhizomes, bulbs and plant exudates. Latex is a milky sap containing proteins, carbohydrates, lipids, amino acids, vitamins, several enzymes and other essential inorganic salts. This study was conducted in 2016 to purify the galactose-specific lectin from the latex of *Euphorbia tortilis* and determine the hemeagglutinating activity of latex lectin using hemagglutination assay. The lectin from the latex of *Euphorbia tortilis* was purified in a single step by affinity chromatography on galactose-sepharose 6B followed by elution with 0.2 M lactose. The purity of latex lectin was determined using SDS-PAGE. The non-denatured lectin resolved as a single band at M_r 68 kDa, whereas the denatured lectin resolved as three bands at M_r 38, 33, and 31 kDa. The resulting galactose-specific latex lectin shows hemeagglutinating activity of 2560, 5120 and 5120 HU/mg, respectively.

Keywords: *Euphorbia tortilis*, Affinity chromatography, Latex, Lectin, Hemagglutination

INTRODUCTION

Lectins are a group of carbohydrate-binding proteins of non-immune origin, capable of specific recognition of and reversible binding to carbohydrate moieties of complex glycoconjugate without chemically modifying them. They agglutinate cells and/or precipitate glycoconjugates. Lectins can be found in animals, plants, and microorganisms. Plant lectins are regarded as carbohydrate-binding proteins although differing in their molecular structures, carbohydrate-binding specificities and biochemical activities. Some may bind mannose-containing oligosaccharides while others bind galactose-containing structures.[1] Plant lectins are involved in defense mechanism to protect the host plant against pathogens and herbivores. Lectin proteins are capable of recognizing and binding glycoconjugates present on the surface of microorganisms or exposed along the intestinal tract of insect or mammalian herbivores. Lectins recognize the glycans, which perform intrinsic functions: providing structural components, modifying protein properties and extrinsic functions: directing trafficking of glycoconjugates, mediating and modulating the cell adhesion and cell signaling.[2] Stillmark working with castor bean extracts first demonstrated the hemeagglutination (lectin activity) in 1888. Many members of the lectinic protein family agglutinate (clump together) red blood cells. Some lectins are so specific in agglutinating certain blood types that they were used in the characterization of the human blood groups A, B, and O. The ABO blood groups are determined by the presence of different terminal sugars on glycans of red blood cells.[3] Although lectins can be found in many different organisms, the majority of lectins in

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research today are isolated from various parts of plants such as seeds, barks, leaves, bulbs, rhizomes and latex. Plant lectins which are able to agglutinate erythrocytes were previously called phytohemagglutinins, phytoagglutinins or plant agglutinin.[4] The plant lectins have ability to trigger the proliferation and differentiation of many T lymphocytes. These are often known as polyclonal activators of mitogens, because of their ability to induce mitosis of the cell population. The plant lectins concanavalin A (Con A) and phytohemagglutinin (PHA) are particularly potent mitogens for T cells. Another plant lectin, pokeweed mitogen, activates both T and B cells.[5]

Latex is a biological fluid and can be occurred in a wide range of plant diversity. It is a colloidal suspension found in specialized living cells or vessels called laticifers. The laticifers are internal secretory structures which are articulated or non-articulated, depending on the observation of terminal walls, and present in cortex, pith, wood, embryos, leaves, fruits and seedlings. The latex can be leaked out from the plant in response to physical damage. Euphorbiaceae is one of the largest and most diverse families of the angiosperms known to have laticifers. It contains a wide array of biochemicals such as rubber, oils, waxes, hydrocarbons, resins, sterols, triterpenes, balsams and other biochemicals. The cytoplasmic remain of the latex after removing the agglomerative particles by centrifugation is referred as "serum". The serum contains a variety of cellular components such as proteins, enzymes, free carbohydrates, lipids, free amino acids, vitamins and other essential inorganic salts.[6, 7]

Lectin interaction with certain carbohydrate is very specific. This interaction is as specific as the enzyme-substrate, or antigen-antibody interaction. Many lectins have multiple binding sites but some have single binding sites. Depending on the number of sugar-binding sites, lectins are able to interact specifically with carbohydrates or glycoproteins in solution or linked to cell membranes. Because of this ability, lectins can be easily detected through agglutination assays. The hemagglutination assay uses chemically or enzyematically or untreated human erythrocytes of different blood groups and known concentration of lectin protein to visualize the erythrocyte agglutinating property of lectins.[8]

MATERIALS AND METHODS

Plant materials

Euphorbia tortilis latex was collected from the plant growing in experimental garden of School of Life Sciences of University of Hyderabad, India.

Chemicals

Protein molecular mass standards for SDS-PAGE (phosphorylase b, 97.4 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 29 kDa; soyabean trypsin inhibitor, 20 kDa; lysozyme, 14.3 kDa) were purchased from Genei, Bangalore, India. Divinylsulfone and sepharose-6B were purchased from Sigma Chemical Company, USA. All other chemicals/reagents used were of analytical grade manufactured from India by different firms.

Preparation of galactose-sepharose

Packed sepharose 6B (100 g wet weight) was suspended in 100 ml 0.5 M sodium carbonate buffer (pH 11.0) and 10 ml divinylsulphone was added. The suspension was kept at room temperature for 70 minutes with slow stirring and the activated gel was thoroughly washed on a glass filter with distilled water. It was then suspended in 100 ml of a 10 % solution (w/v) of galactose in carbonate buffer (pH 10.0) and left overnight in the cold room (4-6°C). The coupled gel was blocked with 2-mercaptoethanol (0.5 ml/25ml gel in 0.5 M bicarbonate buffer, pH 8.5) for 3 hours at room temperature and the resulting product was washed on a glass filter with one liter of carbonate buffer followed by two liters of water and suspended in PBS.[9]

Isolation of latex serum

Latex was collected into centrifuge tubes by incision the mature stems of plants and frozen in liquid nitrogen. The frozen latex was thawed and centrifuged at 12,000 rpm for one hour at 4°C. The latex was separated into two fractions; the top layer containing coagulated low-density particles and a lower portion with large volume of clear "serum". The clear serum was removed with a long needle syringe, piercing through the upper coagulated layer. The separated serum was used for lectin purification.

Purification of Euphorbia tortilis latex lectin

Two milliliters of latex serum (49.6 mg/ml) was applied to a column of the galactose-sepharose 6B, previously equilibrated with saline at the flow rate of 15 ml/hr. The column was washed with saline until the effluent absorbance was less than 0.02 at 280 nm. The lectin was eluted with 0.2 M lactose in saline. The affinity chromatography was performed in the cold room where the temperature was maintained at 4°C. The protein-rich fractions were pooled and dialyzed against saline. The purity of the protein-rich fractions was verified on SDS-PAGE.

Protein estimation

Protein was estimated following Lowry *et al*., with minor modifications.[10] Reagent A was 4% sodium carbonate in 0.2 N sodium hydroxide, B was 1% cupric sulphate, C was 2% sodium tartarate and D was 1N Folin's reagent. The working reagent is a mixture of A, B and C in a ratio of 23:1:1 and used within 24 hours of preparation. One milliliter of the working reagent was added to 1 ml of the protein sample, mixed well and allowed to stand for 10 minutes.

The 0.2 ml of reagent D was added rapidly while vortex the sample. After 30 minutes, the sample absorbance was recorded at 750 nm. Bovine serum albumin (BSA) was used as a standard protein (5-50 µg). Protein in column chromatography fractions was detected by the absorbance at 280 nm.[11]

Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecylsulphate-polyacrylamide gel electrophoresis for proteins was performed following the method of Laemmli with minor modifications.[12] The separation of proteins was performed in 11% resolving gel with 4% stacking gel. Both the resolving and stacking gels contained 2.4% bis-acrylamide as a cross-linker and 0.1% SDS. The final buffer concentrations were 0.45 M Tris-HCl (pH 8.9) in resolving gel and 0.2 M Tris-HCl (pH 6.7) in stacking gel. Ammonium per-sulphate and N,N,N,N-tetramethylethylene diamine (TEMED) were used as polymerizing reagents in final concentrations of 0.05% and 0.1%, respectively. The electrode buffer comprised of 6.025 g Tris and 14.4 g glycine in one litre of distilled water (pH 8.3). The sample buffer constitutes 0.062 M Tris-HCl (pH 6.7), 10% glycerol, 2% SDS, 5% β-mercaptoethanol and 0.001% of bromo-phenol blue.

The samples were incubated for one minute in boiling water with the sample buffer. The samples were loaded in the gel of 8×8× 0.1 cm dimension which was polymerized in plain glass plates and was fixed to Broviga (India) mini-vertical slab-gel apparatus. Gels were run at room temperature at a voltage of 75 and 100 DC (direct current) for stacking and resolving gel, respectively. Electro-phoresis was carried out until the bromo-phenol blue marker reached about 3-4 mm from the bottom of the gel. Then the gels were removed, fixed in the fixative (50% methanol, 12% acetic acid and 0.0185% formaldehyde) and stained in Coomassie Brilliant Blue R for detection of proteins.

Hemagglutination assay

The hemeagglutinating activity of the lectin was determined according to the method described by Lis and Sharon with minor modifications.[13]

Preparation of Alsevere's solution

The Alsevere's solution was prepared by dissolving 2.05 g of glucose, 0.8 g of sodium

citrate and 0.42 g of sodium chloride in 80 ml of distilled water. The pH was adjusted to 6.1 with 1% citric acid and the volume was made up to 100 ml with distilled water. The solution was autoclaved, cooled and stored at 4°C.

Preparation of erythrocytes

Venous blood was added to an equal volume of Alsevere's solution. The blood suspension can be stored as long as two weeks in the Alsevere's solution.The erythrocytes were isolated from the stock blood suspension by centrifugation at room temperature using a table-top centrifuge (1000×g for 5 minutes). The packed erythrocytes at the 1000×g considered as 100%. The packed cells were washed with cold saline (0.9% NaCl) by centrifugation for 3-4 times (5 ml saline for each millilitre of packed erythrocytes) and finally 4% cells were made in saline.

Preparation of trypsin treated erythrocytes

The erythrocytes were treated with trypsin on the day of assay. The packed erythrocytes (0.5 ml) was made up to 12.5% with PBS (phosphate buffer saline) and then incubated with 5 ml of 1% trypsin for one hour at 37°C. The trypsin treated erythrocytes were washed 5-6 times with PBS to remove the traces of trypsin and then made it up to 4% erythrocytes with PBS.

Agglutination assay

Five microlitre of the purified protein sample and 16 µl of crude protein sample were serially diluted with PBS in a microtitre plate and 100 µl of trypsinized 4% erythrocytes were added to each well. The agglutination was observed visually after incubation of the plate for one hour at 37°C. Then the specific activity and fold purification of the protein was calculated.

RESULTS

The *Euphorbia tortilis* latex agglutinin (ETLA) was purified in a single step with a sharp peak from the affinity column (Fig. 1) with four folds increased in purity and amounts to 46.4% of the total protein of latex serum (Table 1).

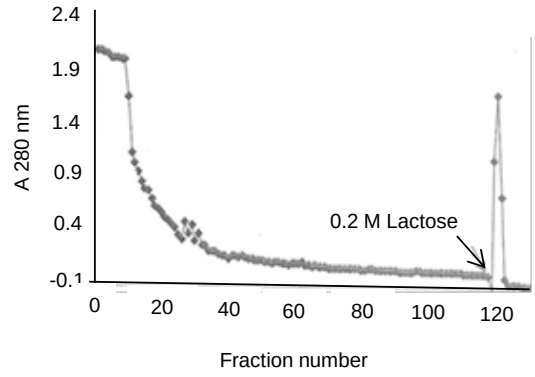

Fig. 1. Affinity chromatography of *Euphorbia tortilis* latex agglutinin (ETLA) on sepharose 6B

Table 1. Purification of *Euphorbia tortilis* latex agglutinin (ETLA)

Purification step	Protein (mg)	Total activity	Specific activity* (HU/mg)**	Yield (%)	Fold purification
Latex serum	99.2	126976	1280	100	1
Gal-sepharose 6B fraction	11.5	58880	5120	46.37	4

* Specific activity is expressed as titre the reciprocal of maximal dilution of protein that gives visible agglutination with 1% trypsinized human O^+ group erythrocytes.

**HU is Hemagglutination unit

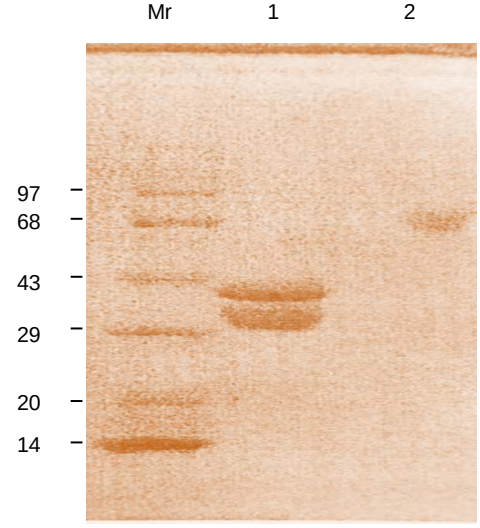

Fig. 2. SDS-PAGE of *Euphorbia tortilis* latex agglutinin (ETLA) on 10% gel in the presence (lane 1) and absence (lane 2) of β-mercaptoethanol

The lectin was analyzed by SDS-PAGE both the presence and absence of β-mecapto-ethanol. The non-denatured lectin resolved as a single band at M_r 68 kDa, whereas the denatured lectin resolved as three bands at M_r 38, 33, and 31 kDa (Figure 2).

Table 2. Specific activity of *Euphorbia tortilis* latex serum and *E. tortilis* lectin with trypsinized human erythrocytes.

Material	A^+	B^+	O^+
Latex serum	1280	2560	1280
Lectin	2560	5120	5120

It indicated that the lectin was composed of three different subunits. In hemeagglutinating assay, agglutination was observed visually after one-hour incubation period. The ETLA agglutinates the trypsinized erythrocytes of human A^+, B^+ and O^+ groups with a specific activity of 2560, 5120 and 5120, respectively (Table 2).

DISCUSSION

The studies of lectins, which have been isolated from the latex of some Euphorbiaceae species, suggested that plant lattices are rich source of lectins. Until now, lectins from latex have been reported from six genera, i.e. *Euphorbia*, *Hevea*, *Hura*, *Monadenium*, *Pedilanthus* and *Synadenium*. Except *Hevea* lectin, all are galactose-specific. In this study, a galactose-specific lectin (ETLA) was isolated from the latex of *Euphorbia tortilis* (Spiral cactus). The method used to purify this lectin by affinity chromatography on sepharose 6B column was simple and very efficient, yielding about 1.92 mg/ml of purified lectin protein. According to Lowry's method, the concentration of purified lectin protein was 3.87% of the total protein of latex serum. The amounts of the lectins found in the latex of Euphorbia species is about 5% of the total latex serum proteins. Analyses carried out with denaturing and non-denaturing treatments by SDS-PAGE showed that galactose-specific latex lectin is an oligomer of 63 kDa, constituted by three 38, 33 and 31 kDa polypeptides. This finding proposed that ETLA is a trimeric molecule. The molecular weight of the lectins isolated from lattices of genus Euphorbia ranged from 60 kDa to 67 kDa, and the unit weights from 27 kDa to 38 kD.[14]

Erythrocytes agglutination is a distinct property of lectins. Therefore, it is used to detect lectin activity using hemagglutination assay with human and animal erythrocytes. The hemeagglutinating activity was tested on intact and trypsinized O^+ human erythrocytes, showing specific activity to the minimum concentration of the crude latex serum protein and ETLA. Agglutination of red blood cells indicates the presence of antigen on the outer surface of human red blood cells' membrane. Additional agglutination assay of the hemeagglutinating activity was performed to investigate the molecule's selectivity against different blood types. The hemagglutination was determined for A^+, B^+ and O^+ erythrocytes by means of double serial dilution (1:2 up to 1: 256). Both the crude protein isolated from latex serum and ETLA give visual agglutination of all human erythrocytes. Although, the ETLA did not present blood group-specific selectivity for all human red blood cells, more preference was presented towards B+ and O+ erythrocytes.

This can be explained by the fact that the A, B and O blood groups are composed of N-acetylgalactosamine, N-acetylglucosamine, fucose and galactose. In A-type individuals, an N-acetylgalactosamine (GalNAc) residue is added to the terminal galactose (Gal) residue, while in B-type and O-type individuals, a Gal residue is appended. The minimum concentrations of ETLA required to agglutinate trypsinized erythrocytes of human A^+, B^+ and O^+ blood groups are 146, 73.4 and 73.4 ng/ml, respectively. This finding indicated that ETLA resembles other Euphorbiaceae lectins in sugar specificity, blood group non-specificity with preference towards O^+ group erythrocytes[15]. Lectins can bind specifically to specific sugars present on the surface of human red blood cells, which causes agglutination of

particular blood types. Because of their properties, lectins are often used in blood grouping reactants, which can identify blood groups A, B, AB and O, and their sub-groups. For example, a lectin from *Dolichus biflorus* is used to identify cells that belong to the A1 blood group.[16]

The effect of ETLA on human erythrocyte agglutination is concerned with the presence of sugar-binding activity and specificity. Among the several sugar-conjugated molecules, ETLA specifically recognize and bind to the galactose moieties. This property can be used to detect specific glycans on intact glycoproteins either in a gel-blotting format or on tissues. Because of its ability to bind free and cell-bound carbohydrates in a selective manner, ETLA covalently coupled with insoluble matrix can be used in affinity chromatography for purification of polysaccharides and glycoproteins.

Some lectins such as Concanavalin A and Jacalin have been commercially used in affinity chromatography for purifying glycoproteins. It has been known that Euphorbia species have ability to resist environmental stresses such as drought, heat, herbivores and microbial attack. This is primarily because of milky white latex-like sap. Water can be stored as milky latex and the lectins in the latex protect the host plant against herbivores and microbes.

Lectins play vital role in plant defense against bacteria by means of interaction between lectins and bacterial cell wall peptidoglycan. A homodimeric galactose-specific latex lectin (EanTH) purified from *Euphorbia antiquorum* (Shar-Zaung-Gyi) had bacteriostatic action on *Salmonella typhimurium*, *Staphylococcus aureus* and *S. epidermidis*. Bacterial lectins can also inactivate human intestinal cells.[17]

The latex of Euphorbia species have been known to be toxic to invertebrates and higher animals. However, the latex extract of *Euphorbia tortilis* was non-toxic towards normal cell line H_9C_2. Morever, anti-HIV compound (phorbol ester prostratin) was detected in latex extract of *E. tortilis*.[18]

Conclusion

In this study, the detected galactose-specific latex lectin (ETLA) resembles its counterpart Euphorbiaceae lectins, in respect to sugar specificity, blood group non-specificity. Hence, it could be used in detection of cell surface glycans and investigation of other biological activities rather than identification of different blood groups.

Competing interests

The author declares that there is no competing interest.

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