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A novel homodimeric lectin from *Astragalus mongholicus* with antifungal activity

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Abstract

A novel lectin (AMML) was isolated from a Chinese herb, i.e., the roots of *Astragalus mongholicus*, using a combination of ammonium sulfate fraction and ion exchange chromatographies. The molecular mass of intact AMML was determined to be 66,396 Da by MALDI-TOF mass spectrometry and 61.8 kDa by gel filtration, respectively. AMML was a dimeric protein composed of two identical subunits each with a molecular mass of 29.6 kDa. The lectin was a glycoprotein with a neutral carbohydrate content of 19.6%. The purified lectin hemagglutinated both rabbit and human erythrocytes, and showed preference for blood types O (native) and AB (trypsin-treated). Among various carbohydrates tested, the lectin was best inhibited by D-galactose and its derivatives with pronounced preference for lactose (3.13 mM). N-terminal amino acid sequence of AMML was determined as ESGINLQGDATL ANN. The optimal pH range for lectin activity was between pH 4.5 and 7.5, and the lectin was active up to 65 °C. It also exerted antifungal activity against *Botrytis cincerea*, *Fusarium oxysporum*, *Colletorichum* sp., and *Drechslera turcia* but not against *Rhizoctonia solani* and *Mycosphaerella arachidicola*.

Keywords: Antifungal activity; *Astragalus mongholicus*; *Astragalus mongholicus*; Characterization; Deglycosylation; Galactose-specific; Homodimer; Hemagglutinating activity; Lectin; Purification; Glycoprotein; Astragali radix

Lectins constitute a class of proteins which possess at least one noncatalytic domain capable of specific recognition and reversible binding to carbohydrate [1]. They have attracted great interest because of their various biological activities, such as cell agglutination, antiproliferative, antitumor, immunomodulatory, antifungal, and antiviral [2–7]. So far, a number of lectins have been isolated and characterized from leguminous species mainly on the basis of hemagglutinating activity [8–13].

Not much information is available in the literature regarding the bioactive proteins in Chinese herbs [14]. Astragali radix, the dried roots of *Astragalus membranaceus* (Fisch.) Bunge or *Astragalus mongholicus* Bunge [*Astragalus membranaceus* (Fisch.) Bunge var. *mongholicus* (Bunge)] (family Leguminosae), have been used as antiperspirant, adiuretic or atonic under the name of Huangqi in China [15]. Much attention has been given to study the indigenous compounds such as polysaccharide, astragaloside, isoflavonoids, triterpene saponins, γ-aminobutyric acid, and various trace elements in this medicinal herb [16–18]. Although Astragali radix has been used for a long time as a component of oriental traditional medicine in China, Japan, and Korea, there are no reports on the

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biological activities of its proteins [18,19]. The present investigation was devoted to trace for bioactive proteins from proteinaceous components in Astragali radix. A novel protein from the roots of *A. mongholicus* Bunge was purified and was found to possess interesting activities, including hemagglutinating activity and antifungal activity toward selected fungal species.

Materials and methods

Materials

QAE Sephadex A-25 was purchased from Pharmacia (Pharmacia, Uppsala, Sweden). Superdex 75 was purchased from Amersham Biosciences AB (Uppsala, Sweden). Econo-Pac Ion Exchange Cartridges (Econo-Pac CM) and Econo-Pac High Capacity Ion Exchange Cartridges (Econo-Pac High Q) were obtained from Bio-Rad (USA). Sugars were purchased from Amresco and Sigma. Endo-β-*N*-acetylglucosaminidase F (PNGase F) was purchased from Sigma (Product Number G 5166). All other chemicals used were analytical grade reagents unless otherwise stated. Rabbit blood was obtained by puncturing the marginal ear veins of healthy rabbits. Typed human red blood cells (A, AB, B, and O) were obtained from healthy donors at the Hospital of China Agricultural University. Three-year-old Astragali radix was obtained from Beijing Tongrentang Group, and identified as *A. membranaceus* (Fisch.) Bunge var. *mongholicus* (Bunge) P.K. Hsiao (i.e., *A. mongholicus* Bunge) [15].

Extraction and purification of lectin in A. mongholicus

Astragali radix (10 g) was broken into pieces (approx 1 mm), added to a 6-fold volume (60 ml) of 50 mM phosphate buffer (pH 7.2) and extracted at 4 °C for 16 h. Supernatant obtained by centrifugation (10,000*g* for 20 min) was then used for lectin purification. Proteins in the supernatant were fractionated by ammonium sulfate precipitation (20–60% saturation). The precipitate was resuspended and dialyzed extensively against 10 mM phosphate buffer (pH 7.2) before ion exchange chromatography on a QAE Sephadex A-25 column (2.6 × 60 cm) pre-equilibrated and eluted with the same buffer. After elution of unadsorbed materials, the column was eluted with 0.25 M NaCl in 10 mM phosphate buffer (pH 7.2) at a flow rate of 1.25 ml/min to collect adsorbed fractions with low hemagglutinating activity. The unabsorbed fraction (Q1) with high hemagglutinating activity was applied to an Econo-Pac CM ion exchange column pre-equilibrated with 10 mM acetate-Na buffer (pH 4.5). Linear gradient elution from 0 to 0.3 M NaCl concentration resulted in two protein peaks (C1 and C2). The C1 peak was found to contain hemagglutinating activity and was subsequently subjected to Econo-Pac High Q column pre-equilibrated with 10 mM Tris–HCl buffer, pH 8.0. Linear gradient elution from 0 to 0.3 M NaCl concentration resulted in two peaks (H1 and H2). The second peak (H2) containing major hemagglutinating activity was pooled and dialyzed against 10 mM Tris–HCl (pH 8.0). The purified lectin (designated AMML)[1] was used in the following assays. The protein elution profiles were monitored at 280 nm.

SDS–polyacrylamide gel electrophoresis and protein determination

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed using 12.5% (w/v) acrylamide in gels as described by Laemmli [20]. Protein bands were visualized by coomassie brilliant blue R-250 or silver staining. The molecular mass standard used was the low molecular weight calibration kit (Amersham Biosciences): phophorylase b (97.0 kDa), albumin (66.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa). Glycoproteins were detected by periodic acid Schiff (PAS) staining of gels after the SDS–PAGE [21]. The electrophoretic patterns of AMML were also examined using SDS–PAGE in the absence and presence of β-mercaptoethanol.

Protein concentration was determined according to the method of Lowry et al. [22] using BSA (bovine serum albumin) as standard.

Deglycosylation of the lectin by endo-β-N-acetylglucosaminidase F

Lectin (100 μg) was dissolved in 90 μl denaturing buffer (0.5% SDS, 1% (v/v) β-mercaptoethanol). The sample solution was denatured by boiling for 5 min. After addition of 10 μl reaction buffer (50 mM phosphate, pH 7.2), 10 μl of 15% (v/v) Triton X-100, and 100 U endo-β-*N*-acetylglucosaminidase F (PNGase F), the sample was incubated at 37 °C for 24 h. The reaction was terminated by boiling for 5 min and PAGE loading buffer was added to the reaction mixture. The molecular mass and the purity of the lectin with and without deglycosylation were analyzed by SDS–PAGE (12.5% gel).

Molecular mass determination and neutral sugar content

Molecular mass of the intact AMML was measured by gel filtration on a Superdex 75 column (1.0 × 40.0 cm)

[1] *Abbreviations used*: AMML, *A. mongholicus* Bunge [*A. membranaceus* (Fisch.) Bunge var. *mongholicus* (Bunge)] lectin; BSA, bovine serum albumin; EDTA, ethylenediaminetetracetic acid; HU, hemagglutination unit; MALDI-TOF MS, matrix assisted laser desorption ionization-time of flight mass spectrometry; PAS, periodic acid Schiff; PBS, 10 mM phosphate-buffered saline (pH 7.2), 0.15 M NaCl; PVDF, polyvinylidene difluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

previously equilibrated with 10 mM phosphate buffer (pH 7.0). The protein was eluted (0.25 ml/min) with the same buffer. Molecular standards from Sigma were used to calibrate the column, they were phosphorylase b (97.0 kDa), albumin bovine V (68.0 kDa), albumin (45.0 kDa), chymotrypsinogen A (25.7 kDa), and cytchrome *c* (12.3 kDa). Molecular mass of AMML was also estimated by laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry on a AXIMA-CFR plus mass spectrometer (Shimadzu, Kyoto, Japan), equipped with a 337.1-nm nitrogen laser. Data from 100 laser shots were averaged for each spectrum. Each spectrum was internally calibrated by aldolase and albumin.

Estimation of carbohydrate content of the protein samples was done by the phenol sulphuric acid method [23], with D-glucose as standard.

Amino acid analysis and N-terminal sequence

The purified lectin was hydrolyzed in a PicoTag workstation using HCl vapor (6 M) at 110 °C for 24 h in the presence of 0.01% phenol and 0.003% β-mercaptoethanol. Amino acids were analyzed using a HP 1100 amino acid analyzer. Tryptophan was also determined after precolumn deviatization. AMML was suspended in 4 M NaOH to a known concentration ranging from 20 to 25 mg/ml, sealed under argon and hydrolyzed for 16 h at 110 °C.

Following SDS–PAGE, the lectin was transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, USA) stained with coomassie brilliant blue R-250. Band corresponding to the lectin was then excised from the membrane. N-terminal sequence was determined by automated Edman degradation using a PROCISE amino acid sequencer (Applied Biosystems, USA) at Peking University, Beijing. N-terminal sequence homology was analyzed using the BLAST database search.

Preparation of erythrocytes and hemagglutination assay

After centrifugation at 2000*g* for 10 min, the erythrocytes obtained were washed four times with PBS (10 mM phosphate-buffered saline, pH 7.2; 0.15 M NaCl). Next, the pellet was resuspended in PBS to give a 2% (w/v) suspension. The erythrocytes suspension (2%) was then incubated for 1 h at room temperature with trypsin (1 mg/ml in PBS). After incubation, the erythrocytes were washed four times with PBS.

Trypsin-treated rabbit erythrocytes were used for hemagglutination assay throughout the experiment unless otherwise stated. A serial 2-fold dilution of the protein solution in microtiter U-bottomed 96-well plates (50 μl) was mixed with 50 μl of a 2% (w/v) suspension of rabbit erythrocytes in PBS at room temperature. Results were read after about 1 h when the blank had fully sedimented. The hemagglutination titer, defined as the reciprocal of the highest dilution exhibiting complete hemagglutination, was expressed as one hemagglutination unit (HU). Specific activity is the number of hemagglutination units per mg of protein.

Effects of pH and temperature on hemagglutinating activity

To study pH stability of the lectin, the lectin solution was incubated for 30 min in 0.1 M different buffers with pH ranging from 2.5 to 11: sodium citrate buffer (pH 2.5–5.5), phosphate buffer (pH 6.0–7.5), Tris–HCl buffer (pH 8.0–9.0) and glycine–NaOH (pH 9.5–11). As described previously, the residual hemagglutinating activity was measured at room temperature.

Effect of temperature on hemagglutinating activity of the lectin was examined after the lectin solution was incubated for 30 min at 37, 40, 50, 60, 65, 70, 75, 80, and 85 °C. Fifty microliters of aliquots were rapidly cooled on ice and the residual hemagglutinating activity was assayed.

Effect of EDTA and divalent cations on hemagglutinating activity

To investigate the role of metal ions on hemagglutination, the lectin was stripped of metal ions by adding 50 μl of 25 mM EDTA to 50 μl of the lectin (1 mg/ml). The extent of hemagglutination was determined after 30 min incubation at room temperature.

Carbohydrate specificity of the lectin

A solution of the purified lectin (25 μl) with four hemagglutination units was mixed with an equal volume of a serial twofold dilution of the carbohydrate sample to be tested. After incubation at room temperature for 30 min, the mixture was mixed with a 2% suspension of trypsin-treated rabbit erythrocytes. The minimal concentration of the carbohydrate in the final reaction mixture capable of completely inhibiting four hemagglutination units of the lectin was recorded.

Assay of antifungal activity and ribonuclease activity

Assay of antifungal activity and quantitative assay to determine the IC_{50} of antifungal activity were performed as described by Wang et al. [11]. The selected fungi, i.e., *Botrytis cinerea*, *Fusarium oxysporum*, *Colletorichum* sp., *Drechslera turcia*, *Rhizoctonia solani*, and *Mycosphaerella arachidicola*, were cultured in 100 × 15 mm petri dishes containing 10 ml potato dextrose agar. After the mycelial colony had developed at a distance of 0.5 cm away from the rim of the mycelial colony were placed sterile blank paper disks (0.625 cm in diameter). Aliquot

of the lectin solution was added to the disks. The petri dishes were then incubated at 23 °C for 72 h until mycelia growth had enveloped disks containing the control and formed zones of inhibition around disks containing samples with antifungal activity.

The activity of *ribonuclease* (RNase) toward tRNA was assayed by determining the generation of acid-soluble, UV-absorbing species with the method of Fong et al. [24]. The protein was incubated with 200 μg of yeast tRNA in 150 μg of 100 mM Mes[2-(*N*-Morpholino)ethane sulfonic acid] (pH 6.0) at 37 °C for 1 h. One unit of enzymatic activity is defined as the amount of enzyme that brings about one unit of increase in OD_{260} per minute in the acid-soluble fraction per milliliter of reaction mixture under the specified condition.

Results

Purification of the lectin

Protein yield and specific hemagglutinating activity of the lectin at each stage of purification are presented in Table 1. Crude extract of proteins from *A. mongholicus* contained relatively high levels of hemagglutinating activity against trypsin-treated rabbit erythrocytes. Fractionation of the crude extract by precipitation with ammonium sulfate (20–60% saturation) increased the specific hemagglutinating activity 1.42-fold. The lectin could not bind to a QAE Sephadex A-25 column in 10 mM phosphate buffer (pH 7.2) (Fig. 1A). The unadsorbed proteins (Q1) demonstrated predominantly hemagglutinating activity. On the other hand, the adsorbed fractions Q2, Q3, and Q4 showed low hemagglutinating activity. Fraction C1 derived from Q1 by ion exchange chromatography on Econo-Pac CM possessed hemagglutinating activity (Fig. 1B). Chromatography of C1 on Econo-Pac High Q yielded a second peak (H2) with major hemagglutinating activity. It demonstrated a molecular mass of 29.6 kDa in SDS–PAGE and was regarded as the purified lectin (Figs. 1C and 2A). There was approximately 14.4-folds increase in specific hemagglutinating activity when the crude extract was subjected

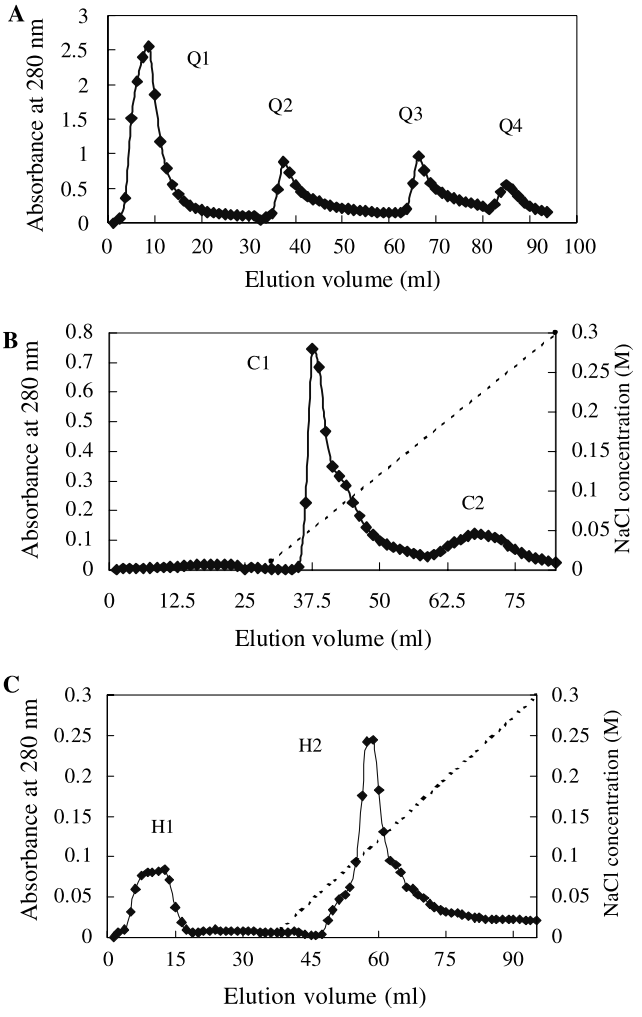


Fig. 1. Ion exchange chromatographies of *A. mongholicus* lectin (AMML). The elution profiles were monitored at 280 nm. Fractions (1.25 ml/tube) were collected at a 1.25 ml/min flow rate. The active fractions were detected by hemagglutination assay. Conditions are described under Materials and methods. (A) Ion exchange chromatography of a fraction of ammonium sulfate precipitation (20–60%) on a QAE Sephadex A-25 column. (B) Ion exchange chromatography of fraction Q1 on an Econo-Pac CM column. (C) Ion exchange chromatography of fraction C1 on an Econo-Pac High Q column. Peak H1 was eluted with a 10 mM Tris–HCl buffer (pH 8.0). Peak H2 (represents purified lectin) was eluted with a linear NaCl concentration gradient (0–0.3 M) in 10 mM Tris–HCl buffer (pH 8.0).

Table 1
Purification summary of AMML

Purification step	Total hemagglutinating activity (HU)[b]	Total protein (mg)[c]	Specific activity (HU/mg)	Purification folds	Recovery of activity (%)
Crude extract[a]	4000	108	37	1	100
Ammonium sulfate precipitation (20–60%)	3360	63.7	52.7	1.42	84
QAE Sephadex A-25	2340	32.9	71.1	1.92	58.5
Econo-Pac CM	1200	8.2	146.1	3.95	30
Econo-Pac High Q	640	1.2	533.3	14.4	16

[a] Crude extract from 10 g *A. mongholicus*.
[b] HU, hemagglutinating unit, i.e., the reciprocal of the highest exhibiting complete hemagglutination. Measured with trypsin-treated rabbit erythrocytes.
[c] Protein was measured by the method of Lowry et al. [22].

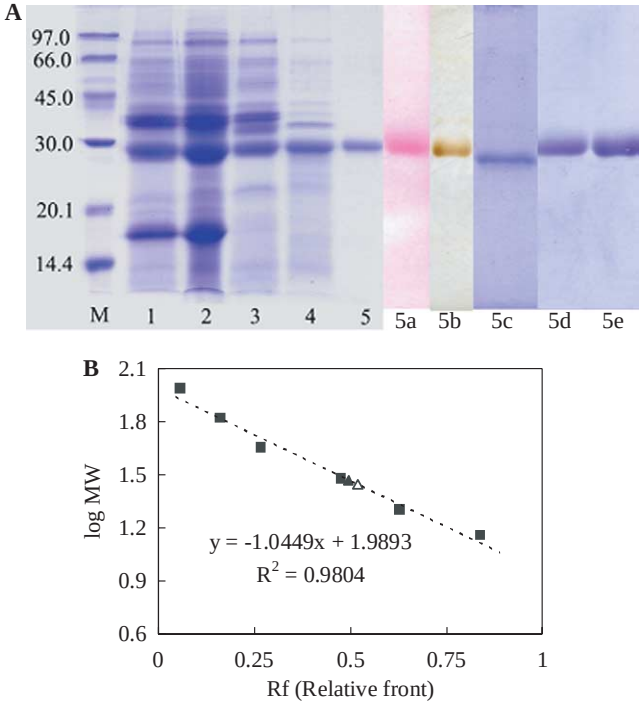


Fig. 2. (A) SDS–PAGE of the purification steps of AMML. Lane M, Amersham Biosciences low molecular weight marks (in kDa); lane 1, crude extract; lane 2, ammonium sulfate precipitation (20–60%); lane 3, an active fraction (Q1) from QAE Sephadex A-25 chromatography; lane 4, an active fraction (C1) from Econo-Pac CM ion exchange chromatography; lane 5, an active fraction (H2) from Econo-Pac High Q chromatography; lane 5a, periodic acid Schiff (PAS) staining of AMML; lane 5b, silver staining of AMML; lane 5c, the deglycosylated form of AMML by PNGase F; lane 5d, SDS–PAGE with β-mercaptoethanol; lane 5e, SDS–PAGE without β-mercaptoethanol. (B) Molecular mass estimation of the AMML by SDS–PAGE in 12.5% gel. The molecular weight size markers (■), pure AMML (▲), and its deglycosylated form (Δ) are indicated.

to various purification steps until a purified lectin was obtained (Table 1). About 1.2 mg of purified lectin was obtained from 10 g Astragali radix. This purified lectin will simply be referred as AMML (*A. membranaceus* var. *mongholicus* lectin) hereafter.

Molecular mass determination and glycoprotein nature

The purified AMML moved as a single symmetrical peak upon gel filtration chromatography. Relative molecular mass of the native AMML was estimated by gel filtration on a calibrated Superdex 75 column (Fig. 3A). A relative molecular mass of 61.8 kDa was calculated for AMML by using $V_e/V_0 = 1.2$ (Fig. 3B). The molecular mass of intact AMML was determined to be 66,396 Da by MALDI-TOF mass spectrometry (Fig. 3C). In SDS–PAGE, the protein exhibited a single band and revealed a molecular mass of 29.6 kDa per monomer (Fig. 2). The silver staining of the SDS–PAGE of the purified AMML is shown in Fig. 2A (lane 5b). The results indicated that a single protein band was formed after silver staining, confirming the homogeneity of the protein. Therefore, it is safe to say that AMML was composed of two identical subunits each with a molecular mass of 29.6 kDa. The electrophoretic patterns of AMML were examined using SDS–PAGE in the presence and absence of β-mercaptoethanol (lanes 5d and e in Fig. 2A). AMML had an apparent molecular mass of 29.6 kDa, as determined by SDS–PAGE under nonreducing and reducing conditions. Thus, two identical polypeptide chains of AMML are held together by only nondisulfide and noncovalent forces.

Periodic acid Schiff (PAS) staining showed that AMML was a glycoprotein (lane 5a in Fig. 2A). The purified lectin when studied for the presence of sugar moiety, showed the presence of 196 μg of sugar/mg of protein. Therefore, AMML is a glycoprotein with 19.6% covalently linked carbohydrate. Endo-β-*N*-acetylglucosaminidase F (PNGase F) treatment of AMML resulted in a protein band shift from 29.6 to 27.9 kDa in SDS–PAGE (lane 5c in Fig. 2A), confirming that AMML is a glycoprotein. PAS staining the lectin after deglycosylation with PNGase F did not result in the formation of the pink bands, indicating that the lectin was deglycosylated. This indicated that the subunits size heterogeneity was a result of difference in glycosylation.

Hemagglutination of rabbit and human erythrocytes

AMML strongly agglutinated rabbit erythrocytes, and showed an evident hemagglutinating specificity toward human (ABO system) erythrocytes (Table 2). The activity against native rabbit erythrocytes occurred immediately after the cells were added to the assay medium containing the lectin. As expected, the sensitivity of the lectin was greatly enhanced following treatment with trypsin. The activity of the lectin was enhanced 2-fold after trypsin treatment (minimum inhibitory concentration = 2.3 μg/ml). Human erythrocytes of native type A (*N*-acetylgalactosamine-galactose-fucose) and O blood groups were also agglutinated, although the effect was more pronounced with erythrocytes of type O. Trypsinization of human erythrocytes caused AMML to lose its main specificity toward types A and O, but enhanced its specificity toward types B (galactose–galactose–fucose) and AB.

Optimum pH and thermal stability of AMML

At 65 °C for 30 min, AMML was fairly stable. However, when heated at 75 °C for 30 min, it lost 50% of its original activity and was totally inactivated when heated at 85 °C for 30 min (Fig. 4A). The hemagglutinating activity of AMML was stable between pH 4.5 and 7.5, but its activity below pH 3.0 and above 10 was completely inhibited (Fig. 4B).

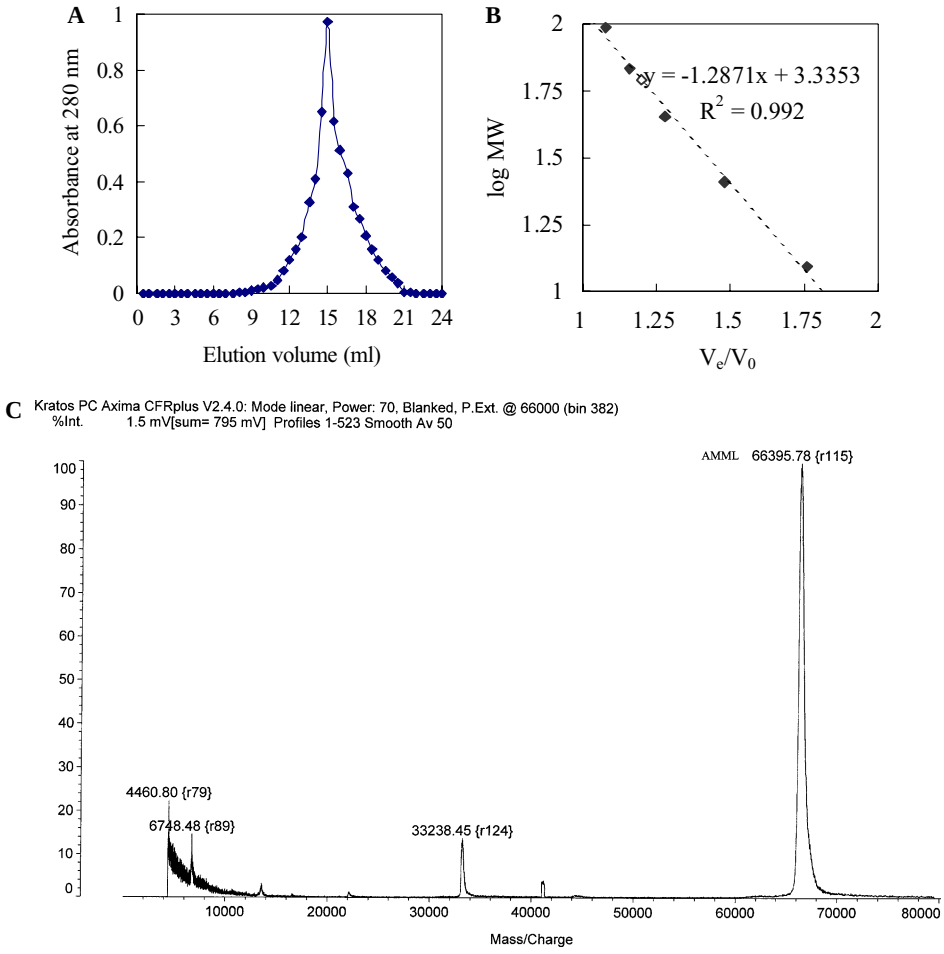


Fig. 3. (A) Gel filtration of AMML. One milliliter of AMML (1.0 mg/ml) in 10 mM phosphate buffer (pH 7.0) was loaded onto a column of Superdex 75 (1.0 × 40.0 cm), which was preequilibrated with the same buffer. 0.5 ml fractions were collected, and the elution was monitored by A_{280nm}. (B) Molecular mass estimation of the AMML by gel filtration on Superdex 75. Ve, volume of eluted protein; V_0, volume of eluted blue dextran. The standards used (◆) were phosphorylase b (97.0 kDa), albumin bovine V (68.0 kDa), albumin (45.0 kDa), chymotrypsinogen A (25.7 kDa), and cytchrome *c* (12.3 kDa). The intact AMML (◇) is indicated. (C) Molecular mass determination of AMML by MALDI-TOF mass spectrometry.

Table 2
Hemagglutinating activity of AMML against human and animal erythrocytes

Erythrocyte source	Hemagglutinating activity (HU)	
	Trypsin-untreated	Trypsin-treated
Rabbit	4	8
Human (type A)	1	ND[a]
Human (type B)	ND[a]	1
Human (type AB)	ND[a]	4
Human (type O)	4	1

[a] The hemagglutinating activity could not be detected. The final lectin concentration was 184 μg/ml in the first well of the U-bottomed 96-well plate.

AMML showed no change in its hemagglutinating activity after treatment with EDTA (data not shown), suggesting that AMML does not require metal ions to be fully active or the metal ions are tightly bound to the lectin molecule.

Carbohydrate specificity of AMML

Carbohydrate-binding specificity of AMML was evaluated by the ability of sugars to inhibit hemagglutination of rabbit erythrocytes (Table 3). AMML was inhibited by 3.13 mM lactose and 7.81 mM galactose, indicating it belongs to the group of galactose plant lectins. D-Raffinose, L-rhamnose, and cellobiose also strongly inhibited the agglutination of rabbit erythrocytes. D-Glucose, D-xylose, and L-arabinose were found to be moderate inhibitors of AMML. The hemagglutinating activity of AMML was not inhibited by D-galacturonic acid, D-fructose, maltose, D-mannose, D-ribose, L-sorbose, and sucrose at 125 mM.

Amino acid composition and N-terminal sequence

A summary of AMML amino acid composition is given in Table 4. Amino acid composition analysis revealed that AMML was rich in Gly (16.0%), Ser (11.0%), Asp (9.9%), and Leu (9.6%). Cys, Met, Trp, and

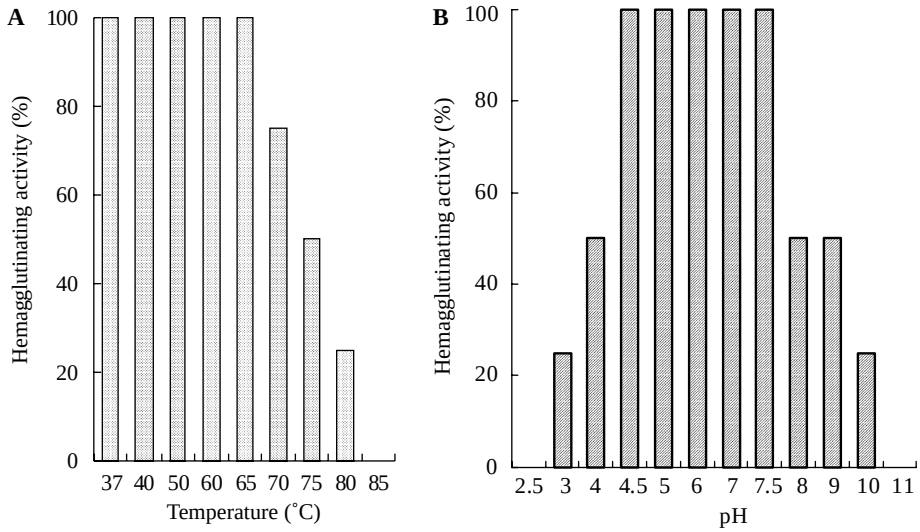


Fig. 4. Thermal (A) and pH stability (B) of AMML. The bars represent the average of five replicates. Full (100%) activity corresponded to 4 HU.

Table 3
Inhibition of hemagglutinating activity of AMML by different carbohydrates

Carbohydrate[a]	Minimum concentration of carbohydrates for inhibition (mM)[b]
Lactose	3.13
N-Acetyl-D-galactosamine	6.25
D-Galactose	7.81
D-Raffinose	12.5
L-Rhamnose	15.63
Cellobiose	25
D-Glucose	62.5
D-Xylose	62.5
L-Arabinose	125

[a] D-Galacturonic acid, D-fructose, maltose, D-mannose, D-ribose, L-sorbose and sucrose at 125 mM carbohydrate were not observed to inhibit 4 HU of lectin activity.
[b] Minimum concentration of carbohydrates required to inhibit 4 HU of lectin activity.

His residues were not detected. The first 15 N-terminal amino acids were determined by automated Edman degradation. AMML possessed a N-terminal sequence Glu-Ser-Gly-Ile-Asn-Leu-Gln-Gly-Asp-Ala-Thr-Leu-Ala-Asn- Asn. The N-terminal sequence of AMML is compared with other proteins in Table 5. By comparing its N-terminal sequence with those available in the data banks, we found that it was not identical to any proteins published so far. However, the above sequence exhibited 53% similarity with the N-terminal sequence of galactose binding lectin from *Arachis hypogaea* [25,26].

Antifungal activity

The purified AMML showed antifungal activity against various fungal species of the phytopathogenic fungi. Especially, it strongly inhibited the growth of *Botrytis cincerea* at the concentration of 20 μg/well (Fig. 5D-1). It inhibited mycelia growth in *Botrytis cinerea* with an IC_{50} of 1.2 μM. The lectin also exterted antifungal activity against *F. oxysporum* (Fig. 5D-2), *Colletorichum* sp., and *D. turcia* at the concentration of 100 μg/well, but was devoid of antifungal activity against *R. solani* and *M. arachidicola* at the concentration of 200 μg/well (data not shown). Besides, AMML had low ribonucleases activity toward yeast tRNA. It hydrolyzed yeast tRNA with an activity of 1.25 U/mg (data not shown).

Table 4
Amino acid composition of AMML

Amino acids	*A. mongholicus* lectin (AMML) (mol/100 of all amino acids)
Asp	9.9
Thr	8.2
Ser	11.0
Glu	6.2
Pro	2.2
Gly	16.0
Ala	7.6
Cys	NF[a]
Val	9.0
Met	NF[a]
Ile	5.4
Leu	9.6
Tyr	2.1
Phe	6.1
His	NF[a]
Lys	1.5
Trp	NF[a]
Arg	5.3

[a] NF, not found.

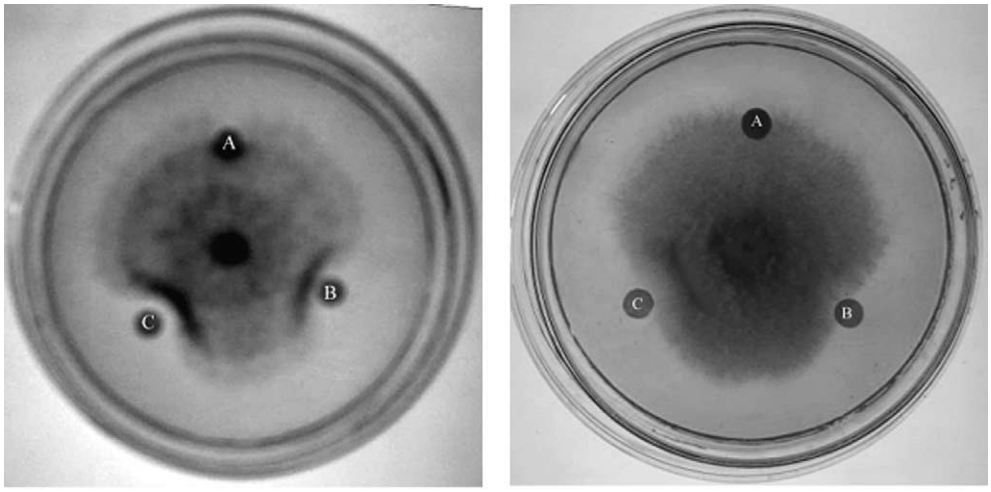
Discussion

Legume lectins are the best-studied group of lectins and hundreds of these proteins have been isolated and

Table 5
Comparison of N-terminal sequence of AMML with other proteins: results of a BLAST search

	Residue No.	N-terminal sequence	Residue No.	Length of protein (No. of amino acid)
AMML	1	ESGINLQGDATLANN	15	260
A. hypogaea galactose binding lectin	18	INLQGDAT[a]	25	246
A. hypogaea galactose binding lectin precursor	22	INLQGDAT	29	248
Ruegeria sp. PR1b	102	INLQGDAPL	110	268
Humicola grisea var. thermoidea β-glucosidase	229	EIGITLNGDATL	240	476

[a] Identical corresponding amino acid residues are underlined.



D-1 D-2

Fig. 5. Antifungal activity of AMML toward *B. cincerea* (D-1) and *F. oxysporum* (D-2). (A) Control (i.e., 10 mM phosphate buffer, pH 7.2), (B) 20 μg (D-1) or 100 μg (D-2) of AMML in 10 mM phosphate buffer (pH 7.2), and (C) 40 μg (D-1) or 200 μg (D-2) of AMML in 10 mM phosphate buffer (pH 7.2).

extensively characterized [8–13]. The genus *Astragalus* member occurs widely in the tropical and subtropical regions over the world and comprises about 278 species, two subspecies, 35 varieties and two forma in China [27]. To our knowledge, the present report represents the first investigation on the purification and characterization of a lectin from the genus *Astragalus*. The lectin (AMML) was purified to homogeneity from the roots of *A. mongholicus* Bunge and was found to be a dimer and composed of two identical subunits with a molecular mass of 29.6 kDa per monomer. In general, the molecular mass of most legume lectin subunits is around 30 kDa, with the fully active lectin forming either dimers or tetramers in nature [9,28]. AMML is a glycoprotein as detected by periodic acid-Schiff staining. This is further confirmed using the method of Dubois et al. [29], which showed a carbohydrate content of 19.6%. This carbohydrate content of AMML is higher than that of lectins from *Leuetzelburgia auriculata* (3.2%), *Erythrina speciosa* (5.5%) [30], *Annona muricata* (8%) [13], *Erythrina indica* (9.5%) [12] and *Talisia esculenta* (18.8%) [31].

Amino acid analysis showed that AMML was rich in Gly, Ser, Asp, and Leu, and did not contain Cys, Met, Trp, and His residues. There are some similarities in amino acid composition between lectins isolated from *A. hypogaea* [26] and *E. indica* [12]. Legume lectins are generally rich in glutamic acid, glutamine, and aspartic acid, while poor in sulfurous amino acids. The absence of cysteines is a characteristic of leguminous lectins, and low or null concentrations of Met and His are very common in vegetable proteins [32]. AMML had an apparent molecular mass of 29.6 kDa, as determined by SDS–PAGE under nonreducing and reducing conditions. The first 15 N-terminal sequences of AMML were determined. The single N-terminal residue is another evidence of the purity of the lectin and the identity of the two subunits. These sequences exhibited only 53% N-terminal sequence similarity with lectin from *A. hypogaea* (Table 5). Its N-terminal sequence suggests that it is a novel lectin.

In this investigation, AMML was a potent agglutinin of native rabbit and human erythrocytes (type O), suggesting that AMML recognizes the structure of saccharides comprising the surface of erythrocyte membranes. AMML failed to agglutinate human blood groups B and AB. This behavior has been observed in other lectins, for example *Sphenostyles stenocarpa* lectins [10]. It is distinctly characterized by its agglutination preference toward the native human type O group and trypsin-treated type AB group. Several sugars were tested for their ability to inhibit agglutination of AMML (Table 3).

The hemagglutinating activity of AMML was preferably inhibited by haptenic sugar D-galactose and lactose with the latter being two times stronger. The concentrations of galactose at 7.81 mM and lactose at 3.13 mM that required for complete inhibition of agglutination of trypsin-treated erythrocytes are common for plant lectins [10,12,25,30,33]. AMML may therefore be regarded as a galactose-specific lectin due to this sugar specificity [34]. Also, the lectin displayed carbohydrate specificity for L-rhamnose and cellobiose (Table 3), indicating that AMML shows plasticity in the carbohydrate-binding site. Similar results are described for some lectins, such as jacalin [35], CPL [36], TDSL [33], which also exhibit plasticity in the carbohydrate-binding region [35,36]. The inhibitory profile of AMML is similar to that of D-galactose-binding lectins, such as the lectin (PRA III) from *A. hypogaea* [26]. However, PRA III does not agglutinate untreated human and rabbit erythrocytes. It is noteworthy that AMML is able to agglutinate untreated human and rabbit erythrocytes. The lectin is characterized by high thermostability and pH stability. AMML retained its full hemagglutinating activity when it was maintained at temperatures up to 65 °C for 30 min. Its thermal stability is comparable to some thermostable lectins treated under similar conditions [29,30].

Lectins are thought to be involved in plant defense system by offering to plant resistance against insects, bacteria and fungi. In particular, chitin-binding lectins seem to have a role in defending plants against fungi and insects. The ability of lectins to inhibit fungal growth differs among fungal species. Studies in vitro demonstrated that a chitinase-free lectin preparation isolated from the stinging nettle *Urtica dioica*, inhibited the growth of *B. cincerea*, *Trichoderma hamatum* and *Phycomyces blakesleanus* [2]. Lectin from *T. esculenta* seeds inhibited the growth of *F. oxysporum*, *Colletotrichum lindemuthianum*, and *Saccharomyces cerevisiae* [31]. Besides, the lectin from *Annona muricata* seeds inhibited the growth of *F. oxysporum*, *F. solani*, and *Colletotrichum musae* at a concentration of 100 μg/ml [13]. AMML in the present study exhibited potent antifungal activity towards the commonly encountered agronomically harmful fungi *B. cincerea*, *F. oxysporum*, *Colletorichum* sp., and *D. turcia*. After adding 3.13 mM lactose and 7.81 mM galactose (the inhibitory sugars of the hemagglutinating activity) in the assay of antifungal activity to *B. cincerea*, lactose, and galactose did not abolish the antifungal effects of the lectin. Similar results are obtained with the lectin Kb-CWL I from *Kluyveromyces bulgaricus* on the yeast strains tested [37]. Therefore, AMML can be a potent candidate in plant genetic engineering for breeding antifungal plants.

In conclusion, a novel lectin with antifungal activity was purified from *A. mongholicus*. AMML is possibly a galactose-binding lectin as judged by its strong lectin activity, carbohydrate specificity and its sequence similarity to the lectin (PRA III) from *A. hypogaea*. It is important to note that galactose-binding lectins with antifungal activity has scarcely been reported.

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