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Isolation and Characterization of a Lactose-Binding Lectin from *Ocimum sanctum*

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ABSTRACT

A novel lectin has been isolated from *Ocimum sanctum* and purified to homogeneity by gel filtration chromatography, which eluted as a single symmetrical peak from a Biogel P-100 column with a molecular mass of 66 kDa. The lectin had a special agglutinating activity with human erythrocytes at a minimum concentration of 0.7 ug/ml. The lectin was stable in the pH range 5-12 and temperature 80 °C for 30 min. *Ocimum sanctum* had shown highest agglutinating activity at pH7 and 25^0C temperature after one hour incubation. Of the various sugars tested, even at 1000 mM sugar concentration, no inhibition was observed. The isolated lectin was found to be lactose-biding lectin sugar moieties and contain 2.6 mg/ml total sugar and 9.3 mg/ml of total protein.

INTRODUCTION

Animals and plants contain numerous carbohydrate binding proteins of non-immune origin called lectins with the ability to recognize specific sugars existing on cell surface. These lectins have attracted great interest due to their various physiological roles in cell agglutination (Khan *et al.,* 2007). Plant lectins are widely used for the detection, segregation and characterization of glycoconjugates based on their carbohydrate binding properties (Peumans and Damme, 1998). Lectins present on the cell surface of squamous cells, which act as antigens, these lectin-binding profiles may be useful in differentiating benign and malignant tumors (Lalwani *et al.,* 1996). Extensive study of sequence homology and 3-D structure of various plant lectins suggests that they are conserved throughout evolution and thus may play, yet unknown, important physiological roles (Etzler *et al.,* 1992). Some of the lectins are significant reagents for identification of cell surface receptors in various bacteria, protozoa, and higher organisms (Etaler, 1978). These lectins are used for typing bacteria, fungi and protozoa. Bacterial lectins resemble plant lectins in carbohydrate specificity and relative thermo stability (Mirelman, 1986). One of the major advantages of applying lectins in microbiology is that cellular or surface receptor sites can be partially characterized by hapten inhibition (Damjanov, 1987).

Ocimum sanctum an medicinal plant in ancient literature belongs to the family Labiatae, characterized by square stem and specific aroma. In India, the plant is grown throughout the country from Andaman and Nicobar islands to the Himalayas (Kirtikar and Basu, 1996). The leaves of plant are considered to be holy and often form a consistent part of the Hindu spiritual rituals (Ghosh, 1995).

The fresh leaf of *O. sanctum* is consumed with the traditional belief that it enhances immunity. This claim has been investigated in experimental animals (Godhwani *et al.,* 1988). In addition, scientific evidence pertaining to various other medicinal properties of this plant is abundantly available (Shankar Mondal *et al.,* 2009), which are generally attributed to the presence of various small molecular chemical constituents. However, not much information is available regarding the bioactive proteins from this herb. We hypothesize that carbohydrate binding protein(s) in the leaves of *Ocimum sanctum* may add evidence-based medicinal value to the large repertoire.

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MATERIALS AND METHODS

Preparation of plant smoothie

Ocimum sanctum leaves were collected from K L University campus, Vijayawada, Andhra Pradesh, India or were procured from local market of Vijayawada, Andhra Pradesh. *Ocimum sanctum* leaves were cleaned thoroughly with distilled water to remove contaminants and dust and were made into a smoothie (Vemuri *et al.,* 2010) with the help of a mortar and pestle and collected into a sterile test tube. These tubes were centrifuged at 10,000 rpm for 3-5 minutes. The supernatant was separated and stored for analysis against blood samples.

Human blood collection

All blood group samples were obtained from NRI medical college, Mangalagiri, Andhra Pradesh, India. Blood samples were collected from volunteers after obtaining the consent from the patients and are without any physiological, neurological disorders or somatic disorders. They had normal body mass index and were free from any lipid or carbohydrate metabolism disorders.

Hemagglutination Assay

In order to test the activity of lectin, hemagglutin assay (Mercedes *et al.,* 1998) was performed using human blood samples of all blood group types. Briefly, 100ul of RBC's fixed in 3% glutaraldehyde were incubated with carbohydrate specific antibody (1:1000 dilution) and 0-10mM of lectin in PBS. Blood samples were incubated with carbohydrate specific antibody and with carbohydrate specific antibody alone as negative control. The plates were incubated for 1 h at room temperature under continuous mixing and were examined by ELISA (Thermo Multiskan EX) at 595nm and 620nm. One hemagglutination unit is defined as the lowest concentration of lectin that causes visible erythrocyte agglutination.

The positive and the negative control were Concanavalin A and PBS respectively. The highest agglutinating activity was determined by the highest titer value obtained by statistical analysis; data are representative of at least three independent experiments.

Protein Content Determination

The total protein concentration of the crude extract was determined using Lowry's method (Lowry *et al.,* 1951) for protein estimation. The bovine serum albumin (BSA) was used as standard. A standard curve was obtained by plotting various concentrations of protein standard (BSA) against their corresponding absorbance. The acquired curve was then used to calculate the total protein content mg/ml.

Carbohydrate Content Determination

Phenol-sulfuric acid method (Dubois *et al.,* 1956) was used to determine the total carbohydrate content of lectin using glucose as standard. To determine the type of lectin Molisch, Iodine, Benedict's, Selwinoffs, Bials and Osazone test, were performed followed by the confirmatory test for the presence of specific sugar moiety.

Gel permeation chromatography

The precipitate from the ammonium sulphate fractionation was re-dissolved in PBS (pH.7.2) and was subjected to gel permeation (Mourao *et al.,* 1999) through the equilibrated Biogel P-100 column (1X5cm) eluted with PBS (pH 7.2). Then 3mL of the sample was loaded to the column and a 1mL fraction of the eluted sample was collected at a flow rate of 0.20 ml/min. The eluted fractions showing agglutination activity were pooled and concentrated either by ethanol precipitation or concentrator plus (Eppendorff). All fractions of the purification procedure were analyzed using SDS-PAGE to confirm the presence of the target protein.

Gel Electrophoresis

Various fractions (flow-through, wash, elution) collected before, during, and after protein elution were diluted with milliQ ultrapure water before gel electrophoresis and protein determination. SDS–PAGE was performed with Laemmle's system. The protein bands were visualized by staining the gel with Coomassie brilliant blue R-250. Glycoproteins were detected by Ezee blue direct (Merck Biosciences) staining of gels.

Denaturation studies and pH profile

To study pH stability of the lectin (He *et al.,* 2015), purified lectin was incubated at room temperature (24°C) in 20mg/ml of different pH buffers ranging from pH 3 to 12: NaH_2PO_4-citric acid buffer (pH 3.0-5.5), sodium phosphate buffer (pH 6.0-7.5), Tris–HCl buffer (pH 8.0-9.0) and glycine-NaOH buffer (pH 9.5-12). After incubation for 30min, the residual hemagglutinating activity was calculated.

To study the effect of temperature on hemagglutinating activity (Singh *et al.,* 2013), purified lectin was incubated for 30min at 20, 30, 40, 50, 60, 70, 80, 85, 90 and 95°C in PBS (pH 7.4). After incubation, aliquots were rapidly cooled on ice and the residual hemagglutinating activity was checked. The results were expressed as percentage of residual activity relative to the control.

Hapten Inhibition Assay

To study the change in agglutination activity in presence of soluble sugars, hapten inhibition assay was carried out (Nagaraja *et al.,* 2006) in all the blood types. The following sugars, with initial concentration of 250, 500 and 1000 mM, were used: D(+) xylose, α-lactose, D(+) maltose, D(+)glucose, β-D(-) fructose, D(+) galactose. Multiwell microtiter plates were used to carry the test at 595nm. 50µL of sugar solution was added to 50 µL of blood and 75µL of 10µg/ml lectin solution. The total volume was made up to 200µL with distilled water and the whole mixture was incubated at room temperature for 1 hour.

Effect of chelating agent and metal ions

For the test of hemagglutin activity in the presence of EDTA, the purified lectin was dialyzed against 5 mM EDTA at

4 °C for 16 h and then submitted to hemagglutin assay. Effect of metal ion on hemagglutin activity was studied (Borrebaeck *et al.*, 1981) by submitting the EDTA-treated lectin to two-fold serial dilutions in PBS with or without 5 mM $CaCl_2$ and 5 mM $MgCl_2$.

RESULTS AND DISCUSSION

Extract of *Ocimum sanctum* leaves contains relatively high hemagglutinating activity that could potentially be inhibited by D-lactose and its derivatives. The total protein concentration and carbohydrate content of the *Ocimum sanctum* extract as listed in table 1 was performed according to Lowry's and Dubois method.

Table 1: Protein and carbohydrate content of *Ocimum sanctum* lectin .

S.No	Common Name	Botanical Name	Parts used	Lowry Assay (mg)	Phenol Sulphuric acid method (mg)
1	Tulsi	*Ocimum sanctum*	Flowers	9.3	2.6

Gel permeation chromatography

Lectin was purified from the cleared lysate using Biogel P-100 (Biorad). 1ml of Resin per purification was washed with 4ml B-P100 bind buffer (300mM NaCl, 50mM sodium phosphate buffer, 10mM imidazole, pH8.0), the beads were allowed to settle by centrifugation and the top buffer layer was removed. The bound fraction was eluted isocratically and the eluted fractions containing lectin were pooled and analysed (figure 1). Similar results were reported (Manpreet Kaur *et al.*, 2006) for visible haemagglutination of lectin isolated from Oyster mushroom, Pleurotus sajor-Caju.

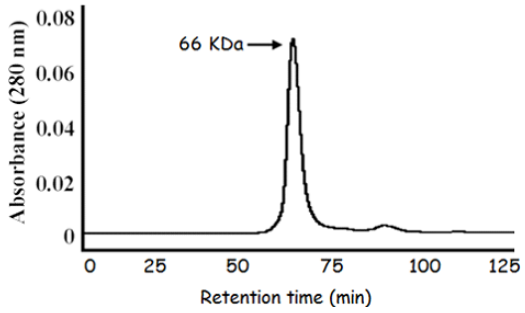

Fig. 1: purification of *Ocimum sanctum* lectin by gel chromatography on biogel P-100 column.

Gel Electrophoresis

The purified lectin was separated on 12% polyacrylamide gel according. 15 µL sample, 2x SDS loading dye were heated at 99°C and 15 µL were loaded per slot in BioRad PAGE apparatus. Dependent on the subsequent visualization of proteins, several different standards were used. 5 µL PageRuler Protein Standard was used for colorimetric detection, the purified *Ocimum sanctum* gave a single band of 66KDa suggesting the purity of the preparation (figure 2). Earlier reports (Etlzer, 1985) have ascertained that lectins are glycoproteins consisting of subunits ranging in molecular mass from 25 to 35KDa, arranged as dimers or tetramers and existing as multiple isoforms sharing similar biochemical properties. This suggests that the purified *Ocimum sanctum* could be a dimeric glycoprotein with identical subunits of 33KDa.

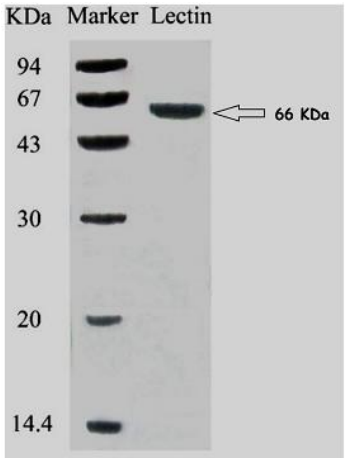

Fig. 2: SDS-PAGE results of lectin gel filtrate fraction which represents purified lectin on 12% polyacrylamide gel along with medium range protein marker (merck biosciences).

Denaturation, pH optimal studies on lectin activity

The results of thermal denaturation of *Ocimum sanctum* showed that the lectin remained significantly stable below 65 °C for more than 90 min without losing its hemagglutinating activity. Above 65 °C lectin activity was gradually lost and was totally inactivated at 80^0C towards all human blood types. Suseelan et al. have also reported that when black gram lectin was heated from room temperature to 100 °C, a linear relationship was observed for percent residual activity vs. temperature (Suseelan *et al.*, 1997). Similarly, the ability of lectin to agglutinate blood type was not affected even after 2 hours at pH 6 and stable till pH 10, while on the other hand, the lectin activity decreased at pH 11 after 30 minutes for blood types (figure 3).

Similar results were earlier reported (Chandrika and Shaila, 1987) in determining the pH activity of lectin from the seeds of *Mimosa invisa* L.

Hapten Inhibition Assay

As reported earlier (Xu *et al.*, 2007), hapten inhibition assays using equimolar concentrations of different simple sugars demonstrated that the agglutination activity of the *Ocimum sanctum* was not affected by any of the sugars tested in the present study. This might be due to the fact that used lectin could possess multiple binding sites and hence it can relate with a wide variety of sugars.

Effect of chelating agent and metal ions

As reported earlier (Sadananada *et al.*, 2013), similar kind of studies were conducted to report the incubation of lectin with 5mM EDTA does not inhibited or decreased the activity of hemagglutinating activity even after adding the divalent

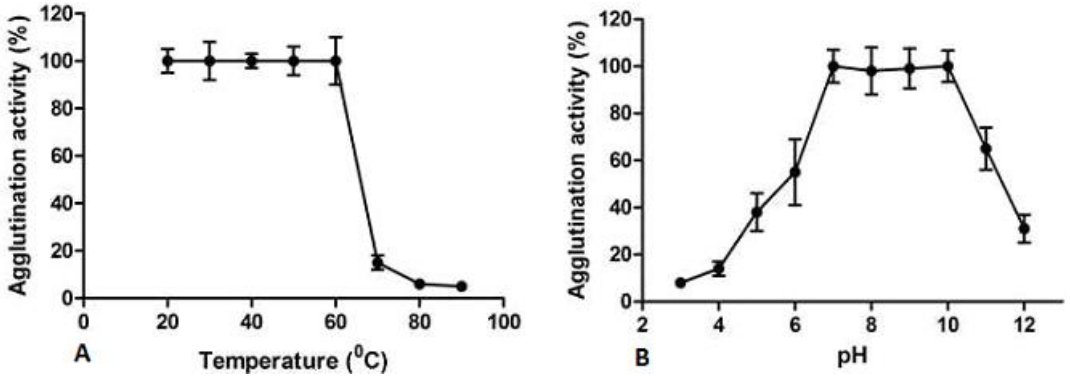

Fig. 3: Thermal stability and pH stability of lectin on the agglutinating (A): Lectin was incubated at various temperatures for 30 min then rapidly cooled on ice. The residual hemagglutination activity was tested at room temperature. The hemagglutination activity of an untreated sample, tested at room temperature, represented 100 % activity. (B): Lectin was incubated at room temperature (24 ℃) in 20 mg/ml of different pH buffers ranging from pH 3 to 12. The titer value obtained at pH 8.0 represented 100% activity.

cations such as Ca^{2+}, Mg^{2+} has not influenced on hemagglutinating activity, this results suggest that EDTA metal ion has no effect on *Ocimum sanctum* lectin, suggesting that any divalent cations are not essential for the hemagglutin activity. Formerly, as reported (Suseelan *et al.*, 1997) blackgram lectin do not show any metal ion requirement. Therefore, it was concluded that metal ions were not required for hemagglutin activity.

CONCLUSION

Prospective work might include expanding our studies to completely establish the role of other properties and functions of *Ocimum sanctum* lectin. The ease of preparation in purified form of lectins and their unique properties to specifically attach with carbohydrate moieties opens the potential use as diagnostic markers. In conclusion, a *Ocimum sanctum* lectin of 66KDa a homo-dimeric glycoprotein of identical subunits was identified and characterized. Further investigation is required to study antiproliferative, antimicrobial and anti-fungal activities of *Ocimum sanctum* lectin.

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