Acta Biochimica Polonica

Vol. 56 No. 4/2009, 649–654

on-line at: www.actabp.pl

Regular paper

Novel galactonic acid-binding hexameric lectin from *Hibiscus mutabilis* seeds with antiproliferative and potent HIV-1 reverse transcriptase inhibitory activities

Sze Kwan Lam✉ and Tzi Bun Ng✉

School of Biomedical Sciences, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, China

Received: 05 June, 2009; revised: 05 October, 2009; accepted: 13 November, 2009
available on-line: 04 December, 2009

A hexameric 150-kDa lectin was isolated from dried *Hibiscus mutabilis* seeds using a chromatographic protocol that involved ion exchange chromatography on SP-Sepharose, and gel filtration on Superdex 75 and Superdex 200. The lectin was not adsorbed on SP-Sepharose and was eluted from the Superdex 75 column in the void volume. It was eluted in the first peak from Superdex 200. It was strongly adsorbed on DEAE-cellulose and Q-Sepharose and could not be easily desorbed. The hemagglutinating activity of the lectin, which was stable at pH 4–7 and up to 50ºC, could be inhibited by 25 mM galactonic acid. This is the first report of a galactonic acid-binding lectin. It potently inhibited HIV-1 reverse transcriptase with an IC_{50} of 0.2 μM. It exhibited weak antiproliferative activity towards both hepatoma HepG2 cells (40% inhibition) and breast cancer MCF-7 cells (50% inhibition) at 100 μM concentration of the lectin. It did not inhibit mycelial growth of a number of fungi tested.

Keywords: *Hibiscus mutabilis*, hexameric lectin, galactonic acid

INTRODUCTION

Lectins are carbohydrate-binding proteins present in a diversity of organisms including humans, vertebrates and invertebrates, plants, and fungi (De Hoff *et al.*, 2009; Jin *et al.*, 2009). They display a host of biological activities such as antitumor (Lam *et al.*, 2009), antifungal (Yan *et al.*, 2005), and antiviral (Leung *et al.*, 2008) activities. Based on their carbohydrate binding specificity they can be divided into (i) mannose binding, (ii) glucose and mannose binding, (iii) N-acetylglucosamine binding, (iv) galactose binding, (v) sialic acid binding, (vi) fucose binding, etc.

Hibiscus mutabilis belongs to the family Malvaceae. It is commonly known as Confederate rose or cotton rosemallow. Only few publications about *H. mutabilis* were found in a *PubMed* search. An aqueous extract of *H. mutabilis* exerted anti-herpes simplex virus-II action (Zheng, 1989). *H. mutabilis* is one of the greening tree species planted in a polluted factory area in Shanghai (Yang *et al.*, 2004). The benzene extract of its flowers did not markedly affect pregnancy (Kholkute *et al.*, 1977). Pigments are found in *H. mutabilis* flowers (Yeh *et al.*, 1958).

According to the Herbalist's Manual, *H. mutabilis* leaves aid detoxification, reduce swellings and alleviate pain. *H. mutabilis* leaves can be used to treat carbuncles, dermal swelling or inflammation, scalds, conjunctivitis, external and internal trauma and herpes zoster, its flowers to treat continuous menstrual flow and scalds, and its roots to treat skin ulcer. However, there is no information about its seeds.

In view of the paucity of information on *H. mutabilis*, especially with regard to its proteinaceous constituents, the present investigation was undertaken to isolate a lectin from its seeds. The lectin is multimeric and manifests highly potent HIV-1 re-

✉Corresponding author: Sze Kwan Lam: e-mail: lamszekwan@yahoo.com.hk; Tzi Bun Ng: e-mail: b021770@mailserv.cuhk.edu.hk; School of Biomedical Sciences, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, China; phone: 852-2609 8031; fax: 852-2603 5123.

Abbreviations: SDS/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis.

verse transcriptase inhibitory activity and a unique sugar specificity. The results of the present study constitute an addition to the scanty literature on hexameric lectins which contrasts sharply with the voluminous data on lectins with a lower molecular mass and a smaller number of subunits.

MATERIALS AND METHODS

Purification of lectin. The seeds were authenticated by Professor Shiuying Hu, Honorary Professor of Chinese Medicine, The Chinese University of Hong Kong, and deposited with the voucher number NG2009003 in Lab 302, Basic Medical Science Building, The Chinese University of Hong Kong. Dried seeds of *H. mutabilis* (420 g) were collected and extracted by homogenization in distilled water (5 ml/g seeds). Following centrifugation at 20 000 × *g* for 30 min at 4°C, ammonium acetate buffer (1 M, pH 4.6) was added to the supernatant until the final concentration of ammonium acetate reached 20 mM. The supernatant was then applied on a 5 cm × 15 cm column of SP-Sepharose (GE Healthcare). Unadsorbed proteins were eluted with 20 mM ammonium acetate buffer (pH 4.6). Adsorbed proteins were eluted with 1 M NaCl added to the 20 mM ammonium acetate buffer. The unadsorbed fraction was dialyzed, lyophylized, and then subjected to chromatography in 100 mM ammonium acetate buffer (pH 4.6) on a Superdex 75 HR10/300 G/L column using an AKTA Purifier (GE Healthcare). The column had been calibrated with molecular mass markers, including Blue Dextran 2000 (to determine void volume), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), myoglobulin (17.6 kDa), ribonuclease A (13.7 kDa), aprotinin (6.5 kDa), and vitamin B12 (1.3 kDa) (GE Healthcare). The first fraction was collected, lyophylized, and then subjected to chromatography in 100 mM ammonium acetate buffer (pH 4.6) on a Superdex 200 HR 10/300 GL column using an AKTA Purifier. The column had been calibrated with molecular mass markers, including Blue Dextran 2000 (to determine void volume), thyroglobulin (669 kDa), ferritin (440 kDa), immunoglobulin G (150 kDa), bovine serum albumin (67 kDa), β-lactoglobulin (35 kDa), ribonuclease A (13.7 kDa), and aprotinin (6.5 kDa) (GE Healthcare). The first fraction represented purified lectin.

Molecular mass determination using sodium dodecyl sulfate/polyacrylamide gel electrophoresis, gel filtration, and N-terminal amino acid sequencing. The purified lectin (30 μg), heated for 10 min or unheated, SDS and β-mercaptoethanol treated or untreated, was subjected to SDS/PAGE. The gel was stained with Coomassie Blue R-250. Gel filtration on a fast protein liquid chromatography Superdex 200 HR 10/300 GL column (GE Healthcare) using an AKTA Purifier (GE Healthcare) was conducted to determine the molecular mass of the lectin. The *H. mutabilis* lectin, IgG, and a mixture of the lectin and IgG were separately chromatographed on a Superdex 200 HR 10/300 GL column to find out if the lectin has the same molecular mass as IgG. The N-terminal sequence of the lectin was determined as described in Lam *et al*. (2009).

Protein determination. Protein concentration was determined by Bradford reagent (dye-binding method) using bovine serum albumin as standard.

Assay for hemagglutinating activity. In the assay, a serial twofold dilution of the lectin solution (1.28 mg/ml) and concanavalin A (as positive control) in microtiter U-plates (50 μl) was mixed with 50 μl of a 2% suspension of red blood cells (from rabbit, human (A, B, AB and O groups) and Sprague-Dawley rat) in phosphate-buffered saline (pH 7.2) at 20°C. The results were read after about 30 min when the blank had fully sedimented. One hemagglutination unit is the reciprocal of the highest dilution that is capable of inhibiting hemagglutination. Specific activity is the number of hemagglutination unit per milligram protein (Wong & Ng, 2006).

Inhibition of lectin-induced hemagglutination by divalent metal ions. The purified lectin was demetallized by treatment with EDTA before incubation in a water bath at room temperature for 15 min in the presence of one of the following salts: $ZnSO_4$, $CaCl_2$, $FeSO_4$, $MgCl_2$, $MgSO_4$, $CuCl_2$, and $CuSO_4$, all at 5 mM. Hemagglutinating assay was then performed (Wong & Ng, 2006).

Inhibition of lectin-induced hemagglutination by carbohydrates. The hemagglutinating inhibition tests were performed to investigate inhibition of lectin-induced hemagglutination by various carbohydrates (Wong & Ng, 2006). The carbohydrates tested included galactonic acid, mannitol, D-glucosamine, D-xylose, sucrose, α-lactose, D-fructose, D-mannose, D-glucuronic acid, D-glucose, D-sorbitol, and sialic acid. Serial twofold dilutions of sugar samples (25 μl of 400 mM) in phosphate-buffered saline were performed. All of the dilutions were mixed with 25 μl of lectin with 16 hemagglutination units and incubated for 15 min at room temperature. Finally, 50 μl of 2% rabbit erythrocyte suspension was added. The minimal concentrations of the aforementioned carbohydrates required to bring about inhibition of hemagglutination were recorded.

Effects of temperature and pH on lectin-induced hemagglutination. A solution of the purified lectin (50 μg/ml) was incubated at various temperatures (4°C, 10–100°C) or in buffers at various pH values (pH 1–14) for 15 min. It was then cooled down

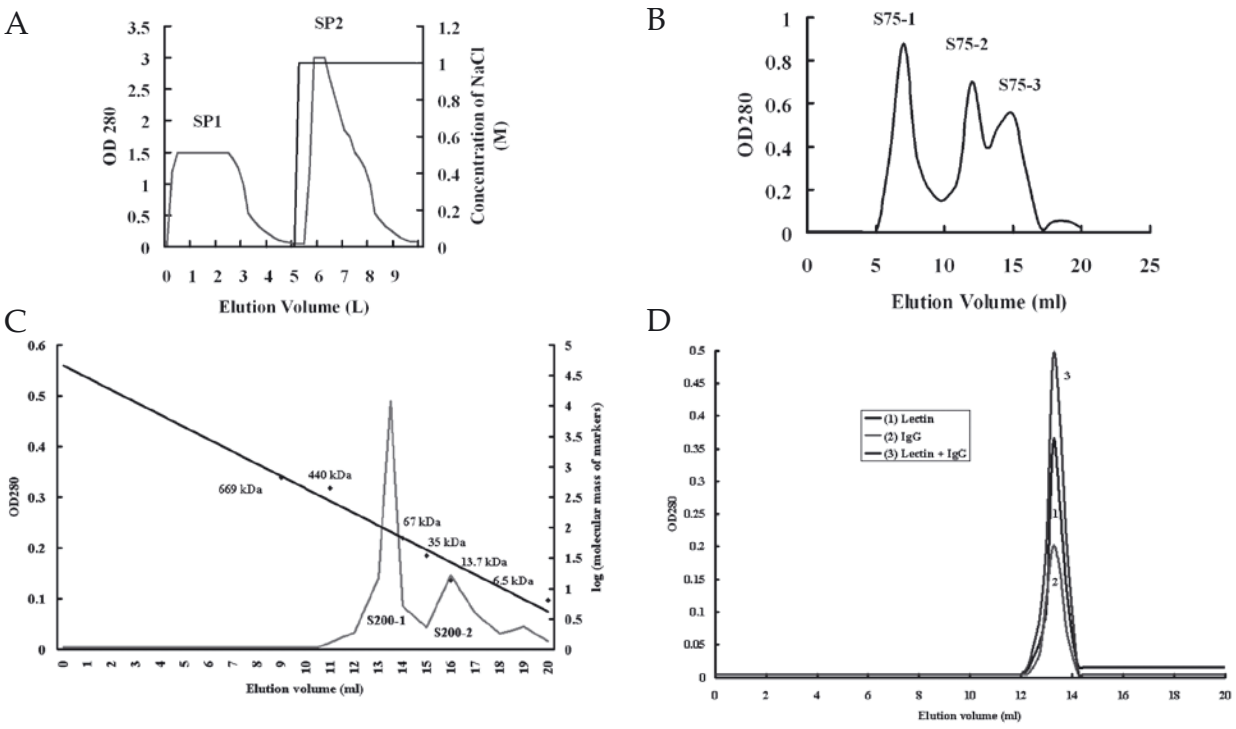

Figure 1. Purification of *H. mutabilis* lectin.
A. Ion exchange chromatography of *H. mutabilis* extract on SP-Sepharose. The vertical line in the right half of the chromatogram indicates the use of 1 M NaCl to elute fractions SP2. Flow rate = 10 ml/min. **B**. Gel filtration of SP1 fraction on Superdex 75. Flow rate = 0.5 ml/min. **C**. Gel filtration of fraction S75-1 on Superdex 200. S200-1 represents purified *H. mutabilis* lectin. Flow rate = 0.5 ml/min. **D**. Gel filtration of *H. mutabilis* lectin, IgG (150 kDa), and a mixture of the lectin and IgG separately on Superdex 200. Flow rate = 0.5 ml/min.

to room temperature or neutralized to pH 7, immediately before the hemagglutination assay (Wong & Ng, 2006).

Assay of antifungal activity. The assay for antifungal activity toward the pathogenic fungi *Mycosphaerella arachidicola, Fusarium oxysporum, Helminthosporium maydis, Valsa mali* and *Rhizoctonia solani* was carried out as described in Lam and Ng (2009). Nystatin (Sigma) was used as positive control.

Assay of HIV-1 reverse transcriptase inhibitory activity. The assay of lectin for the ability to inhibit HIV-1 reverse transcriptase was carried out by using an enzyme-linked immunosorbent assay kit from Boehringer Mannheim (Germany) as described in Lam and Ng (2009), in view of the observation that some lectins exhibit this activity (Lam *et al.*, 2009). *Capparis spinosa* lectin was used as a positive control.

Assay of antiproliferative activity toward tumor cells. The assay of the antiproliferative activity of the lectin was carried out by testing its inhibitory effect on the growth of human hepatoma HepG2 cells and human breast cancer MCF-7 cells as described in Lam and Ng (2009). Doxorubicin and *Rachycentron canadum* lectin (Ngai & Ng, 2007) were used as positive and negative control, respectively.

RESULTS

Isolation of lectin

The *H. mutabilis* seed extract was resolved on SP-Sepharose into two fractions of different sizes, an unadsorbed fraction SP1 with hemagglutinating activity and an adsorbed fraction SP2 without hemagglutinating activity (Fig. 1A). Fraction SP1 was dialyzed, and lyophylized before separation on Superdex 75 into three major fractions. S75-1, with hemagglutinating activity eluted in the void volume, and two larger fractions, S75-2 and S75-3, without such activity (Fig. 1B). Fraction S75-1 was lyophylized before being separated on Superdex 200 into two major fractions, S200-1, a larger fraction with hemagglutinating activity, and a smaller fraction S200-2 without activity (Fig. 1C). The lectin, IgG, and a mixture of the lectin and IgG were eluted from the Superdex 200 HR 10/300 GL column with the same elution volume (Fig. 1D) indicating that the lectin has the same molecular mass as IgG (150 kDa). Fraction S200-1, with a native molecular mass of 150 kDa, represented purified lectin as evidenced by a single 24-kDa band in SDS/PAGE, indicating that the lectin was a hexamer (Fig. 2B). An SDS/PAGE analysis of crude extract was performed indicating the lectin

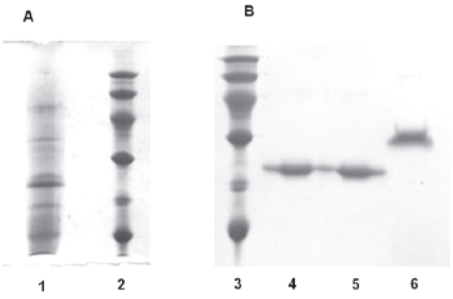

Figure 2. SDS of subsequent steps of *H. mutabilis* lectin purification.
A. Lane 1: *H. mutabilis* crude extract. Lane 2: molecular mass markers including phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14.4 kDa). **B**. Lane 3: molecular mass markers. Lane 4: Fraction S200-1 (30 μg) representing *H. mutabilis* lectin (heated, SDS/β-mercaptoethanol treated). Lane 5: unheated purified lectin (30 μg) treated with SDS/β-mercaptoethanol. Lane 6: purified lectin (30 μg) without heat or SDS/β-mercaptoethanol treatment.

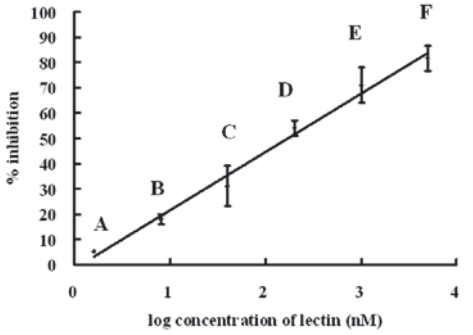

Figure 3. Inhibition of HIV-1 reverse transcriptase by *H. mutabilis* lectin.
Percent inhibition compared to a control without the protein is indicated. Values are expressed as the mean ±S.D. (N=3). Different letters on the same straight line indicate statistically significant difference ($P<0.05$) between the data upon analysis using ANOVA followed by Duncan's multiple range test.

is one of the major protein components (Fig. 2A). Unheated, SDS/β-mercaptoethanol treated purified lectin showed the same electrophoretic mobility as the heated, SDS/β-mercaptoethanol treated purified lectin in SDS/PAGE, while the electrophoretic mobility of unheated, SDS/β-mercaptoethanol untreated purified lectin was lower (Fig. 2B). The *H. mutabilis* lectin was obtained with a specific hemagglutinating activity of 4000 unit/mg for rabbit blood. There was no hemagglutinating activity for rat blood and human blood. The lectin yield was 17 mg from 420 g seeds. The recovery of hemagglutinating activity was 34% (Table 1). Its N-terminal sequence, ACVAPLDEAACAAAK, resembled none of the lectins reported so far. The homogeneity of the lectin was disclosed by a single peak in each Edman degradation cycle during amino acid sequencing.

Table 1. Yields from 420 g *Hibiscus mutabilis* seeds and specific hemagglutinating activities (ha) at different stages of purification of *Hibiscus mutabilis* lectin

Column	Chromatographic fraction	Yield (mg)	Specific ha (unit/mg)	Total ha (unit)	Recovery of ha (%)	Purification fold of ha
–	Crude extract	202	1025	207052	100	1
SP-Sepharose	S1	78	2108	164423	79	2.1
Superdex 75	S75-1	39	2632	102648	50	3.0
Superdex 200	S200-1	17	4000	68000	33	3.9

Hemagglutinating activity of lectin

The hemagglutinating activity of the lectin could be inhibited by galactonic acid at 25 mM concentration There was no inhibition when the following sugars were tested up to 100 mM: D-glucosamine, mannitol, D-xylose, sucrose, D-fucose, D-raffinose, α-lactose, D-fructose, L-arabinose, D-galacturonic acid, D-galactose, D-mannose, D-glucuronic acid, D-glucose, D-sorbitol, and sialic acid. The hemagglutinating activity of the lectin was stable in the pH range 4–7. About 50% activity was retained at pH 3 and pH 8–9. The activity was completely eliminated at pH 1–2, and 10–14. The activity was stable in the temperature range 0–50°C, reduced to half at 60°C, and destroyed at 70°C. Of the various salts tested, only manganese chloride and manganese sulfate restored the activity of the lectin (not shown).

Other biological activities of isolated lectin

The lectin inhibited HIV-1 reverse transcriptase with an IC_{50} of 200 nM (Fig. 3). There was no inhibitory effect on mycelial growth of *Mycosphaerella arachidicola, Fusarium oxysporum, Helminthosporium maydis, Valsa mali*, or *Rhizoctonia solani* at 100 μM lectin. The lectin exhibited weak antiproliferative activity toward tumor cells. There was about 50% growth inhibition of MCF-7 cells and about 40% growth inhibition of HepG2 cells when the cells were treated with 100 μM lectin. *Rachycentron canadum* lectin showed no antiproliferative effect. By comparison, the positive control doxorubicin exhibited an antiproliferative activ-

ity toward these tumor cells with an IC_{50} of 5 μM and 10 μM, respectively.

DISCUSSION

To the best of our knowledge, there are only seven hexameric lectins reported in the literature, including those from *Axinella corrugata* (Dresch *et al.*, 2008), *Ganoderma lucidum* (Thakur *et al.*, 2007), *Helix pomatia* (Sanchez *et al.*, 2006), *Ctenopharyngodon idellus* (Lam & Ng, 2002), *Trimeresurus albolabris* venom (Du *et al.*, 2002), *Lactarius rufus* (Panchak & Antoniuk, 2007), and *Araucaria angustifolia* lectin (Datta *et al.*, 1991). Even fewer examples of octameric lectins, e.g. *Anguilla japonica* lectin (Mistry *et al.*, 2001), and only a single decameric lectin purified from *Araucaria angustifolia* (Datta *et al.*, 1991) have been reported. All hexameric lectins reported are stable at around pH 4 to pH 8 and around 50°C to 65°C. The *H. mutabilis* lectin resembles them in pH stability and thermostability.

Many lectins manifest binding to one sugar (Sultan *et al.*, 2009) or two sugars (Devi *et al.*, 2009). In some cases, lectins can be inhibited by more than two sugars (Wang *et al.*, 2000; 2002; Lam *et al.*, 2009). It is worth noting that the *H. mutabilis* lectin is the first galactonic acid-binding lectin reported to date. This lectin is capable of agglutinating rabbit, but not rat or human red blood cells. As yet, there is no evidence that galactonic acid is present on the surface of rabbit erythrocytes. Thus, the lectin may also recognize some other sugars on the surface of rabbit erythrocytes that have not been tested in this study.

Some lectins are adsorbed on anion-exchangers (Sultan *et al.*, 2009) or cation-exchangers (Sharma *et al.*, 2009). Some are adsorbed on both types of exchangers (Xu *et al.*, 2007). The *H. mutabilis* lectin is not adsorbed on a cation exchanger (SP-Sepharose), and it is tightly bound and cannot be dislodged from anion-exchangers by 2 M NaCl in pH 4.6 ammonium acetate buffer (DEAE-cellulose and Q-Sepharose). The purification scheme mainly depends on gel filtration, which is different from the previously reported lectins.

Information pertaining to the biological activities of hexameric lectins is meager. The *A. corrugata* lectin displays a chemotactic effect on rat neutrophiles (Dresch *et al.*, 2008). The *C. idellus* lectin is mitogenic toward murine splenocytes and peritoneal exudate cells (Lam & Ng, 2002). There are no reports on the biological activities of other hexameric, octameric and decameric lectins.

The *H. mutabilis* lectin potently inhibited HIV-1 reverse transcriptase with an IC_{50} of 0.2 μM. Its activity is much stronger than that of other lectins which display an IC_{50} of 3–35 μM, including pinto bean lectin (Wong *et al.*, 2006) and *Xerocomus spadiceus* lectin (Liu *et al.*, 2004). Its potency is similar to that of *C. spinosa* lectin (Lam *et al.*, 2009). The mechanism of inhibition is probably protein–protein interaction, HIV-1 protease inhibits the homologous reverse transcriptase by protein–protein interaction (Böttcher & Grosse, 1997).

Not all lectins showed antiproliferative activity on tumor cells, e.g. *Rachycentron canadum* lectin (Ngai & Ng, 2007). Some lectins exhibit antiproliferative or antitumor activity (Xu *et al.*, 2007). The *H. mutabilis* lectin at 100 μM concentration produced only about 50% inhibition of proliferation in HepG2 and MCF-7 cells. There are only a small number of reports on antifungal lectins, e.g. *Artocarpus genus* lectin can inhibit *Fusarium moniliforme* and *Saccharomyces cerevisiae* (Trindade *et al.*, 2006). The *H. mutabilis* lectin is similar to most other lectins that are devoid of antifungal activity.

The N-terminal amino acid sequence of *H. mutabilis* lectin is ACVAPLDEAACAAAK. To date, no lectins have been reported with this partial sequence.

The subunits of a protein in the quaternary structure must be in non-covalent association (Devlin, 1997). The subunits in dimeric or tetrameric leguminous lectins comprise a flat six-stranded β-sheet and a curved seven-stranded β-sheet linked by loops of different lengths (Banerjee *et al.*, 1994). The monomer of hexameric *Helix pomatia* agglutinin is composed of a six-stranded antiparallel β-sandwich. The β-strands are linked and produce a hairpin at one extremity of the β-sandwich (Sanchez *et al.*, 2006). The subunit arrangement of *H. mutabilis* lectin probably resembles that of hexameric *Helix pomatia* agglutinin. SDS/β-mercaptoethanol treatment resulted in a higher electrophoretic mobility of the lectin indicating its multimeric nature.

All in all, the reported *H. mutabilis* lectin is unique in its high molecular mass, multimeric nature, sugar specificity, novel N-terminal amino acid sequence and extremely strong adsorption to anionic exchangers including DEAE-cellulose and Q-Sepharose.

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