Original article

Isolation, Purification and Characterization of a Lectin from *Ocimum basilicum* Seeds (OBSL) with a Complex Sugar Specificity

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ARTICLE INFO

Article history:
Received 2022 May 16th
Reviewed 2022 June 12th
Accepted 2022 June l9th

Keywords:
Ocimum basilicum; Lamiaceae; Lectin; agglutinin; hemagglutination; characterization.

Abstract

Lectins are defined as the carbohydrate-binding proteins of non-immune origin that can interact and precipitates glycoconjugates from their solutions. Due to these features, many applications have been discovered for these proteins. The present investigation has been devoted to optimising a protocol for the extraction, purification and characterization of a lectin from the seeds of the medicinal plant *Ocimum basilicum* (OBSL). Different extraction procedures were employed, including varying buffers with variable pHs. Precipitation of the seeds' crude protein extract with salting-out using solid ammonium sulphate (AmS) at 40, 60 and 80% concentrations resulted in the segregation of the lectins into at least three isoforms. The lectin was purified to apparent electrophoretic homogeneity by fetuin-agarose affinity resin. The lectin was a trimer composed of three subunits of different molecular weights. Scanning the three AmS precipitated protein fractions at the UV ranges, indicates that the protein is rich in tryptophan rather than tyrosine. The lectin haemagglutination pattern studies showed that OBSL had a better affinity for the B+ human blood group, sheep and rabbit erythrocytes as compared to other human and animal blood types. The lectin was not inhibited by any simple sugar indicating its classification as a lectin with complex sugar specificity. OBSL was stable within the pH range from 2.5 to 10.5 showing two pH activity optima. It was optimally active from 40 to 50 °C. This study represents the first-ever report on this plant lectin and might stimulate further studies to extensively characterize this protein.

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1. Introduction

Ocimum basilicumL. is an important medicinal plant and culinary herb. It is often known as sweet basil, or great basil and has been in use as a folk medicinal plant for the treatment of many diseases (Simon *et al.* 1999). The basils, belong to the genus Ocimum and the mint family Lamiaceae. Species of Lamiaceae are valued for their pharmaceutical properties; for example, the aromatic oils produced in their leaves are used as antioxidants(Vieira & Simon 2000), antifungal, insect repelling, and toxic activities (Bais *et al.* 2002). Lectins are a unique class of proteins or glycoproteins broadly found in nature, which selectively recognizes and reversibly binds to sugars and glycoconjugates through their affinity sites by hydrogen bondings, hydrophobic interactions and Van der Waals forces(Misevic & Garbarino 2021). For a long time,

these proteins were termed hemagglutinins, due to their capacity to agglutinate red blood cells. This term was coined to include all proteins from different sources, non-immune basis and with the capacity to bind carbohydrates, with or without recognition capacity for blood type (Santos *et al.* 2014). Lectins are generally classified according to their structure and specificity for carbohydrates. They bind reversibly with specific sugars. Some lectins may also contain another binding site that can interact with a noncarbohydrate ligand of hydrophobic nature (Osman & Konozy 2017). Thus, lectins have various specificities that are linked to their capacity to recognize and interact with acetylaminocarbohydrates, aminocarbohydrates, sialic acid, hexoses, pentoses, and many other sugars, and precipitate polysaccharides, glycoproteins and glycolipids bearing specific sugars. They have many biologically significant activities such as the ability to agglutinate red blood cells, lymphocytes, fibroblasts, spermatozoa, fungal, bacterial, and plant cells (Singh *et al.* 1999). Some plant lectins have also recently shown to possess anti-inflammatory, anti-nociceptive and antiulcer properties (Konozy *et al.* 2022). In our laboratory, we have detected strong hemagglutinating activity in the seeds of *Ocimum basilicum* and tempted us to isolate, purify and characterize this protein, therefore this work was undertaken.

2. MATERIALS AND METHODS

2.1 Materials and Reagents

Ocimum basilicum was authenticated by a plant taxonomy expert. Good quality seeds were collected locally and used for the isolation of lectin. Typed human blood cells (A, B, AB, and O) were obtained from healthy donors. All other reagents were either of analytical grade or the highest quality available. The present study was conducted at the laboratory of glycobiology and proteomics, Africa city of technology, Khartoum, Sudan.

2.2 Extraction and Isolation of Lectin

Seventeen grams of dry *Ocimum basilicum* seeds were pulverized into a fine powder with a coffee mill. The seeds powder was defatted and depigmented with N-hexane at room temperature (10 mL/g) (Konozy *et al.* 2002). The supernatant was decanted to remove hexane and then washed with at least two folds of hexane till no further colour was noticed in the washings. Washed seed powder was left at room temperature overnight for complete evaporation of hexane. The resultant 15 g of the hexane dried powder was divided into 4 lots each of 3.5 g were stirred with different buffers namely 50mM Tris-buffered saline (TBS) pH 7.4; 0.9% Saline; 50mM Phosphate buffered saline (PBS) pH 7.5 and 50mM Acetate buffer pH 5.5 at 4°C for 4hr. The extract was centrifugation at 10000*rpm* for 30 min. The clear supernatant was then used for lectin activity evaluation and protein content determination. Proteins in the supernatant were fractionated by salting out technique using solid ammonium sulphate (40, 60and 80% saturation) (Ahmed *et al.* 2019). Obtained precipitated protein was dissolved in a minimal amount of buffer and dialyzed exhaustively against distilled water till free of ammonium sulphate. These protein fractions were termed henceforth Fr40, Fr60 and Fr80 according to the ammonium sulphate added to obtain respective concentrations. The dialyzed fractions were lyophilized to dryness and stored at -20°C till further analysis.

2.3 Purification of the lectin

Five milligrams of crude seed extract were loaded onto the affinity column of fetuin-agarose. The protein was recycled at least 3 times, the column was washed from unbound protein with at least a 12-bed volume using 50mM Tris-HCl pH 7.5 buffer till the column eluents were dropped to below OD_{280nm} 0.02. The bound lectin was eluted using 3% acetic acid prepared in 150mM NaCl. 3mL fractions were collected and those fractions with protein readings were pooled and dialyzed exhaustively against distilled water, lyophilized to dryness and preserved at -20 °C till further use.

2.4 Determination of Protein Content

Protein content in different samples was determined according to Bradford's method using bovine serum albumin (BSA) as the standard (Bradford 1976).

2.5 Native and SDS-PAGE

Native-PAGE and SDS-PAGE for studying purity as well as subunits structure of lectin respectively were performed according to references (Reisfeld *et al.* 1962; Laemmli 1970) respectively.

2.6 Hemagglutination assay (HA) and lectins blood group specificity

The hemagglutinating activities of the extracts at the various stages of purification were performed in 96-well (U- or V-shaped) plates. Serially two-fold dilution lectins solution obtained from crude extract, Ammonium sulfate precipitated fractions and subsequent purifications stages were mixed with an equal volume of a 2 % suspension of human RBCs of all types, and animal erythrocytes (sheep, rabbit, donkey, camel, cow and goat). The hemagglutinating activity was tested with intact and trypsinized human/animal erythrocytes (Lis & Sharon 1972). In brief: the obtained raw blood was centrifuged at room temperature at 2500*rpm* for 5 min. the supernatant was plasma was decanted and the cells pellets (erythrocytes) were washed 4-5times with 150mM NaCl at 2500*rpm* for 5 min. 2% erythrocytes solutions were prepared and used for the hemagglutination detection of lectin. For the trypsinized erythrocytes, trypsin was added to erythrocytes at a final concentration of 0.1% (w/v) in 50mM Tris-HCl pH 7.5, the mixture was incubated at 37 ℃ for 1 hour. The trypsinized erythrocytes were washed 4-5 times with 150mM NaCl to remove the enzyme. The erythrocytes solution was then made into 2% in 150mM NaCl and used for the assay. Lectin samples were mixed with trypsin-treated or untreated erythrocytes and the hemagglutination activity was performed at room temperature exactly as shown before and reported after an hour. The hemagglutination titer was defined as the reciprocal of the minimal dilution that was able to induce visible erythrocyte agglutination.

2.7 Lectin sugar specificity assay

The lectin sugar specificity assay towards the different mono, di and tri-sugars was performed by setting up a series of wells containing twice the minimum haemagglutination dose of lectin together with serial dilutions of the sugar under test. This test was carried out using the following sugars galactose, glucose, lactose, maltose, mannose, raffinose, ribose, N-acetyl-D-galactosamine and N-acetyl-D-glucosamine.In the first row of wells of the microtiter plates, 50 µL of carbohydrates (500mM) were added. The carbohydrates were serially diluted with an equal volume of 150mM NaCl. Thereupon, 50 µL of the 4 Units lectin sample was added to each well. The lectin-sugar complexes were incubated at room temperature for 30 minutes before 50µL of a 2% suspension of trypsinized type A human erythrocyte was added. After an hour, the lowest concentration of carbohydrate that was able to inhibit agglutination of erythrocytes at 37 °C was determined. Controls samples were only sugar and lectin or lectin plus saline were also performed (Awadallah *et al.* 2017).

2.8 Effect of pH

To determine the optimal pH for lectin activity, hemagglutination assay was carried out using buffers of different pHs: 100mM glycine pH 2.5,9.5,10.5 and 11.5; 100mM acetate pH 3.5 and 5.5; 100mM Tris-HClpH 4.5, 100mM phosphate pH6.5 and 7.5 and 100mM Tris pH 8. Aliquots of lectin samples were incubated with these buffers at room temperature following the addition of erythrocytes and hemagglutination determination (Osman *et al.* 2016).

2.9 Effect of temperature

The optimal temperature of lectin was carried out at 0, 10, 20, 30, 40, 50, 60, 70, 80, and 90°C. These were performed by incubating aliquots of lectin at respective temperatures for 30min then lectin solutions were immediately cooled in an ice bath, and hemagglutination activity was determined with 2% suspension of trypsinized type A human erythrocyte. The thermal stability of lectin was assessed by incubating samples at 50°C for 30, 40, 50, 60, 70, 80, 90, 100, 110 and 120 min and lectin samples were then assayed for hemagglutination. Lectin activity at any given temperature was compared to control samples incubated at 4°C and expressed as a percentage of relative activity (Osman *et al.* 2016).

2.10 The UV scan of ammonium sulphate precipitated protein fractions:

This was carried out by scanning the ammonium sulphate precipitant protein fractions (Fr40, 60 and 80) in the UV ranges from 250 nm to 300 nm (Anthis & Clore 2013).

3. RESULTS AND DISCUSSION

3.1 protein extracts

The lectin extraction procedure is often started by defatting the finely ground seeds powder with hexane or petroleum ether (Konozy *et al.* 2002). Then followed by polar solvent extraction of the seeds powder to obtain soluble lectin in reasonably detectable quantities. The current work was started by optimising the extraction procedures by using different buffers with different pHs ranging from low acidic to highly basic. Lectin activity and protein contents were simultaneously evaluated to assess suitability and efficiency. The obtained results are depicted in Table 1. Though the major part of lectin was extracted using the acetate buffer as indicated by the obtained specific activity (233 U/mg), the protein content was rather low, secondly, the presence of lectin in acetate buffer rendered the lectin stability very weak, in addition, at this low pH, it becomes challenging to test the lectin activity due to the rapid RBCs hemolysis. Therefore, although the Tris buffer comparatively resulted in a lower specific activity, however, the lectin activity was stable and the protein content was high to enable broad characterization. Therefore the entire purification and characterization were carried out using 50mM Tris buffer pH 7.5. Table 1 exhibits the protein concentrations and the extracted lectin activity using different buffers.

Table: 1 *Protein content and OBSL activity extracted by different buffers*

Extract	Concentration (mg/ml)	Total Protein (mg)	Activity Unit (HU)	Specific Activity ($HUmg^{-1}$)	Total Unit
Crude extracted with PBS buffer	1.5	147	128	87	12800
Crude extracted with Tris-HCL buffer	1.2	122	8	7	800
Crude extracted with 150mM NaCL	0.2	22	8	36	800
Crude extracted with Acetate Buffer	0.3	28	64	233	6400

3.2 protein fractionation by salting out using ammonium sulphate (AmS)

Fractionation by a salting-out technique using solid ammonium sulphate is a recurrent step in many protein purifications (Konozy *et al.* 2002; Ahmed *et al.* 2019) which results firstly in reducing contaminant proteins and secondly in the separation of different isoforms of the desired protein. The major amount of protein was precipitated when the crude extract was brought to 80% AmS followed by Fr40 then Fr60. All of these fractions presented with lectin activity, indicating the presence of OBSL in multiple forms (isoforms), these results are in agreement with several publications where lectins from many plants are usually exist in isoforms (Konozy *et al.* 2002; Osman *et al.* 2016; Awadallah *et al.* 2017). Although lectin was detected in all AmS fractions, Fr40 and Fr60 tended to cause hemolysis of the RBC before their complete settlement in the plate, which made conclusive judgment and calculation of the activity of lectin challenging. Such a problem was not noticed in the case of Fr80. Many plant extractants are known cause RBCs hemolysis among which is the saponin (Jones & Elliott 1969), and since *Ocimum basilicum* is already documented to contain this compound (Habib *et al.* 2016) we can not exclude the presence of these compounds in Fr40 and 60 which might have been the reason behind the noticed hemolysis in these two fractions. Though fractionation of crude extract by AmS resulted in the separation of OBSL into at least three isoforms, however, as depicted in Table 2, no considerable lectin

enhancement/enrichment in these fractions was obtained (poor yield). Therefore the using of AmS fractions were futile and hence we decided to use the crude seed extract for the purification of the OBSL.

Table 2. *Ocimum basilicum seed crude extract fractionating by AmS*

Extract	Volume (mL)	Concentration (mg/ml)	Total Protein (mg)	Activity (HU)	Specific Activity (HUmg-1)	Purification Fold	Total Unit	Yield %
Crude with Tris-HCL	100	1.2	122	8	7	1	800	100
Fr 40%	4.5	2.6	11.9	HEM	-	-	-	-
Fr 60%	6	2.0	11.8	HEM	-	-	-	-
Fr 80%	30	1.2	36.9	16	13	1.9	480	0.6

HEM: RBCs hemolyzed.

3.3 Purification of OBSL

Since none of the tested sugars was successful in inhibiting OBSL hemagglutinating activity. It was logical to try purifying the lectin using fetuin-agarose. Fetuin is a glycoprotein containing sialylated N-linked and O-linked glycans (Carr *et al.* 1993) and would often be used for the purification of lectins with complex sugar specificities (Broekaert *et al.* 1984; Koppe & Rurnow 1988; Sun *et al.* 2007). The bound lectin was eluted from the affinity column with enhancement in the purification fold 37 times Table 3. Upon performing native-PAGE to assess the homogeneity of the preparation native-PAGE was performed which resulted in a single discrete band indicating the purity of our protein (Figure 1-A). The subunit structure was evaluated using SDS-PAGE under the reduced form, where the lectin was split into three subunits of molecular weights 66, 58 and 39kDa (Figure 1-B). Since we did not determine the molecular weight of the native lectin, we can not confirm the number of subunits that shape the full structure of this protein, however, since many lectins from this family are of trimeric compositions, OBSL is likely to be of trimeric native molecular weight of total 163kDa. This native molecular weight is very close to many Lamiaceae lectins, the family of *Ocimum basilicum* (Pérez & Vega 2007).

Table 3: *Purification chart of OBSL*

Extract	**Volume (mL)**	**Concentration (mg/ml)**	**Total Protein (mg)**	**Activity (HU)**	**Specific Activity (HUmg-1)**	**Total Unit**	**Purification Fold**
Crude with Tris-HCl	100	1.2	122	8	7	800	1
Fetuin-agarose	3	2	6	512	256	1536	37

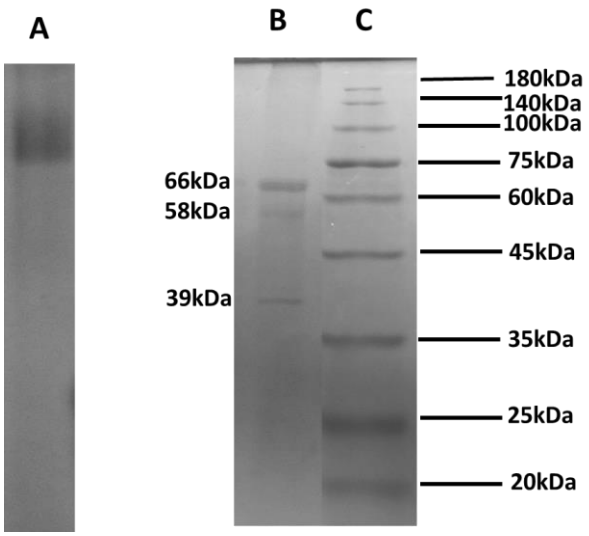

Figure 1: *Native and SDS-PAGE of OBSL*
5µg lectin were loaded and gels were stained with colloidal coomassie blue G-250 (Dyballa & Metzger 2009)

A: OBSL native –PAGE
B: OBSL SDS-PAGE (Subunit molecular weights)
C: Molecular weight markers

3.4 Hemagglutination assay

The OBSL agglutinated fairly the untreated human erythrocytes, with an apparent tendency towards B+ erythrocytes as compared to A, AB and O blood groups. Treatment of erythrocytes with trypsin is known to enhance the agglutination tendency of many lectins (Singh & Walia 2018; Mizgina *et al.* 2021). However, in the present study treatment of erythrocytes with protease suppressed remarkedly the agglutination (Table 4).

Table 4: *Hemagglutination activities for Ocimum basilicum against 2% human erythrocytes*

Blood groub	A^{+ve}	$A^{+ve}{}_{Tryp}$	B^{+ve}	$B^{+ve}{}_{Tryp}$	AB^{+ve}	$AB^{+ve}{}_{Tryp}$	O^{+ve}	$O^{+ve}{}_{Tryp}$
Crude	32	4	256	4	8	4	4	4
Fraction 40%	HEM	HEM	HEM	HEM	HEM	HEM	HEM	HEM
Fraction 60%	HEM	HEM	HEM	HEM	HEM	HEM	HEM	HEM
Fraction 80%	32	2	32	2	512	2	4	2

HEM: RBCs hemolyzed.
The term "Tryp" at superscript indicates trypsin erythrocyte treated with tryp

Table 5: *Hemagglutination activities of OBSL against animal erythrocytes*

Animal Blood	Activity	Unit Activity
Sheep	4	16
Sheep ^{Tryp}	4	16
Goat	3	8
Goat ^{Tryp}	3	8
Cow	3	8
Cow ^{Tryp}	4	16
Donkey	2	4
Donkey ^{Tryp}	2	4
Rabbit	5	32
Rabbit ^{Tryp}	6	64
Camel	2	4
Camel ^{Tryp}	2	4

The term Tryp at superscript indicates erythrocyte treated with trypsin

On the other hand, OBSL showed a high level of agglutination towards animal erythrocytes such as sheep, cow and rabbit blood. Treatment of animal erythrocytes with trypsin did not cause a major change in lectin agglutination (Table 5).

3.5 Carbohydrate specificity of lectin

The carbohydrate specificity of an agglutinin in any plant crude extract is often done by the hapten inhibition tests using a series of simple sugars. Among the different mono, di and trisaccharides we used none were successful in inhibiting the agglutinating activity of OBSL indicating the complex sugar activity of this lectin. The presence of plant lectins with

complicated sugar specificity is not rare. Many plant lectins have failed to be inhibited by simple sugars and therefore have been characterized as complex specificity types of lectins(Bourne *et al.* 2002; Adenike & Eretan 2004; Benevides *et al.* 2012).

3.6 Effect of pH and Temperature

The optimum pH of OBSL was 7.5, after and below which the activity sharply declined. At around pH 8.5, the OBSL lost almost 95% of its activity. However, an interesting result was noticed after this point, as the pH rises the lectin activity again started to shoot up giving other optima between pH 11 to 12 (Figure 2). Lectins with such extreme stability are not scarce, Okabe and his colleagues had shown a lectin from to fog egg to be stable till pH 10, with a loss of less than 5% of its total activity (Okabe *et al.* 1991), whereas Phaseolus vulgaris cv. (Anasazi Beans) was shown to be fairly stable in the pH range from 1 to 14 (Sharma *et al.* 2009).

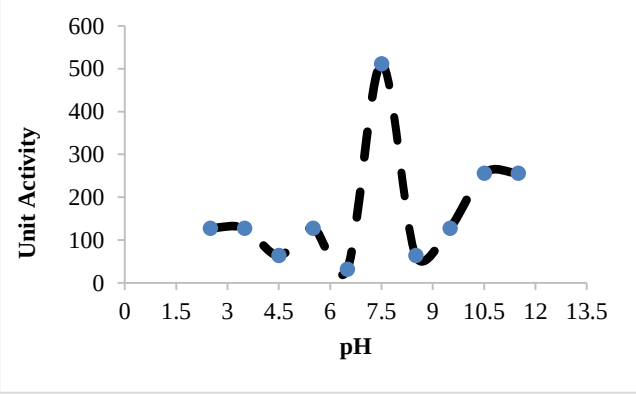

Figure 2. ***Effect of pH on OBSL activity***
Aliquots of lectin samples were incubated with buffers of different pHs (see method section). The mixtures were neutralized by the addition of either 0.1 M NaOH or 0.1 M HCl, and the lectin assay was performed as shown in the methods section.

Upon exposing OBSL to different degrees of temperature ranging from 0 to 100 °C. The lectin exhibited remarkable stability with a broad temperature optimum between 40 to 55 °C. However, at 60 °C the lectin activity suddenly dropped losing more than 85% of its original activity (Figure 3). Lectins with high thermal stability are a common feature of many plant agglutinins. Working with *Apuleia leiocarpa* seeds, Carvalho and her colleagues reported that the purified lectin was thermally stable till 100 °C (de Souza Carvalho *et al.* 2015). Preserving aliquot of OBSL at room temperature for up to 45 days, resulting in no noticeable loss in lectin activity confirming the high stability of OBSL (not shown).

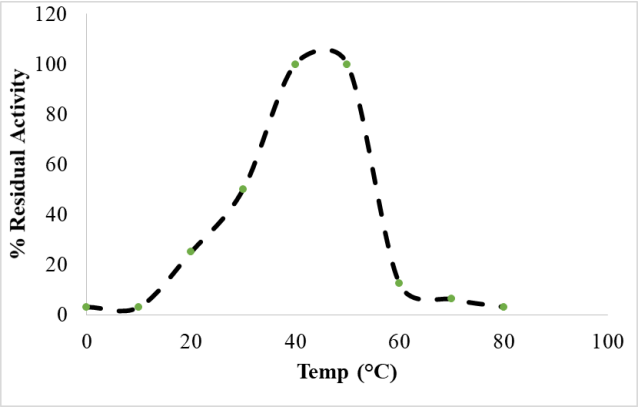

Figure 3. ***Effect of temperature on OBSL activity***

Aliquots of lectin samples were incubated for 30 min at different temperatures ranging from0 °C to 80 °C (see method section). The samples were immediately cooled in and the lectin assay was performed as shown in the methods section

3.7 UV Scan of Ammonium sulphate protein precipitated (AmS) fraction

Scanning of the three ammonium sulphate precipitated fractions (Fr40, Fr60 and Fr80) at the UV range resulted in

interesting results. The three fractions were apparently of protein of similar content to the aromatic amino acid tryptophan. Tryptophan and tyrosine and phenylalanine are known to contribute majorly toward protein absorbance at the UV ranges respectively (Antosiewicz & Shugar 2016). From Figure 4 it is apparent that the three AmS fractions (Fr40, Fr60 and Fr80) are rich in tryptophan rather than tyrosine in which the absorbance maxima were centred at 280nm, a region where absorbance is often due to tryptophan rather than tyrosine which absorbs maximally at around 275nm.

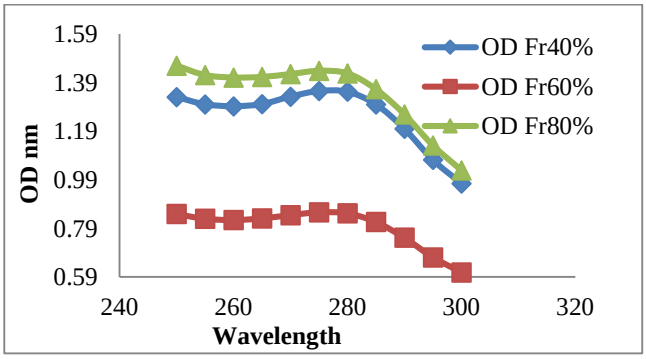

Figure 4: *Ammonium sulphate (AmS) precipitated protein fractions (Fr40, Fr 60 and Fr80) scanned at UV- range (250nm to 300nm).*

4.0 Conclusion

In this study, a lectin with complex sugar specificity was isolated and purified to apparent electrophoretic homogeneity. Fractionation of the seed crude extract by a salting-out technique using solid ammonium sulphate resulted in the isolation of at least three OBSL isoforms. The interesting results obtained with respect to the lectin's high thermal stability and high purification yield may urge further structural and molecular studies that would pave the way in investigating the lectin applications in the biological and medical fields.

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