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Purification and Characterization of Lectins from Mulberry Seeds (*Morus alba* L.)

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Abstract: Three electrophoretically homogeneous lectins were purified from the extract of mulberry seeds. The two lectins i.e. MSL-2 and MLS-3 were purified by gel filtration of 100% ammonium sulfate saturated crude extract followed by ion-exchange chromatography on DEAE-cellulose while another MSL-1 was purified by further chromatography on CM-cellulose column. The purity of the lectins was checked by polyacrylamide disc gel electrophoresis. The molecular masses of MSL-1, MSL-2 and MLS-3 as determined by gel filtration were estimated to be 175, 120 and 89 kDa, respectively. Again molecular masses of MSL-1, MSL-2 and MSL-3 as determined by SDS-polyacrylamide gel electrophoresis were calculated to be 177, 121 and 90 kDa, respectively. SDS-polyacrylamide gel electrophoresis indicated that the lectin, MSL-1 was dimer in nature with two subunits held together by disulfide bonds. The other two lectins, MSL-2 and MSL-3 were tetramer in nature contained four non-identical subunits that were held together by nonionic hydrophobic interaction. The lectins agglutinated specifically rat red blood cells and galactose and galactose containing sugars were found to be the potent inhibitor of agglutination. The lectins were glycoproteins in nature with neutral sugar content of 5.6, 5.3 and 4.5% for MSl-1 MSL-2 and MSL-3, respectively. The sugar composition of the lectins was glucose and mannose for MSL-1 and galactose for both MSL-2 and MSL-3.

Key words: Lectins, moraceae, hemagglutination

INTRODUCTION

Lectins are a group of (glyco)proteins found in a wide variety of plants, animals and microorganisms that interact with glycoconjugates and polysaccharides by binding to specific carbohydrate residues[1,2]. The presence of lectins has been reported in various plants[3-6]. Lectins are currently attracting much interest primarily because they serve as invaluable tools in diverse area of biomedical research[7]. Because of their unique carbohydrates binding properties, lectins are useful for the separation and characterization of glycoproteins, glycopeptides and in the studies of glycolipids; following the changes that occur in the cell surfaces during physiological and pathological processes from cell differentiation to cancer; histochemical studies of cells tissues; tracing neural pathways; typing blood cells and bacteria; fractionation of lymphocytes and bone marrow transplantation. They are also used to stimulate lymphocyte to assess the immuno state of patients and for chromosome analysis in human cytogenetics as well as production of cytokines. In addition, lectins are excellent model for examining the molecular basis of specific reactions that occur between proteins and other types of molecules such as binding of antigen to antibodies, substrate to enzymes, drugs to proteins and hormones and growth factor to cells.

Mulberry (*Morus alba* L.) is the host plant of silkworm, *Bombyx mori* that produces silk. It belongs to the family *Moraceae* under genus *Morus*. It is a deep-rooted perennial woody plant commercially propagated through vegetatively. Mulberry seed contains 37% carbohydrate, 32% fat and 15% protein. We found three lectins of *Morus alba* L. which exhibit high agglutination activity. The present study demonstrated the presence of three lectins in the seeds of *Morus alba* L. and their purification and characterization.

MATERIALS AND METHODS

Mulberry seeds were collected from the Bangladesh Sericulture Research and Training Institute, Rajshahi, Bangladesh in 1998-99. Sephadex G-150, DEAE-cellulose and Sepharose-4B were purchased from Sigma chemicals, USA. All other reagents were used in analytical grade.

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Extraction of crude lectins: All the experimental procedures were carried out at 4-10°C unless otherwise stated. First the mulberry seeds were taken in a mortar and pounded uniformly into fine powder. Pre-cooled petroleum ether (40-60°C) was added in this powder and homogenized uniformly with the homogenizer. Discarding the oily part, the homogenate was filtered through a muslin cloth. The process was repeated two times in order to make quite fat-free. The filtrate was then further clarified by centrifugation at 8,000×g for 10 min. The precipitate obtained after centrifugation were collected, air-dried at room temperature. Water was used as suitable extracting media for preparation of crude protein from the fat-free powder as reported by Clark and Switzer[8]. The fat-free dry powder was mixed uniformly with pre-cooled distilled water (4 mL g^{-1} meal) and kept overnight at 4°C with occasional stirring. The suspension was then filtered through a muslin cloth and centrifuged at 8,000×g for 20 min. The clear supernatant was collected and adjusted to 100% saturation by adding solid ammonium sulfate. The ammonium sulfate precipitate was centrifuged and dissolved in minimum volume of water and dialyzed against 5 mM phosphate buffer (pH 7.6) for 24 h at 4°C. Then it was further centrifuged and clear supernatant was used as crude lectin.

Purification of lectins

Gel filtration: Gel filtration of crude lectin extract was performed on sephadex G-150 using 5 mM phosphate buffer (pH 7.6) at 4°C.

DEAE-cellulose chromatography: The protein fraction obtained by gel filtration was dialyzed against distilled water for 12 h and against 10 mM Tris-HCl buffer (pH 8.4) for 12 h and then loaded onto the DEAE-cellulose column at 4°C. The lectins were eluted from the column with the same buffer containing different concentration of NaCl (0.06, 0.18 and 0.3 M).

CM-cellulose chromatography: The above protein fraction (not pure) obtained from DEAE-cellulose chromatography was dialyzed against distilled water and 5 mM phosphate buffer for 12 h each and then loaded onto the column. The lectin was eluted from the column with the same buffer containing 0.2 M NaCl.

Polyacrylamide disc gel electrophoresis: Polyacrylamide disc gel electrophoresis was conducted at room temperature (pH 8.4) on 7.5% gels as described by Ornstein[9]. 1% amido black was used as staining reagent.

Characterization of lectins

Molecular mass determination by gel filtration: The molecular masses of the lectins were determined by gel filtration on sephadex G-150 (0.75×100 cm) using lysozyme, trypsin inhibitor, α-amylase, bovine serum albumin and β-amylase as reference proteins.

Molecular mass determination by SDS-PAGE: SDS-polyacrylamide gel electrophoresis was conducted on 10% polyacrylamide gel at room temperature (pH 7.2) according to the method of Weber and Osborn[10]. The marker proteins used were the same as those used for gel filtration. 0.1% SDS with 1% β-mercaptoethanol was used as denaturating agents and the proteins were stained with the Coomassie Briliant Blue R-250.

Hemagglutination and carbohydrate inhibition: Hemagglutination activity was performed by using 2% albino rat red blood cells according to the method of Lin *et al.*[11]. Lectin solution (200 μ) in 5 mM phosphate buffer saline, pH 7.2 was mixed with 200 μ of 2% rat red blood cell suspension by gentle stirring. The mixture was incubated at 37°C for an hour. The degree of agglutination was observed under the microscope. The agglutination activity was expressed as titre (the reciprocal of the highest dilution showing visible agglutination). The specific activity was expressed as the titre per mg of protein. The hemagglutination inhibition was performed in the presence of different sugars according to the same procedure as described above.

Affinity chromatography: Affinity chromatography was carried out on Sepharose-4B. After dialysis against 5 mM phosphate buffer saline, pH 7.2, the pure lectins were applied to Sepharose-4B column previously equilibrated with the same buffer at 4°C. The adsorbed lectin was eluted with same buffer containing 0.2 M galactose.

Protein and carbohydrate analysis: The concentrations of proteins were measured by the method of Folin-Lowry[12] using bovine serum albumin as the standard. The presence of sugar in protein was detected by periodic acid Schiff's method[13]. The total neutral sugar contents of the proteins were estimated by phenol-sulfuric acid method[14] with D-glucose as the standard.

According to one-dimensional Thin Layer Chromatography was performed to identify the sugar components in the hydrolyzed protein solution according to Joseph and Murrel method[15] using different standard sugars. The chromatogram was developed with the solvent mixture: Isopropanol, acetic acid and water (3:1:1, v/v/v). Aniline-phthalic acid solution was used as spraying reagent for identifying spots.

RESULTS

Purification of mulberry seed lectins: The 100% ammonium sulfate saturated crude lectin extract after dialysis against 5 mM phosphate buffer, pH 7.6 was applied to sephadex G-150 column at 4°C, which was previously equilibrated with same buffer. Lectins were eluted as one major peak i.e. fraction, F-1. The fraction, F-1 as indicated by solid line was pooled precipitated by 100% ammonium sulfate and purified further by ion-exchange chromatography (Fig.1). The precipitate of F-1 fraction was dissolved in minimum volume of distilled water, dialyzed against distilled water for 12 h and against 10 mM Tris-HCl buffer, pH 8.4 at 4°C for 12 h. After removal of insoluble materials, the clear supernatant was applied to a DEAE-cellulose column at 4°C, previously equilibrated with same buffer. The lectins were eluted were eluted by a linear gradient of NaCl from 0.0 to 0.3 M in the same buffer. The column bound lectins were eluted as a single but broad peak, indicating the presence of more than one components (data not shown). In order to separate these components, the elution was carried out stepwise with increasing concentration of NaCl in the same buffer.

Figure 2 showed that the components of F-1 were separated into three major fractions i.e. F-1a-c, which were eluted with the same buffer containing 0.06, 0.18 and 0.3 M NaCl, respectively. The fractions indicated by solid bar were pooled separately and their homogeneity was checked by palyacrylamide disc gel electrophoresis. It was evident from the electrophoretic pattern (Fig. 3) that the Fraction F-lb and F-1c contained pure protein as they gave single band while F-1a gave two bands on the gel. The fraction F-1a was further purified by CM-cellulose chromatography. All three fractions possessed hemagglutination activities.

The fraction of F-1a as indicated by solid bar was pooled together and dialyzed over night against 5 mM phosphate buffer, pH 6.5 and applied to the CM-cellulose column previously equilibrated with the same buffer at 4°C. Figure 4 showed that fraction F-1a was separated into two peaks, F-1a' and F-1a˝. The major peak F-1a' was eluted with the buffer only where as a small minor peak F-1a˝ was eluted from the column with buffer containing 0.2 M NaCl of these two fractions, only F-1a' exhibited lectin activity. The fraction F-1a' contained pure protein as it gave single band on 7.5% polysaccharide gel (Fig. 3D) The overall purification data of mulberry seed lectins were summarized in Table 1. The fraction, F-1b showed maximum hemagglutinating activity with a purification of

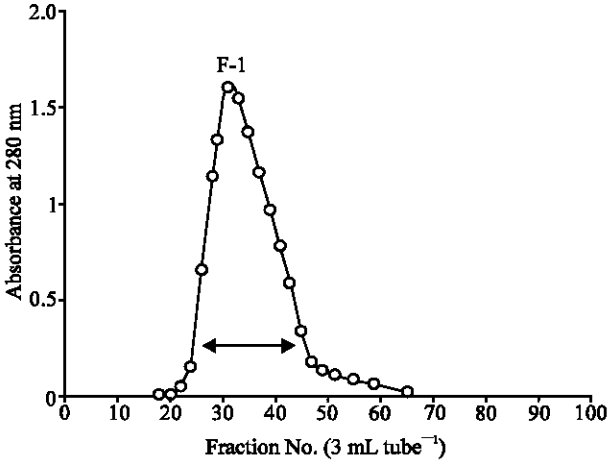

Fig 1: Gel filtration of crude lectin extract on Sephadex G-150. The crude extract (82 mg) was applied to the column (2.5×100 cm), pre-equilibrated with 5 mM phosphate buffer, pH 7.6 at 4°C and developed with same buffer

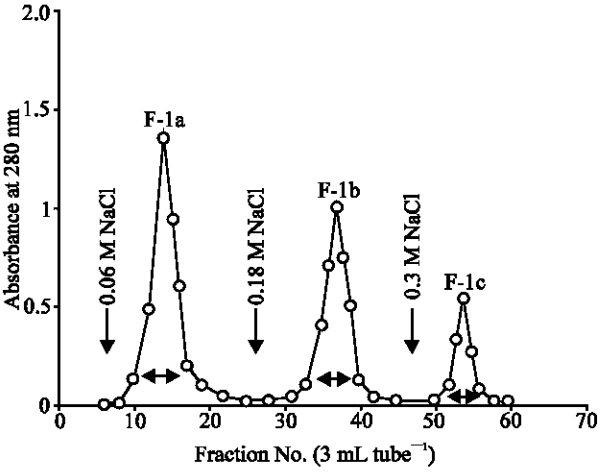

Fig 2: Ion-exchange chromatography of fraction-1 on DEAE cellulose. F-1 (28 mg) obtained from gel filtration was applied to the column (2.1×24 cm), which was previously equilibrated with 10 mM Tris-HCL buffer, pH 8.4 at 4°C ad eluted by step wise increase of NaCl concentration in the same buffer

15.27 fold while F-1a' and F-1c fractions showed 12.47 and 10.68 fold increase in hemagglutinating activities respectively over crude extract. Although over 96.70% protein was lost during purification process and the overall yield of hemagglutinating activity was found to decrease about 43%, the purification fold was increased about 38 fold after each subsequent purification steps.

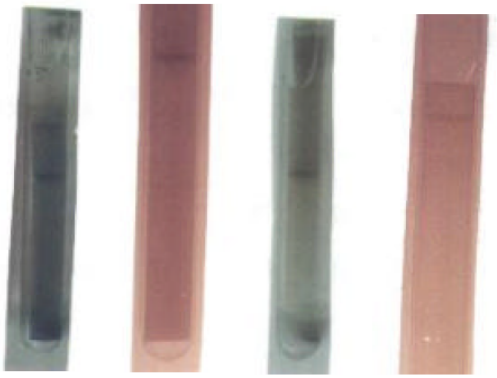

Fig 3: Polyacrylamide disc gel electrophoretic pattern of purified protein at room temperature on 7.5% gel using 1% amido black as using staining reagent. A=F-1a, B=F-1b, C=F-1c and D=F-1a'

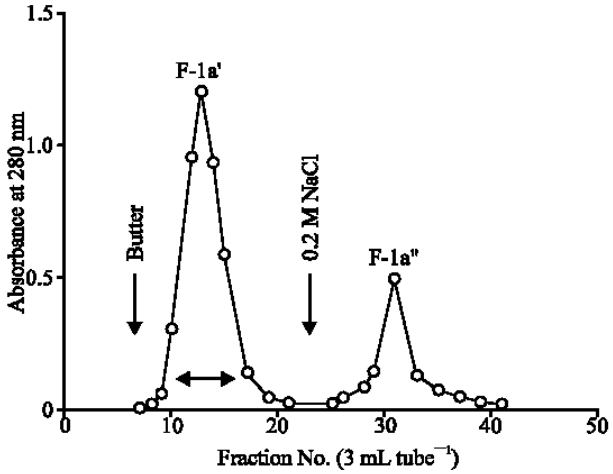

Fig 4: Ion- exchange chromatography of fraction F-1a on CM- cellulose. F-1a (12 mg), obtained from DEAE cellulose chromatography was applied to the column (1.5×15 cm), pre-equilibrated with 5 mM phosphate buffer, pH 6.5 at 4°C and eluted by buffer and 0.2 M NaCl concentration in the same buffer

The yield might be decreased due to the denaturation of proteins during the lengthy purification procedures.

Molecular masses of the lectins and their subunits: The molecular masses of the lectins as determined by gel filtration were estimated to be 175, 120 and 89 kDa for F-1a' (Mulberry seed lectin, i.e. MSL-1), F-1b (Mulberry seed lectin i.e. MSL-2) and F-1c (Mulberry seed lectin i.e. MSL-3), respectively. It was found that in the presence of

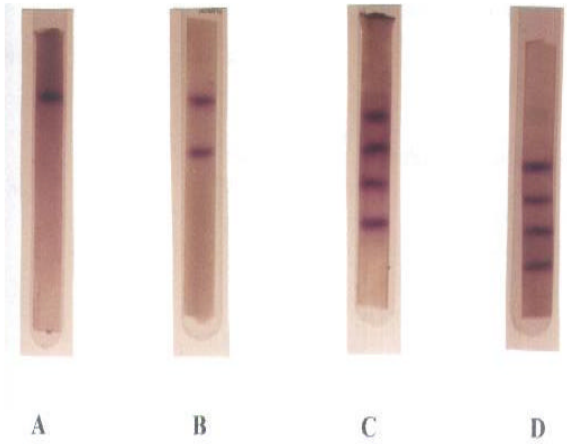

Fig 5: SDS-PAGE pattern of lectins on 10% gel at room temperature (Staining reagent: Coomassie Brilliant Blue). A, F-1a' (MSL-1) in presence of SDS; B, F-1a'(MSL-1) in the presence of SDS and β-mercaptoethanol; C, F-1b (MSL-2) in the presence of SDS; D, F-1c (MSL-3) in the presence of SDS.

0.1% SDS, MSL-1 gave a single band with molecular mass 177 kDa, while MSL-2 and MSL-3 gave four distinct bands on SDS-PAGE (Fig.5). In the presence of 0.1% SDS and 1% β-mercaptoethanol, MSL-1 gave a strong and weak bands, corresponding to molecular masses of 110 and 70 kDa, respectively while MSL-2 and MSL-3 gave four bands with molecular masses of 42, 35, 25 and 19 and 35.5, 22.5, 17 and 15 kDa, respectively. The molecular masses of MSL-2 and MSL-3 as determined by summation of molecular masses of the subunits were found to 121 and 90 kDa, respectively.

Affinity to Sepharose-4B: The binding abilities of mulberry seed lectins to sepharose-4B were examined by chromatographic procedure. All three lectins, MSL-1, MSL-2 and MSL-3 firmly bound to sepharose-4B in 5 mM phosphate buffer, pH 7.2 at room temperature and eluted from the column as sharp peak by 0.2 M galactose in the same buffer (data not shown).

Hemagglutination properties: The lectins, MSL-1 and MSL-3 agglutinated the albino rat red blood cells a minimum protein concentration of 9, 12.75 and 19.25 μg mL^{-1}, respectively was needed for visible agglutination. It is evident from the results that galactose, methy-α-D-galactopyranoside, methy-β-D-galactopyranoside, lactose and D-raffinose are the most potent inhibitors for all three lectins; the β-anomers were found to be slightly more potent inhibitors than α-anomers (Table 2).

Table 1: purification of mulberry seed lectins

Fraction		Total protein (mg)	Total hemagglutinating activity (titre)	Specific activity (titre mg^{-1})	Yield (%)	Purification (Fold)
Crude extract		600	4230	7.05	100.00	1.00
100% $(NH_4)_2SO_4$ saturated		200	3344	15.20	79.06	2.16
After gel filtration		80	2840	35.53	67.05	5.04
DEAE	F-1a	12.1	685	45.46	12.10	6.45
-cellulose	F-1b	7.6	818	107.10	19.30	15.27
fraction	F-1c	4.7	362	75.38	8.54	10.68
CM-cellulose	F-1a'	7.4	639	87.98	15.13	12.47

Table 2: Hemagglutination inhibition assay of mulberry seed lectins.

Protein	Sugar	Concentration (mM) Max.	Min.	Inhibition
MSL-1	D-glucose	110	-	NI
	D-glucosamine-HCl	110	-	NI
	N-acetyl-α-D-glucosamine	110	-	NI
	Galactose	-	20	I
	Methyl-α-D-galactopyranoside	-	28	I
	Methyl-β-D-galactopyranoside	-	15	I
	N-acetyl-α-D-galactosamine	110	-	NI
	Lactose	-	20	I
	D-Mannose	110	-	NI
	D-Raffinose	-	25	I
MSL-2	D-glucose	110	-	NI
	D-glucosamine-HCl	110	-	NI
	N-acetyl-α- D-glucosamine	110	-	NI
	Galactose	-	20	I
	Methyl-α-D-galactopyranoside	-	25	I
	Methyl-β-D-galactopyranoside	-	20	I
	N-acetyl-α-D-galactosamine	110	-	NI
	Lactose	-	20	I
	D-Mannose	120	-	NI
	D-Raffinose	-	20	I
MSL-3	D-glucose	110	-	NI
	D-glucosamine-HCl	120	-	NI
	N-acetyl-α-D-glucosamine	110	-	NI
	Galactose	-	20	I
	Methyl-α-D-galactopyranoside	-	25	I
	Methyl-β-D-galactopyranoside	-	18	I
	N-acetyl-α-D-galactosamine	110	-	NI
	Lactose	-	20	I
	D-Mannose	120	-	NI
	D-Raffinose	-	25	I

NI, No inhibition; I, inhibition

Lectin concentration and carbohydrate contents: The purified lectins in the aqueous solution gave absorption maximum ~276-280 nm and minimum ~244-248 nm. The absorbance of 1.0 at 280 nm for MSL-1, MSL-2 and MSL-3 were found to be equal to 0.90, 0.85 and 0.77 mg protein, respectively by Lowry method. Similar results were obtained from drying the protein under vacuum. The neutral sugar contents of the lectins, MSL-1, MSL-2 and MSL-3 were found to 5.6, 5.3 and 4.5%, respectively. The sugar composition of the lectins as identified by TLC was glucose and mannose for MSL-1 and galactose for both MSL-2 and MSL-3.

DISCUSSION

Three lectins have been isolated from the crude extract of mulberry seeds. All lectins are glycoproteins in nature as they gave a yellow-orange color in the presence of phenol-sulfuric acid. The presence of sugar in the lectins was further confirmed by the findings that they produced a pinkish-red band on polyacrylamide gel when gels are stained with periodic acid Schiff's staining reagents after electrophoresis (Data not shown). The lectins specifically agglutinated rat red blood cells and the agglutination by the lectins was inhibited more pronouncedly in the presence of galactose and galactose containing saccharides. This finding was further confirmed from the binding affinity of the lectins to sepharose-4B. It is concluded from the above findings that all these three lectins are galactose specific.

Although, the lectins purified from mulberry seeds are similar in their sugar specificities but quite different in their molecular masses, sub-unit structures, neutral sugar contents and sugar compositions. The three lectins were each found to migrate as single band with slightly different mobilities on polyacrylamide gel. In the presence of SDS, MSL-1 was moved as single band while MSL-2 and MSL-3 gave four bands with different molecular masses on the gel. In the presence of SDS and β-mercaptoethanol, MSL-1 was dissociated into two sub-units. From these results, it is concluded that MSL-1 is a dimeric protein with the two monomers held together by disulfide bonds, while MSL-2 and MSL-3 are heterotrophic; with the monomers held together by nonionic hydrophobic interaction. The lectins purified from plant sources contained mostly four sub-units of two identical pairs, e.g. *Abrus precatorius*[16] and very few contained four sub-units of identical molecular mass, e. g, *Phaseolus vulgaris*[17,18].

In conclusion, the purified lectins, MSL-1, MSL-2 and MSL-3, besides being specific for rat red blood cell agglutination, are members of the galactose-binding lectins.

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