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Isolation and Characterization of a Bioactive Mannose-Binding Protein from the Chinese Chive *Allium tuberosum*

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A mannose-binding protein was isolated from two different cultivars of the Chinese chive *Allium tuberosum* by extraction with 0.2 M NaCl, ammonium sulfate precipitation, and affinity chromatography on mannose agarose and fetuin agarose. It exhibited hemagglutinating activity toward rabbit erythrocytes. The lectin (agglutinin) was adsorbed on the mannose-agarose column, but not on the fetuin-agarose column. This *A. tuberosum* lectin (ATL) is unglycosylated, and not sialic acid binding. Lectins isolated from the two cultivars exhibited the same molecular mass of 25 kDa on gel filtration (Superose 12) and 12.5 kDa on SDS-polyacrylamide gel electrophoresis, indicating that they might be a dimeric protein composed of two identical subunits. The N-terminal amino acid sequence analysis of the lectin of various cultivars of *A. tuberosum* revealed that they were identical and showed 50%, or more, homology to the lectins from *Galanthus nivalis* (family Amaryllidaceae), *Narcissus tazetta* (family Amaryllidaceae), and *Aloe arborescenes* (family Liliaceae).

KEYWORDS: Chinese chive; *Allium tuberosum*; mannose-binding lectin

INTRODUCTION

Lectins are a group of proteins or glycoproteins of nonimmune origin, widely distributed in plants, animals, fungi, and microorganisms, with the ability to recognize and bind specific sugar(s) and to agglutinate blood cells (*1*). In general, they are composed of identical or different subunits, usually with one sugar-binding site per subunit (*2*). However, the recent study of function−structure relationship in monocot mannose-binding lectins by molecular modeling indicated that the number of active mannose-binding sites per monomer varies between three and zero (*3*). The specific endogenous role of plant lectin is unknown. Two main functions, as storage (such as, those that are rich in seeds, in vegetative storage tissues) and as defense (such as anti-insect, anti-fungal, anti-microbial, as well as toxic to birds and mammals) have been proposed and recognized on the basis of some observations and experimental results (*4*−*6*). The defensive role of lectins stimulates the research on possible application of lectins in crop protection. The immense advancement of genetic engineering technology makes it feasible to breed strains of resistance with the useful genes of lectin (*7*). Other biological activities of lectins that have been uncovered include not only immunomodulatory activity toward mammalian cells of fungal lectins (*8*−*10*), mistletoe lectins (*11*, *12*), and *Urtica dioica* agglutinin (*13*), but also activity against HIV-1 virus, Herpes simplex virus, and cytomegalovirus (*14*−*18*).

The mannose-binding lectins have been isolated from the leaves and bulbs of a nonedible plant, the Chinese daffodil *Narcissus tazetta* (Amaryllidaceae, Monocot). The *Narcissus tazetta* lectins (NTL) agglutinate rabbit red blood cells and display anti-bovine immunodeficiency virus activity but did not possess strong cytotoxic activity against normal and cancer cell lines (*19*−*21*). The present study will be focused on the isolation and characterization of the mannose-binding lectin in an edible plant, the Chinese chive, *Allium tuberosum* Rottler, which has not been investigated before. The Chinese chive, also known as Lazy Fellow's vegetable, is a favorite kitchen vegetable in Chinese cuisine. It is a next of kin of garlic and the onion family. However, it differs from garlic and onion in that its underground bulbs are scarcely developed, but the aerial shoots grow up single or in groups of two or three from a brownish rhizome (*22*). There are two cultivars on the market. One is normally grown under sunshine with dark green leaves (designated as JC, a short form for Jiou Cai), whereas the other is grown in dark for blanching. This pale yellow cultivar is designated as JH (a short form for Jiou Hwang), which is even more tender and delicious than JC. The sautéed young flowering stalk of JC (designed as JCT, a short for Jiou Cai Tai) usually presents a delicious dish during Chinese festivals. This perennial plant is not only edible, but is also considered a Chinese medicinal herb with various usages such as antiinflammatory, improving blood circulation, and tonic to liver, as well as being effective for the treatment of bruise by taking internally and/or applying externally (*23*).

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MATERIALS AND METHODS

Isolation of *Allium tuberosum* Lectin. One kilogram of fresh *Allium tuberosum* (JH cultivar) was purchased from the local market. The whole plant was rinsed and extracted with 0.2 M NaCl (2 mL/g). The homogenate was centrifuged, and ammonium sulfate [$(NH_4)_2SO_4$] was added slowly (up to 176 g/L) to the resulting supernatant to make 30% saturation. The resulting precipitate at 30% $(NH_4)_2SO_4$ saturation was discarded. The supernatant was further precipitated with the gradual addition of $(NH_4)_2SO_4$ (up to 356 g/L) to make a final 80% saturation. The resulting precipitate was dissolved in distilled water, and the solution was dialyzed extensively against distilled water and then lyophilized to yield a crude powder.

The crude powder was dissolved in 20 mM Mes buffer (pH 6) and applied on a mannose-agarose (Sigma) column, which was previously equilibrated and eluted with the same buffer. After the unadsorbed proteins were eluted, the adsorbed proteins were eluted by 0.2 M mannose in the eluting buffer and finally with 2 M NaCl. The adsorbed proteins were reloaded on a fetuin-agarose column that was preequilibrated with Tris−Cl buffer (45 mM, pH 8.15). The protein in the breakthrough peak was collected and precipitated with 10% cold trichloroacetic acid (TCA, Sigma) overnight at 4 °C. The TCA precipitate was solubilized in Tris−Cl buffer (45 mM, pH 8.15). The pH of the resulting supernatant was readjusted to 8 by Tris−Cl (1.5 M, pH 8.8). The solution was finally desalted through PD10 gel filtration (Pharmacia) and lyophilized.

Hemagglutinating Activity Test. The assay was carried out in a U-shaped microtiter plate as described previously (*19*). The hemagglutination titer is defined as the reciprocal of the highest dilution exhibiting hemagglutination and is equivalent to one hemagglutination unit. Specific hemagglutinating activity is expressed as the number of hemagglutinating units per mg of protein.

Test of Inhibition of Lectin-Induced Hemagglutination by Various Carbohydrates. A solution of lectin (25 μL of 0.8 mg/mL) with 8 hemagglutination units was mixed with an equal volume of a serial 2-fold dilution of the carbohydrate sample to be tested. After incubation at room temperature (with gentle shaking) for 30 min, the mixture was mixed with an equal volume (50 μL) of 2% suspension of rabbit red blood cells (without shaking). Concanavalin A was used as a positive control, and phosphate-buffered saline was used as a negative control. After 1 h of standing at room temperature, the minimal concentration of the carbohydrate in the final reaction mixture capable of completely inhibiting 8 hemagglutination units of the lectin was calculated from the results (*19*).

Molecular Size Estimation by Gel Filtration and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). The molecular weight of the protein was estimated by using gel filtration on a Superose 12 FPLC column (Pharmacia) in 150 mM ammonium bicarbonate (pH8), which had been calibrated with bovine serum albumin (molecular mass 66 kDa), trypsinogen (24 kDa), cytochrome C (12.4 kDa), aprotinin (6.5 kDa), and cytidine (246 Da).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to check the purity as detailed by Laemmli (*24*). It is a discontinuous system with 15% separating gel (pH 8.9) and 4% stacking gel (pH 6.8). Prior to electrophoresis, all the samples were boiled for 5 min with sample loading buffer which contained 2% SDS in the presence of 2% 2-mercaptoethanol. Coomassie Brilliant Blue R-250 was used to stain the gels for 30 min after electrophoresis. The de-staining solution was 10% acetic acid. The molecular mass of samples was deduced by comparing their molecular weight markers (low molecular range, Sigma, St. Louis, Mo.)

Analysis of N-terminal Amino Acid Sequence. Isolated *A. tuberosum* lectin (ATL) on gel was blotted onto a poly(vinylidene difluoride) membrane (PVDF) in a modified Dunn's Buffer (consisted of 10 mM $NaHCO_3$, 3 mM Na_2CO_3, pH 9.9, with 0.02% SDS) at a constant voltage (30 V) in a mini trans-blot cell (Bio-Rad) for $1^1/_2$ h at 4 °C. The 12.5 kDa subunit was cut out and analyzed by an HP G1000A Edman degradation unit and an HP-1000 HPLC system.

Glycoprotein Detection using Periodic Acid−Schiff (PAS) Staining. The presence of carbohydrates in the lectin was tested by periodic acid−Schiff staining after western blotting onto an immobilized membrane (PVDF). The protein on PVDF membrane was first fixed in trichloroacetic acid (12.5%). The oxidation of the carbohydrate was performed by reacting with an oxidizing reagent containing 1% periodic acid in 3% acetic acid solution. The resulting membrane then reacted with the basic Fuchsin-sulfite in Schiff's reagent (Sigma) in dark to yield a pink color complex against a white background.

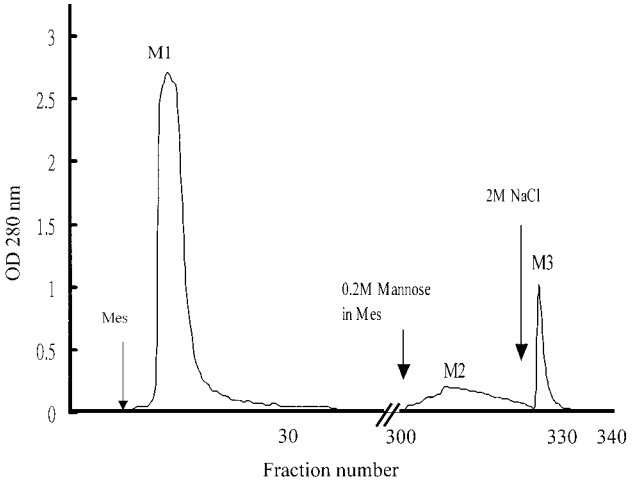
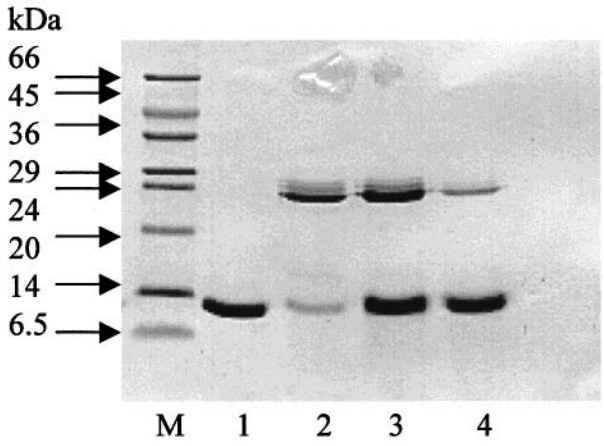


Figure 1. Affinity chromatography of *Allium tuberosum* (JH cultivar) extract on a mannose-agarose column (1.5 × 8 cm) that had been equilibrated and was eluted with Mes buffer (20 mM, pH 6). After unadsorbed materials (M1) had been eluted completely with the buffer (until there was no more absorbance at 280 nm), the adsorbed materials (M2 and M3) were eluted with 0.2 M mannose in Mes buffer and 2 M NaCL, respectively. Because there was only a minute amount of M2 and M3, the crude powder was repeatedly loaded on the column and eluted for 8 times (designated as // to indicate the pool of 8 eluants) in order to obtain a substantial amount of M2 and M3 fractions for further purification. The arrows indicate the points at which the buffers were changed.

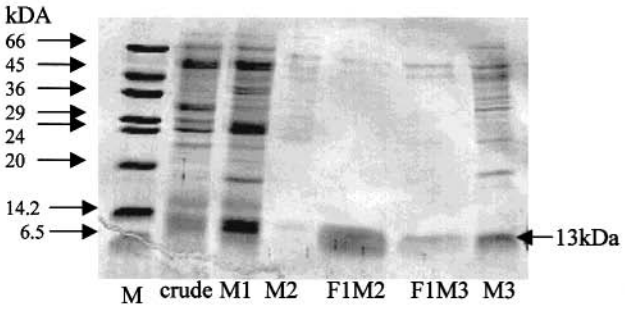
RESULTS AND DISCUSSION

The mannose-agarose affinity column adsorbed a very small peak (M2) which was eluted with 0.2 M mannose in Mes buffer (20 mM, pH 6). All the strongly mannose-agarose bound protein (M3 fraction) was then eluted with 2 M NaCl (**Figure 1**). The adsorbed material was first attempted to be purified by FPLC-gel filtration (Superose 12). However, the eluant appeared to contain a few proteins at the same retention time for the molecular mass of 24−26 kDa. Both M2 and M3 fractions were reapplied onto a Fetuin-agarose (Sigma) column, which was preequilibrated with 45 mM Tris Buffer (pH 8.15). The breakthrough peak (F1) was precipitated with 10% trichloroacetic acid (Sigma) and solubilized in the eluting buffer (Tris−Cl, 45 mM, pH 8.15). A single band with slightly less than 13 kDa was detected by SDS-PAGE (**Figure 2**) in the supernatant of this solution. The SDS-PAGE patterns of the lectin-containing fractions at different stages of purification are shown in **Figure 3**. The lectins are not glycoproteins because they did not react with the Schiff's Reagent. The protein isolated from all samples of JC, JH, and JCT using very similar purification procedures exhibited the same molecular mass of 25 kDa on gel filtration and 12.5 kDa on SDS-PAGE, indicating that they might be a dimeric protein composed of two identical subunits (**Figure 2**). Their N-terminal amino acid sequences are identical (**Table 1**). The purified protein (ATL) ranged from 0.2% to 0.5% of the total extractable protein in JCT, JH, and JC respectively (**Table 2**). The specific hemagglutinating activity was increased about 40-fold as the purification proceeded as demonstrated by ATL extracted from the JH cultivar (**Table 3**).

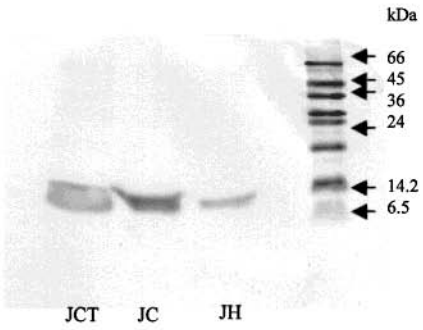


M = marker
Lane 1 = ATL purified from fetuin-agarose column
Lanes, 2,3 and 4 = mannose binding proteins from JH, JC, and JCT, respectively, purified after mannose agarose column and gel filtered through Superose 12 column.

Figure 2. Comparison of ATL purified from fetuin-agarose and gel filtration chromatography after affinity chromatography on a mannose-agarose column. M, low-range marker from Sigma; lane 1, ATL purified from fetuin-agarose column; lanes, 2, 3, and 4 are mannose binding proteins from *Allium tuberosum*, JH, JC, and JCT, respectively, purified after gel filtered through Superose 12-FPLC column (Pharmacia).



M= marker (Sigma, low range)
Crude=crude extract
M1= the breakthrough peak of mannose-agarose column
M2= the adsorbed peak of MA column eluted with 0.2M mannose in MES buffer
M3= the adsorbed peak of MA column eluted with 2M NaCl
F1= the breakthrough peak of fetuin-agarose column, eluted with 45mM Tris buffer (pH=8.15) and treated with 10% trichloroacetic acid.



The purified ATL from JH, JC, and JCT

Figure 3. SDS-polyacrylamide gel electrophoresis of *Allium tuberosum* chromatographic fractions during the course of purification (left), and purified ATL (right). M, low-range marker from Sigma; M1, the breakthrough peak of mannose-agarose (MA) column; M2, the adsorbed peak of MA column eluted with 0.2 M mannose in Mes buffer; M3, the adsorbed peak of MA column eluted with 2 M NaCL. F1M2 and F1M3 denote the 10% trichloroacetic acid treated fractions from breakthrough peak of fetuin-agarose column eluted with Tris buffer (45 mM, pH8.15) after M2 and M3 fractions were loaded on an affinity fetuin-agarose column, respectively. JH, JC, and JCT were the purified ATL.

The lectins isolated from JH, JC, and JCT are expected to have the same physicochemical properties and can be isolated by mannose-agarose and fetuin-agarose affinity columns. It is demonstrated that they are very strongly bound to the mannose-agarose column, therefore, it is concluded that they would be desorbed better by a higher concentration of mannose than that currently used (0.2 M) or by a buffer with higher ionic strength. It is also indicated that they are nonsialic acid-binding by not being bound to the fetuin-agarose column. ATL is also moderately cross-reactive to the rabbit antiserum against lectins of *Narcissus tazetta* (NTL), after employing immuno-blot detection of its western blot by BCIP (5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt) and NBT (nitroblue tetrazolium chloride) (data not shown). As expected, the ATL closely resembles those mannose-binding lectins isolated from garlic (*25*) and onion (*26*) (**Table 2**), because they are in the same family, Alliaceae. Furthermore, their N-terminal amino acid sequences show 50% or more homologous to that of the other monocot mannose-specific binding lectins, such as NTL, GNA (family Amaryllidaceae) (*27*), and AAA (family Liliaceae) (*28*) which are from the nonedible plants. It is not surprising that the epitopes on ATL cross-react moderately with antibody against NTL.

The minimum concentrations of D(+)-mannose and methyl α-D-mannopyranoside needed to completely inhibit 8 hemagglutinating units of lectin (containing 20 μg of lectin) from JH were 500 mM and 250 mM, respectively. The minimum amount of mannan (polysaccharide made up of mannose) required to

Table 1. N-Terminal Amino Acid Sequences of ATLs with Comparison to Some Other Monocot Mannose-Binding Lectins in the Families Alliaceae, Amaryllidaceae, and Liliaceae

family	lectin	sequence
Alliaceae	ATL (JCT)	RNVLLNGEGLYAGQSLEVGHYKYIMQDDDN[a]
Alliaceae	ATL (JC)	RNVLLNGEGLYAGQS[a]
Alliaceae	ATL (JH)	RNVLLNGEGLYAGQSLEVGH[a]
Alliaceae	ASA I	RNLLTNGEGLYAGQSLNVEPYHFIMQEDCN[b]
Alliaceae	ACA	RNVLVNNEGLYAGQSLVVEQYTFIMQFDCN[c]
Amaryllidaceae	GNA	DNILYSGETLSTGEFLNYGSFVFIMQEDCN[d]
Amaryllidaceae	NTL	DNILYSGETLYSGQFLNYGDYRFIMQADDN[e]
Liliaceae	AAA	DNILYSSEVLHENQYISYGPYEFIMQHDCN[f]

[a] From the present study, ATL, *Allium tuberosum* lectin. [b] ASA I, *Allium sativum* agglutinin domain I (*25*). [c] ACA, *Allium cepa* agglutinin (*26*). [d] GNA, *Galanthus nivalis* agglutinin (*27*). [e] NTL, *Narcissus tazetta* lectin (*21*). [f] AAA, *Aloe arborescens* agglutinin (*28*).

Table 2. Summary of the Yield of ATL from Flowering Stalk (JCT) and Two Different Cultivars of *Allium tuberosum* (JH and JC)

fraction	JCT protein mg/kg leaves (%)[a]	JH protein mg/kg leaves (%)[a]	JC protein mg/kg leaves (%)[a]
crude powder	682	1265	1024
M1 (nonmannose-binding protein)	492 (72.1)	806 (63.7)	726 (70.9)
M2 (mannose-binding protein I)	3.6 (0.53)	3.2 (0.25)	3.6 (0.35)
M3 (mannose-binding protein II)	19.2 (2.8)	140 (11.1)	39.4 (3.84)
purified ATL from M2 and M3	1.6 (0.23)	6.8 (0.54)	4.2 (0.4)

[a] As % of the total extractable protein.

Table 3. Specific Hemagglutinating Activities of Lectin-Containing Fractions in *Allium tuberosum* (JH cultivar) as Purification Proceeded

fractions of JH on affinity column	specific hemagglutinating activity (U/mg protein)
total extractable protein (JH)	100
M1 (nonmannose-binding protein)	47
M2 (mannose-binding protein I)	572
M3 (mannose-binding protein II)	1927
purified ATL from M2 and M3	3657

completely inhibit 8 hemagglutinating units of lectin was 2 μg. The following sugars were devoid of any inhibitory effect when tested: up to 1 M of methyl α-D-glucopyranoside, and up to 0.4 M of D-glucosamine, *N*-acetyl-D-glucosamine, D-fructose, L(−)-fucose, and D-galactose.

Allium tuberosum lectin (ATL) shares with the other monocot mannose-binding lectins many common physicochemical properties, such as exclusive binding specificity toward mannose and marked amino acid sequence homology. Therefore, ATL belongs to the superfamily of mannose-binding lectins of monocots (*26*). Monocot mannose-binding lectins exhibit some important biological activities such as antiviral (*16*, *17*) and insecticidal (*1*, *4*, *7*) effects, which have been exploited for biotechnological applications. ATL may also possess some of these bioactivities and may deserve further investigation for its biotechnological potential. The traditional medicinal value of *A. tuberosum* may not be solely due to this particular bioactive protein isolated in minute amounts. In mammals, mannose-binding lectins play an important role in the prevention of bacterial infections and cancer metastases (*29*). Furthermore, the mannose-binding lectin from the dicotyledon *Dolichos lablab* has shown its unique function in preserving hematopoietic progenitors in suspension culture (*30*). We expect the monocot mannose-binding lectins may have the function similar to that of dicot. Scientific and medical potential awaits reinvestigation to show whether such bioactivities are significantly useful.

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