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Sialic acid binding lectins from leaf of mulberry (*Morus alba*)

Sunanta Ratanapo [a], Wayakorn Ngamjunyaporn [b], Montri Chulavatnatol [b,*]

[a] *Department of Biochemistry, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand*
[b] *Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok 10400, Thailand*

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Abstract

Two new lectins, MLL 1 and MLL 2, were purified from the leaves of mulberry (*Morus alba*) by a procedure consisting of ammonium sulfate precipitation, affinity chromatography on a *N*-acetylgalactosamine-agarose column, gel filtration on a Sephacryl S-200 column, anion-exchange chromatography using a DEAE-Sephacel column and gel filtration on a Sephadex G-75 column. Both MLL 1 and MLL 2 were found to be glycoproteins with neutral sugar contents of 8.8 and 40%, respectively. They showed the same native molecular weight of 51 000. Each consisted of subunit with molecular weight of 16 500. They were highly specific for *N*-glycolylneuraminic acid. © 1998 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Lectins; Sialic acid; *N*-glycolylneuraminic acid; *Morus alba*; Mulberry

1. Introduction

Lectins are multivalent carbohydrate-binding proteins or glycoproteins of non-immune origin and found in various organisms [1,2]. In addition to their many biological roles, they are also widely used as biochemical tools in many fields of research [3–5]. Sialic acids which are often found as terminal sugars of glycoconjugates play important roles in many biological recognition mechanisms [6]. Various derivatives of sialic acids and their different glycosidic linkages to oligosaccharides contribute to the remarkable diversity of glycoconjugates on the cell surfaces of some pathogenic bacteria [7,8] and human cancer [9,10]. Lectins with specificity to different forms of sialic acids therefore have been increasingly used as diagnostic tools for identifying pathogenic bacteria and

Abbreviations: MLL, mulberry leaf lectin; NeuGc, *N*-glycolylneuraminic acid; NeuAc, *N*-acetylneuraminic acid; GalNAc, *N*-acetylgalactosamine; ManNAc, *N*-acetylmannosamine; Gal, galactose; Glc, glucose; Man, mannose; BSM, bovine submaxillary mucin; HA, hemagglutination activity; TBS, tris-buffered saline; $AmSO_4$, ammonium sulfate; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

* Corresponding author. Tel.: +662-2455195; fax: +662-2480375; e-mail: scmcl@mahidol.ac.th.

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malignant tumors. Thus search for new lectins with specificities for different sialic acids is of an interest. Most sialic acid-specific lectins so far have been purified from invertebrates [11] but few are from plants [12–14].

Leaves from mulberry plant (*Morus alba*) are the most preferred feed for silkworm, *Bombyx mori*. From our preliminary study, crude extract of the leaf was found to induce the agglutination of certain animal erythrocytes and the hemagglutination could be inhibited by *N*-glycolylneuraminic acid [15]. We describe here the purification and characterization of two sialic acid binding lectins from leaves of mulberry.

2. Material and methods

2.1. Purification of mulberry leaf lectin

Mulberry leaf lectin was extracted from the leaves of *M. alba*, locally called Mon-noi. Fresh leaves were homogenized in ice-cold 50 mM Tris–HCl, pH 7.5, containing 0.3 M NaCl, 20 mM diethyldithiocarbamic acid, 5% glycerol, and 2% polyvinylpyrrolidone. The buffer used was 3 ml g^{-1} of the fresh leaves. The homogenate was filtered through a layer of cheesecloth and stored at 20°C for 24 h. After thawing, it was centrifuged at 8000 × *g*, for 40 min at 4°C. The supernatant was collected and ammonium sulfate was added to 70% saturation. The resulting precipitate was recovered by centrifugation at 8000 × *g* for 40 min, redissolved in tris-buffered saline, TBS (50 mM Tris–HCl, pH 7.5 containing 0.3 M NaCl) and dialysed against the buffer overnight at 4°C. The solution was then centrifuged at 13,000 × *g* for 15 min and the supernatant was collected and stored at −20°C.

A column of *N*-acetylgalactosamine-agarose (1.5 × 10 cm) was equilibrated with TBS at flow rate 10 ml h^{-1}. An aliquot of the dialysed ammonium sulfate fraction containing 210 mg protein was applied onto the column. The column was then washed with the buffer until the absorbance at 280 nm of the effluent was negligible. The lectin was then eluted with TBS containing 20 mM *N*-acetylgalactosamine. Each fraction (2 ml) was dialysed against TBS and the fractions exhibiting the hemagglutination activity were combined and concentrated. The pooled preparation containing ≈ 16 mg protein was applied to a Sephacryl S-200 column (1.5 × 71 cm) which was pre-equilibrated with 50 mM Tris–HCl, pH 7.5 containing 0.15 M NaCl. Elution was carried out using the same buffer at a flow rate of 30 ml h^{-1} and fractions of 3 ml each were collected. The fractions exhibiting the hemagglutination activity were combined, concentrated and dialysed against the 50 mM Tris–HCl, pH 7.5.

An aliquot of the pooled preparation containing 15 mg protein was applied to a DEAE-Sephacel column (2 × 9 cm) which was pre-equilibrated with 20 mM Tris–HCl, pH 7.5 at flow rate 20 ml min^{-1}. Two major peaks of the lectin activities were recovered. One was found in the unbound fractions and the other was eluted stepwise with the buffer containing NaCl. The active fractions of the bound lectin were combined, concentrated, chromatographed onto a Sephadex G-25 (1.2 × 48 cm) column to remove the salt and then re-chromatographed onto the DEAE-Sephacel column. The lectin was then eluted stepwise with the buffer containing NaCl. Then the active fractions were concentrated and passed through a column of Sephadex G-75 (1.2 × 48 cm) which was pre-equilibrated with 50 mM Tris–HCl, pH 7.5. The active protein peak was collected. The native molecular weight of each purified lectin was determined using a calibration curve established for the Sephadex G-75 column by using the following molecular weight markers: bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (29 000) and cytochrome C (12 400).

2.2. Preparation of erythrocyte suspension

Sheep blood was collected in 15% (v/v) acid citrate-dextrose anticoagulant (75 mM trisodium citrate, 38 mM citric acid, and 120 mM dextrose) and stored at 4°C up to 2 weeks. An erythrocyte suspension was freshly prepared by washing the erythrocytes three times with ten volumes of washing buffer (0.10 M Tris–HCl, pH 7.5 containing 50 mM NaCl) and suspended in the same buffer as 2% suspension (v/v).

2.3. Hemagglutination and hemagglutination inhibition assay

Hemagglutination reaction and hemagglutination inhibition by carbohydrates and glycoproteins were performed in a microwell plate 96 U (Nunc) according to Ratanapo and Chulavatnatol [16] using 2% (v/v) sheep erythrocyte suspension. The lectin activity was expressed in unit (U) of hemagglutination activity (HA) which was the reciprocal of the highest dilution of lectin giving complete agglutination. The inhibition of lectin activity was recorded as the minimum concentration of carbohydrate or glycoprotein required to completely inhibit hemagglutination.

2.4. Carbohydrate content analysis

Neutral sugar content of the purified lectin was determined in a scaled down version by phenol-sulfuric method of Dubois et al. [17] using mannose as standard.

2.5. Sodium dodecylsulfate-polyacrylamide gel electrophoresis

Slab gel electrophoresis was performed by the method of Laemmli [18], using 12% polyacrylamide gel in the presence of 0.1% sodium dodecylsulfate. Protein bands were then detected with Silver stain (Biorad). Molecular weight markers used were bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (29 000), trypsinogen (24 000), trypsinogen inhibitor (20 100), and cytochrome C (12 400).

2.6. Protein determination

Protein concentration was determined by the method of Lowry et al. [19].

3. Results

3.1. Purification of mulberry leaf lectin

Crude extract of *M. alba* was initially found to agglutinate human and various animal erythrocytes including sheep, goose, and rabbit (data not shown). However, sheep erythrocyte was chosen for convenience to follow the agglutinating activity. Almost all the lectin hemagglutination activity (87%) was recovered in the precipitate at 70% saturation of ammonium sulfate (Table 1). Based on a preliminary test showing that hemagglutination induced by the crude extract could be inhibited by *N*-acetylgalactosamine, affinity chromatography of the redissolved ammonium sulfate fraction was carried out using an *N*-acetylgalactosamine-agarose column. After washing, the lectin activity was eluted from the column using a buffer containing 20 mM *N*-acetylgalactosamine (Fig. 1). In this purification step, the specificity activity of the lectin increased 97 folds (Table 1). In the next purification step using a Sephacryl S-200 column, the lectin activity coincided with the second protein peak (Fig. 2) and the purification improved to 126 folds. When the lectin was further purified using a DEAE-Sephacel column, two major protein peaks showing the lectin activity were recovered: one (MLL 1) was eluted as

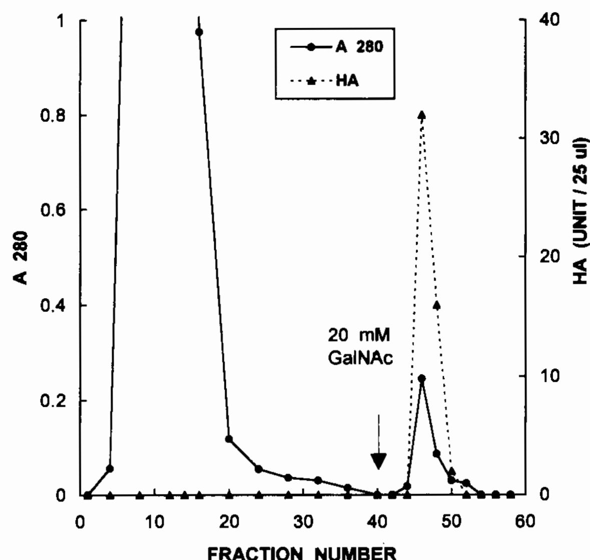

Fig. 1. Elution profile of MLL on an *N*-acetylgalactosamine-agarose column. The 0–70% ammonium sulfate fraction (210 mg protein) was applied to the column (1.5 × 10 cm), washed with TBS and eluted with the buffer containing 20 mM *N*-acetylgalactosamine. The fraction volume was 3 ml. The active fractions from number 46 to 52 were pooled.

Table 1
Purification of MLL from mulberry leaves

Fraction	Volume (ml)	Protein (mg)	Total activity ($U \times 10^{-4}$)	Specific activity ($U\ mg^{-1}$)	Purification (fold)	Recovery (%)
Crude extract	276	2,263	141.21	624	1	100
0–70% $AmSO_4$	30	1,425	122.88	862.3	1.4	87
GalNAc-agarose	6	16.2	98.30	60 679	97.3	69.6
Sephacryl S200	30	15.1	118.78	78 662.3	126.1	84.1
DEAE-Sephacel						
unbound (MLL 1)	48	10.3	12.32	11 961.2	19.2	8.7
bound (MLL 2)	24	4.8	3.07	6395.8	10.2	2.2
Sephadex G-75						
MLL 1	15	2.9	18.84	64 965.5	104.1	13.3
MLL 2	21	4.0	4.86	12 150	19.5	3.4

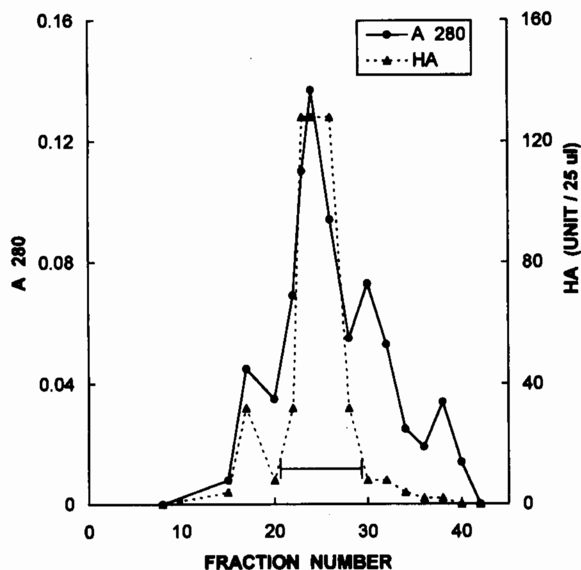

Fig. 2. Elution profile of MLL on a sephacryl S-200 column. An aliquot (16 mg protein) of the active fractions from *N*-acetylgalactosamine-agarose column was applied to a Sephacryl S-200 column (1.5 × 71 cm) pre-equilibrated with 50 mM Tris–HCl, pH 7.5 containing 0.15 M NaCl. The active fractions from number 21 to 29 were pooled.

unbound fractions and the other (MLL 2) was eluted by 0.13 M NaCl (Fig. 3). The MLL 2 was separated from some minor contaminated proteins after being rechromatographed onto the same column (Fig. 4). By Sephadex G-75, both MLL 1 and MLL 2 were separated from other contaminant and both lectins exhibited the same native molecular wieght of 51 000 (Fig. 5a and b). The purified mulberry leaf lectins were called MLL 1 and MLL 2, respectively. The purification fold of MLL 1 was 104 while of MLL 2 was 19. Yields of 1.28 mg MLL 1 and 1.77 mg MLL 2 were obtained from 1 g of the crude extract protein or 55.2 g of the mulberry leaves.

3.2. Molecular properties of the mulberry leaf lectins

By sodium dodecylsulfate-polyacrylamide gel electrophoresis, both MLL 1 and MLL 2 showed an intense band with a relative molecular weight of 16 500 (Fig. 6). Both MLL 1 and MLL 2 were found to be glycoproteins with the neutral sugar contents of 8.8 and 40% of the protein (w/w), respectively.

3.3. Carbohydrate-binding specificity of the mulberry leaf lectins

Various sugars and glycoproteins were tested for the ability to inhibit the agglutination of sheep erythrocytes caused by the lectins. Both MLL 1 and MLL 2 activities were most effectively inhibited by NeuGc at 0.78 and 1.56 mM, respectively (Table 2). GalNAc and D-Gal at higher concentrations also inhibited the lectin activities. While MLL 2 activity was inhibited by lactose, MLL 1 was not. By contrast, NeuNAc, GlcNAc, ManNAc, D-galactosamine, D-glucosamine, and D-mannosamine and D-Glc were not inhibitory at 100 mM. Bovine submaxillary mucin was a more potent inhibitory glycoprotein than fetuin. While α_1-acid glycoprotein was inhibitory only to MLL 2, thyroglobulin were not inhibitory to either MLL 1 or MLL 2.

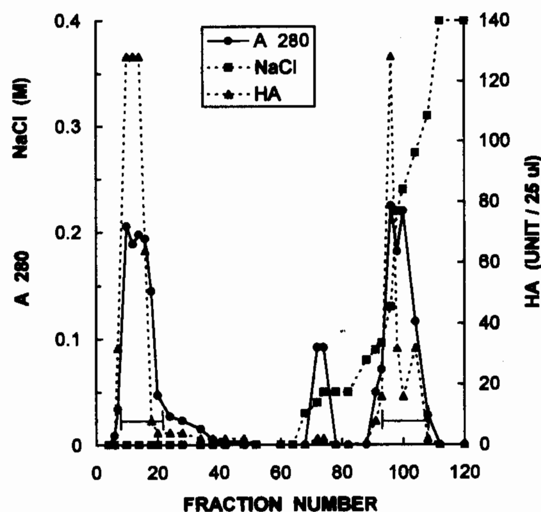

Fig. 3. Elution profile of MLLs on a DEAE-Sephacel column. An aliquot (15 mg protein) of the MLL obtained from the Sephacryl S-200 column was subjected to a DEAE-Sephacel column (2 × 9 cm) pre-equilibrated with 20 mM Tris–HCl, pH 8.0. The unbound active fractions from number 7 to 22 were pooled (MLL 1). The bound active fractions from number 91 to 107 were pooled (MLL 2).

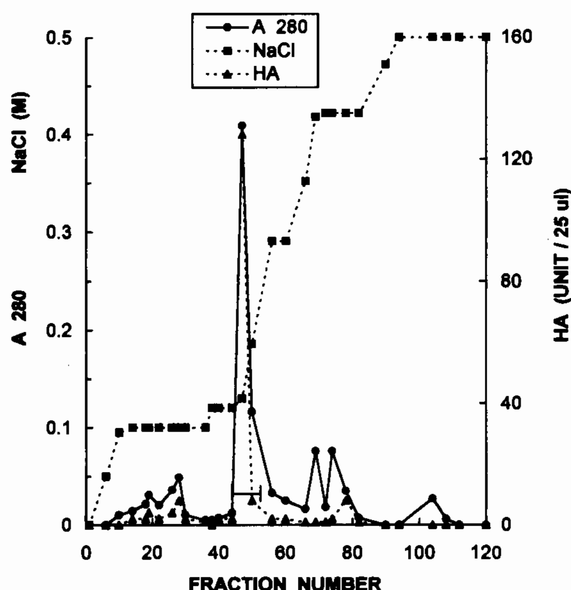

Fig. 4. Elution profile of rechromatographed MLL 2 (from Fig. 3) on the DEAE-Sephacel column. The lectin was eluted stepwise with the buffer containing NaCl. The active fractions from number 45 to 52 were pooled.

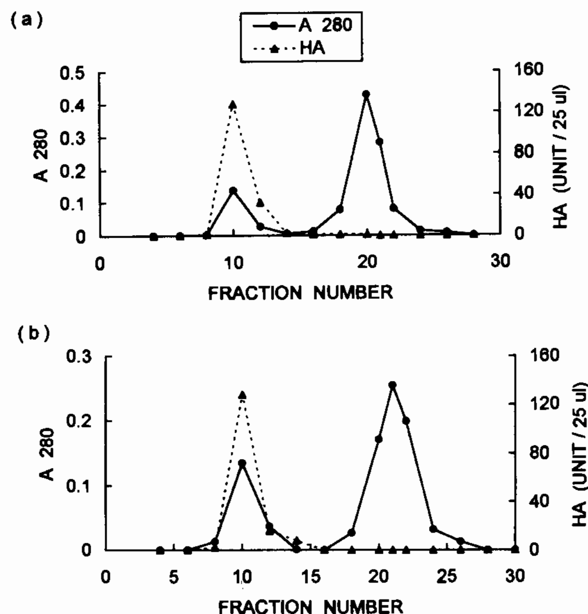

Fig. 5. Elution profile of MLL 1 and MLL 2 on Sephadex G-75 column. The unbound MLL1 from Fig. 3 and the MLL 2 from Fig. 4 were separately applied to a Sephadex G-75 column (1.2 × 48 cm) pre-equilibrated with 50 mM Tris–HCl, pH 7.5 containing 0.15 M NaCl. (a) The elution profile of MLL 1. (b) The elution profile of MLL 2.

4. Discussion

To one knowledge, this is the first report on the presence of lectins in mulberry plant. The purified mulberry leaf lectins called, MLL1 and MLL2 are the first plant lectins having specificity for NeuGc (Table 2). Among sialic acid binding lectins from plants, none is specific to NeuGc. Wheat germ agglutinin is a plant lectin known for many years to have specificity for sialic acid [12] but this specificity is due to the structural similarity of sialic acid to *N*-acetylglucosamine, its preferred ligand. A lectin isolated from elderberry, *Sambucus nigra* bark, has been shown to bind to a sequence NeuNAc $\alpha 2 \rightarrow 6$ D-Gal/D-GalNAc with high specificity but does not bind to either NeuNAc or NeuGc [13]. A leukoagglutinin from seeds of a leguminous plant, *Maackia amurensis*, binds preferrentially to terminal sialic acid linked $\alpha 2 \rightarrow 3$ to penultimate D-Gal residue but does not bind to free lactose or NeuNAc [14]. The specificity of MLL's to only NeuGc but not to NeuAc is similar to two lectins purified from invertebrates, the marine crab, *Scylla serrata* [20] and the apple snail, *Pila globosa* [21] and to a lectin from mushroom, *Hericium erinaceum* [22]. The affinity of the MLLs for NeuGc can partly explain the observed inhibition by NeuGc-containing glycoproteins (Table 2): BSM (14% of total sialic acids), thyroglobulin (10% of total sialic acids), fetuin and α_1-acid glycoprotein (7% of total sialic acids) [23].

It is also interesting to note that both MLLs require no metal ion for their lectin activities (data not shown). They are different from many lectins from plants and invertebrates which require Ca^{2+} or other divalent cations for their lectin activities [11,24]. However, some lectins which are active in the absence of divalent cations, have been reported for mushroom, *Hericium erinaceum* [22]; cephalochordate, *Branchiostoma lanceolatum* [25]; and conger eel, *Conger myriaster* [26].

Being glycoproteins, the subunit molecular weight of both MLLs may appear bigger than its true size since it would bind less SDS and hence migrate slower than expected. Thus, each MLL is probably a tetramer. Furthermore, MLL 2 having

Table 2
Inhibition of MLL[a]

Purified lectin from Sephadex G-75	Sugar (mM)				Glycoprotein (mg ml^{-1})		
	NeuGc	GalNAc	Gal	Lactose	BSM	Fetuin	α_1-acid glycoprotein
MLL1	0.78	100	100	–	0.39	3.125	–
MLL2	1.56	50	100	50	3.125	6.25	0.0038

[a] NeuNAc, Glc, Man, Glucosamine, GlcNAc, ManNAc at 100 mM and thyroglobulin at 0.5 mg ml^{-1} were not inhibitory.

a higher sugar content than MLL 1 would appear to be fainter than MLL 1 in the protein stain in SDS-PAGE (Fig. 6). The observation that MLL 2 shows a lesser specific activity than MLL 1 (Table 1) may suggest that the lectin activity is probably modulated by glycosylation.

Although many different roles have been proposed for plant lectins [27], the most emphasized lectin function is defence against pathogens [28,29]. Since sialic acids have not been found in plants [6], it is likely that MLLs may be involved in the recognition and/or interaction with other infective agents. This possibility should be further studied.

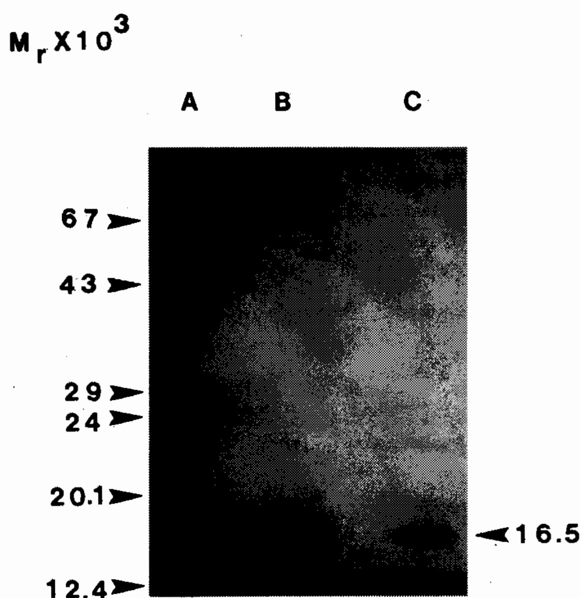

Fig. 6. SDS-PAGE of MLLs using 12% polyacrylamide gel containing 1% SDS. Lane A, Marker proteins; Lane B, MLL 1 (12 μg protein); Lane C, MLL 2 (14 μg protein).

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