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New Mannose-Binding Lectin Isolated from the Rhizome of Sarsaparilla *Smilax glabra* Roxb. (Liliaceae)

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A new mannose-binding lectin, designated SGM2, was isolated from the rhizome of a Chinese medicinal herb *Smilax glabra* (also known as sarsaparilla in general) by saline extraction, ammonium sulfate precipitation and fractionation, and affinity chromatography on fetuin− and mannose-agarose. SGM2 is shown to have a molecular mass of 37 kDa on gel filtration and 12.5 kDa on SDS-PAGE, indicating that it is a trimeric protein composed of three identical subunits. When the first 30 amino acid residues at the N-terminal were compared, SGM2 had ∼40% homology with those of some other monocots. SGM2 had the property of hemagglutinating activity toward rabbit erythrocytes, which could be reversed by mannose and mannose polymers. SGM2 exhibited antiviral activities against both herpes simplex virus type 1 (HSV-1) and respiratory syncytial virus (RSV) with the same EC_{50} of 8.1 μM.

KEYWORDS: Sarsaparilla; *Smilax glabra*; monocot; Liliaceae; mannose-binding lectin; antiviral; herpes simplex virus 1; resiratory syncytial virus

INTRODUCTION

Smilax glabra Roxb., a member of the Liliaceae family, also shared the name of sarsaparilla with other species of *Smilax* (including *S. officinalis*, *S. aristolochiaefolia*, *S. febrifuga*, *S. ornate*, *S. regelii*, and *S. japicanga*) around the world. Some sarsaparilla roots have been used in the West as an ingredient in root beer and other beverages for their foaming properties, whereas *S. glabra* is a very important herbal medicine in southeastern China. The Chinese name for *S. glabra* is tufuling, the root of which has been used in folk medicine for many efficacies, such as anti-inflammatory, antivenereal, and detoxifying activities (*1*). Many phytochemicals have been isolated from the root of tufuling with different bioactivities. Among these, smitilbin, engeletin, astilbin, eurryphin, or resveratrol from *S. glabra* had liver protective functions (*2*). The aqueous extract from the rhizome of *S. glabra* remarkably inhibited primary and secondary inflammation of adjuvant arthritis in rats, presumably through the immunomodulatory action (*3*). The methanol extract of the rhizome of *S. glabra* induced the increase of antioxidant activities in V79-4 cell culture (*4*) and reduced the blood glucose level in both normal and non-insulin-dependent diabetic mice (*5*). The boiling water extract of the rhizome of *S. glabra* also exhibited a wormicidal effect on *Clonorchis sinensis* (*6*). However, information on the protein constituents extracted from the rhizome of *S. glabra* is scant, except one study showing a heterodimeric, non-mannose-binding agglutinin (*7*). In the course of our research on the antiviral property of the water extract of *S. glabra*, we have found a new protein with mannose-binding properties, which has not been reported before. The monocot mannose-binding lectins belong to a superfamily of structurally and evolutionarily related proteins, which share the exclusive specificity for sugar binding, especially for mannose and/or mannan (a mannose polymer) and a number of other biological properties such as agglutinating rabbit erythrocyte, antiviral activity (*8*), and insecticidal effect (*9*). This paper describes the isolation and characterization of the new mannose-binding lectin from the rhizome of *S. glabra*.

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MATERIALS AND METHODS

The fresh tuber of *S. glabra* was purchased from the market in Guangzhou, China, and was identified by the herbalist of The Guangzhou University of Traditional Chinese Medicine. All chemicals used were of analytical or higher grade, unless otherwise specified, and were purchased from Sigma. A bicinchoninic acid (BCA) kit (Pierce) was used for protein assay throughout the experiment.

Isolation of Mannose-Binding Lectin from *S. glabra*. The whole tuber was rinsed, chopped, and extracted with 0.2 M NaCl (2 mL/g). The homogenate was centrifuged, and $(NH_4)_2SO_4$ was added to the resulting supernatant to 30−80% 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 (*11*).

The crude powder was dissolved in Tris-HCl buffer (45 mM, pH 8.15) and applied on a fetuin−agarose column, which was pre-equilibrated with the same buffer. The breakthrough peak (F1) was again loaded on a mannose−agarose column (Sigma) and eluted with MES buffer (20 mM, pH 6.2). After the unadsorbed peak was eluted (F1M1), the bound protein (F1M2) was eluted with 0.2 M mannose in MES buffer. The F1M2 fraction was further purified by a size exclusion technique on a Superdex 75 10/30 column using an AKTA fast protein liquid chromatography (FPLC) system (Amersham-Pharmacia Biotechnology). Total protein concentration was determined according to a bicinchoninic acid (BCA) method (Pierce).

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Hemagglutinating Activity Test. The assay was carried out in a U-shape microtiter plate as described previously (*10*). The hemagglutination titer is defined as the reciprocal of the highest dilution exhibiting hemagglutination and is equivalent to one hemagglutination titer. Specific hemagglutinating activity is expressed as the number of hemagglutinating titer (units) per milligram (mg) of protein.

Test of Inhibition of Lectin-Induced Hemagglutination by Various Carbohydrates. A fixed volume of lectin (25 μL) with 3 hemagglutination units (0.666 mg/mL) 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 (Con A) was used for a positive control, and phosphate-buffered saline used as a negative control. After the mixture had stood for 1 h at room temperature, the minimal concentration of the carbohydrate in the final reaction mixture capable of completely inhibiting 3 hemagglutination units of the lectin was calculated from the results (*10*).

Molecular Size Estimation by Gel Filtration and Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE). The molecular mass of the protein was estimated by gel filtration on a Superdex 75 10/30 column (Pharmacia) using the FPLC system (Pharmacia-Amersham Biotechnology). The column had been calibrated with bovine serum albumin (molecular mass of 66 kDa), trypsinogen (24 kDa), cytochrome *c* (12.4 kDa), aprotinin (6.5 kDa), and cytidine (246 Da). The eluting buffer was 100 mM ammonium bicarbonate (pH 8), and the flow rate was set at 0.4 mL/min.

SDS-PAGE was carried out to check the purity. Coomassie Brilliant Blue R-250 was used to stain the gels for 30 min after electrophoresis. The destaining solution was 10% acetic acid. The molecular masses of samples were deduced by comparing their electrophoretic mobilities with those of molecular weight markers (low molecular range, Sigma Chemical, St. Louis, MO).

Analysis of N-Terminal Amino Acid Sequence. Isolated proteins on gel were blotted onto a poly(vinylidene difluoride) membrane (PVDF, 0.45 μm) (Millipore) in a modified Dunn's buffer at a constant voltage in a mini trans-blot cell (Bio-Rad) at 4 °C for 1.5 h. The target proteins were cut off and analyzed by using an HP G1000A Edman degradation unit and an HP-1000 HPLC system (*11*).

Glycoprotein Detection Using Periodic Acid−Schiff (PAS) Staining. The presence of carbohydrates in the lectin was tested by PAS staining, after Western blotting onto an immobilized membrane (PVDF, 0.45 μm) (Millipore) as described previously (*11*).

Antiviral Assay. Cells were cultivated using Eagle's minimal essential medium (MEM) supplemented with fetal bovine serum (10%) and gentamycin (50 μg/mL). Herpes simplex virus type 1 (HSV-1) was propagated in Vero cells (African green monkey kidney cells, ATCC CCL81), whereas respiratory syncytial virus (RSV) was propagated in HEp-2 cells (human epidermoid carcinoma cells, ATCC CCL23) using a 96-well microtiter plate (Falcon Plastics, Oxnard, CA). After 24 h of incubation at 37 °C in a 5% CO_2 incubator, the culture medium was removed from the monolayer cells. To confluent monolayers of cells in the 96-well microtiter plate were added a cell suspension (0.1 mL) containing 100 times the 50% tissue culture infective dose ($TCID_{50}$) and maintenance medium (0.1 mL) containing appropriate serial 2-fold diluted concentrations of the test sample. The cytotoxicity was evaluated by examining the morphological alterations in the Vero and HEp-2 cells. The results were expressed as the maximum noncytotoxic concentration (MNCC) for cells.

MNCC of the test sample was used as the highest concentration from which a serial 2-fold dilution was made with the culture medium. Acyclovir (ACV, Sigma) and ribavirin (Sigma) were used as the positive controls for HSV-1 and RSV, respectively. Cell controls and virus control were run simultaneously. The cytotoxicity was evaluated by examining the morphological alterations in the cultured cells using a light microscope. The antiviral activities were estimated by cytopathic effect (CPE) reduction assay. The plates were incubated at 37 °C with 5% CO_2 (usually for 4−5 days) until the virus in the control wells showed complete virus-induced CPE as observed under the light microscope. The concentration of the samples required to inhibit 50% of the growth of the cells was determined from the graphic plot (*12*), which is described below.

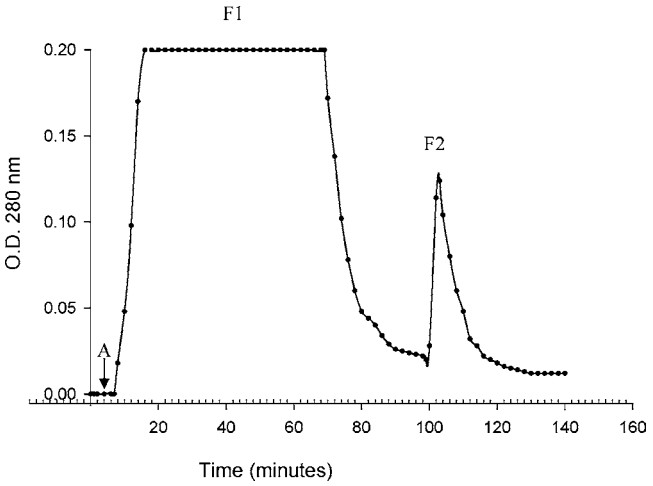

Figure 1. Fetuin–agarose column chromatographic profiles of the extract of *S. glabra* rhizome. The fetuin–agarose column (1.5 × 5 cm) had been equilibrated and was eluted with A (Tris-HCl buffer, 45 mM, pH 8.15). After unadsorbed materials (F1) had been eluted completely with the buffer (until there was no more absorbance at 280 nm), the unadsorbed materials (F1) were concentrated by ultrafiltration and dialyzed with MES buffer and reloaded on a mannose–agarose column (see **Figure 2**). The flow rate was 0.7 mL/min.

For CPE reduction assay, the virus-induced CPE was scored on day three after infection. The reduction of virus multiplication was calculated as the percentage of virus control (reduction of virus multiplication = $CPE_{exptl}/CPE_{virus\ control} \times 100\%$). The concentration reducing CPE by 50% with respect to virus control at day three was estimated from the graphic plot and was defined as the 50% effective concentration (3-day EC_{50}).

RESULTS

About 1 g of crude protein was obtained from 1 kg of wet weight of the fresh rhizome of *S. glabra*, and 74.3% of the total extractable protein was composed of the F1 fraction (**Figure 1**). From the fetuin−agarose affinity column the unadsorbed material (F1) was reloaded on a mannose−agarose column, which was then eluted with MES buffer (20 mM, pH 6.2) to remove F1M1. The fraction that remained in the column (F1M2, ∼0.68% of the total extractable protein) was eluted by 0.2 M mannose in MES buffer (**Figure 2**). The F1M2 fraction was further purified by gel filtration on Superdex 75 HR 10/30 (Pharmacia). The purified protein was concentrated in the descending half of the chromatographic peak, which yielded ∼0.3% of the total extractable protein (**Table 1**; **Figure 4**). The pure protein (SGM2) was a single band at the marker 12.5 kDa when revealed by SDS-PAGE (**Figure 3**), and its molecular mass is ∼37 kDa as determined with a calibrated standard curve by gel filtration on Superdex 75 with the retention time (in minutes) at 25.1 ± 0.02 (mean ± SE, $n = 6$). SGM2 reacts negatively to Schiff's reagent and was considered as an unglycosylated protein.

An analysis of 30 amino acid residues at the N-terminal (**Table 2**) revealed that SGM2 was 43% identical to both mannose-binding lectins from *Galanthus nivalis* (family Amaryllidaceae) and *Narcissus tazetta* (Family Amaryllidaceae), whereas it was 37% identical to those from *Aloe arborescenes* (family Liliaceae) and *Allium tuberosum* (Alliaceae).

SGM2 is a weak agglutinin toward rabbit erythrocytes with a specific hemagglutinating activity of ∼200 units/mg when

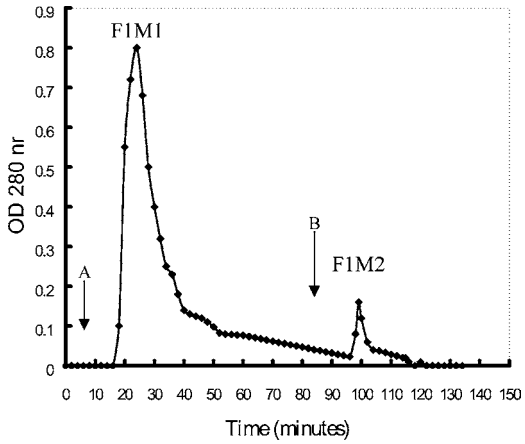

Figure 2. Mannose–agarose column chromatographic profiles of the F1 fraction of *S. glabra* rhizome. The mannose–agarose column (1.5 × 5 cm) had been equilibrated and was eluted with A (MES buffer, 20 mM, pH 6.2). After unadsorbed materials (F1M1) had been eluted completely with the buffer (until there was no more absorbance at 280 nm), the adsorbed materials (F1M2) were eluted with B (0.2 M mannose in MES buffer). The flow rate was 0.7 mL/min.

Table 1. Summary of the Yield[a] and Specific Hemagglutinating Activities of Lectin-Containing Fractions (on a Mannose–Agarose Column) in *S. glabra* as Purification Proceeded

fraction of *S. glabra* on affinity column	protein (mg)	% F1	specific hemagglutinating activity (units/mg of protein)
total protein (F1) loaded on column	56.9	100	800
F1M1	30.7	54	800
F1M2	0.52	0.91	160
F1M2 after gel filtration (SGM2)	0.23	0.4	200

[a] Yield: F1M2 was 0.91% of F1 and F1 was 74.3% of the total crude protein. Therefore, F1M2 was ~0.68% of the total extractable protein, and thus the purified protein (SGM2) was 0.3% of the total extractable protein.

compared to Con A, which has a specific hemagglutinating activity of 12500 units/mg in the same experimental conditions. The minimal concentrations of D-(+)-mannose (Sigma) and methyl α-D-mannopyranoside (Sigma) needed to completely inhibit 3 hemagglutinating units of lectin from SGM2 were similar at 250 mM. The minimal amount of mannan (a polymer of mannose, Sigma) required to completely inhibit 3 hemagglutinating units of lectin (16.65 μg) was 1.56 μg. *N*-Acetyl-D-glucosamine (up to 0.4 M), however, could not reverse the hemagglutinating activity. SGM2 was also moderately cross-reactive to the rabbit antiserum against lectins of *Narcissus tazetta* (NTL), after employing immunoblot detection of its Western blot by 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (BCIP) and nitroblue tetrazolium chloride (NBT) (*11*) (data not shown).

SGM2 exhibited antiviral activity with an EC_{50} of 8.1 μM to both HSV-1 and RSV.

DISCUSSION

In **Table 1**, the specific hemagglutinating activity decreased starting from the crude extract (800 units/mg) to the purified SGM2 (200 units/mg). This is not a common phenomenum in the purification of active proteins. This result could imply that there might be some other lectins present in the crude extract, which have no affinity to mannose−agarose, such as the

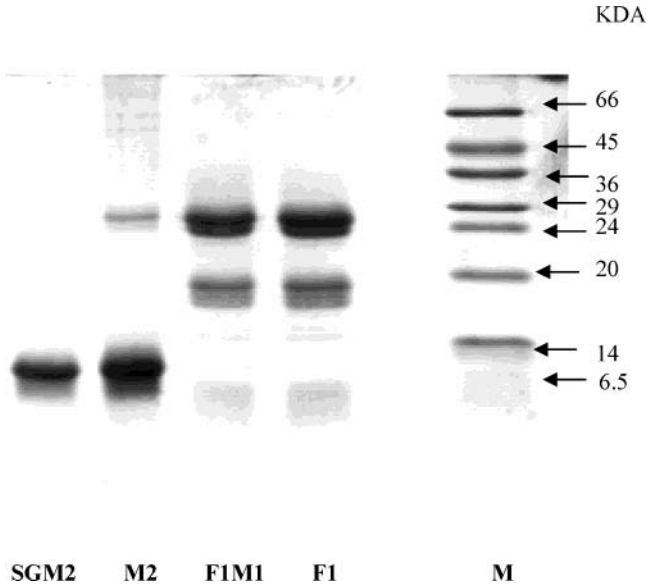

Figure 3. SDS-PAGE of the chromatographic fractions of *S. glabra*: M, marker (Sigma, low range); F1, unadsorbed materials from fetuin–agarose column; F1M1, the breakthrough peak on the mannose–agarose column; F1M2, the adsorbed fraction containing SGM2; SGM2, the purified protein after gel filtration on the Superose 12 column.

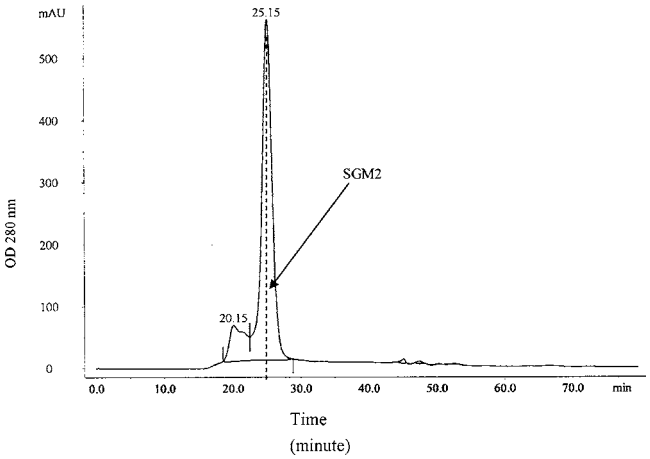

Figure 4. Gel filtration of F1M2 fraction by the AKTA-FPLC system on a Superdex 75 HR 10/30 column. The column was eluted with 100 mM NH_4HCO_3 buffer (pH 8) at a flow rate of 0.4 mL/min. The major peak with an average retention time (in minutes) of 25.1 ± 0.02 (mean ± SE, $n = 6$) contained SGM2. The descending half of the peak was collected as SGM2. The molecular mass of SGM2 was 37 kDa when calibrated with a standard curve.

heterodimeric agglutinin isolated from *S. glabra* as reported by Ng and Yu (*7*). We have found that SGM2 is only a weak agglutinin but that the counterpart, the mannose-unbound fraction (F1M1), has a higher specific hemagglutinating activity than SGM2. From the SDS-PAGE as shown in **Figure 3**, this mannose-binding protein appeared to exist with a glycoprotein moiety having a molecular mass of ~26 kDa by SDS-PAGE. The presence of glycoprotein was confirmed by a PAS stain. The glycoprotein, which coexisted with this mannose-binding protein in the first half of the peak on Superose 12, could make the separation of protein difficult. Fortunately, the protein moiety (SGM2) was concentrated in the descending half of the peak as monitored by SDS-PAGE. For the purpose of increasing the yield, we may try to collect the first half of the peak and separate them again by ionic strength with ion-exchange chromatography.

Table 2. N-Terminal Amino Acid Sequence of SGM2 in Comparison with Certain Other Monocot Mannose-Binding Lectins in Families of Alliaceae, Amaryllidaceae, and Liliaceae

family	lectin	sequence
Liliaceae	SGM2	VNVLYRGESLHVNEALAWDDNLFILQSDCN[a]
Liliaceae	SGA 15 kDa	ENNVLETQESLQSDERLSYQDVSFVMGQDDN[b]
	SGA 17 kDa	ENQVLLTQESLQSDERLSYQDVSFVMQQDCN[b]
Liliaceae	AAA	DNILYSSEVLHENQYISYGPYEFIMQHDCN[c]
Alliaceae	ATL (JCT)	RNVLLNGEGLYAGQSLEVGHYKYIMQDDDN[d]
Amaryllidaceae	GNA	DNILYSGETLSTGEFLNYGSFVFIMQEDCN[c]
Amaryllidaceae	NTL	DNILYMGETLYAGQFLNYGPYKFIIQDDCN[e]

[a] In the present study. SGM2, *S. glabra* mannose-binding lectin. [b] SGA, *S. glabra* agglutinin (*7*). [c] Through computer NCBI search, GNA, *Galanthus nivalis* agglutinin (*13*); AAA, *Aloe arborescens* agglutinin (*23*). [d] ATL, *Allium tuberosum* lectin (*11*). [e] NTL, *Narcissus tazetta* lectin (*24*).

SGM2 is the first, unglycosylated, mannose-binding lectin isolated from the sarsaparilla, *S. glabra*. It is homologous to some extent to some other monocot mannose-specific binding lectins in their N-terminal amino acid sequences, such as GNA (*Galantus nivalis* agglutinin, Amaryllidaceae) (*13*), NTL (*Narcissus tazetta* lectin, Amaryllidaceae) (*24*), AAA (*Aloe arborescens* agglutinin, Liliaceae) (*23*), and ATL (*Allium tuberosum* lectin, Alliaceae) (*11*) (**Table 2**). This protein exists as a trimer consisting of three 12.5 kDa subunits when isolated by affinity columns on fetuin− and mannose−agarose columns, which is greatly different from the SGA (*S. glabra* agglutinin) reported by Ng and Yu (*7*). First, SGM2 is a mannose-binding protein, whereas SGA is not; its hemagglutinating activity is not inhibited by mannose as reported (*7*). Second, SGM2 is a homotrimer of 12.5 kDa subunit with a molecular mass of 37 kDa, whereas SGA is a heterodimeric agglutinin composed of 15 and 17 kDa subunits with a molecular mass of 32 kDa. Third, the N-terminal amino acid residues of SGM2 and SGA are not identical. SGM2 and both subunits of SGA, however, have not more than 30% identity at the first 30 N-terminal amino acid residues (**Table 2**). Nevertheless, SGM2 is closer to the mannose-binding lectins of some other monocot plants with ∼40% or more identity at the N-terminal amino acid sequence (**Table 2**). SGM2 can also cross-react to the NTL antibody against rabbit as ATL (*Allium tuberosum* mannose binding lectin) (*11*) and have similar hemagglutinating activities that could be reversed by mannose and/or mannan, although the potency is much lower than that of NTL (*14*). Furthermore, SGM2 elicits antiviral activity toward herpes simplex virus (type 1) and respiratory syncytial virus in vitro with an EC_{50} of 8.1 μM for both viruses. In comparison, Acyclovir (ACV), the current drug for the treatment of HSV-1, has an EC_{50} of 1 μM and that of Ribavirin for RSV is 12.5 μM under our current antiviral assay system.

Unlike the bulb lectin in Amaryllidaceae (*13*, *14*), which are stored in large quantities in the bulb tissues (up to 10% of the total extractable protein for GNA and 4−5% for NTL), SGM2 is present only in minute amounts (<0.7%) of the total extractable protein in the subcylindrical rhizome of *S. glabra*. The monocot mannose-binding lectins belong to a superfamily of structurally and evolutionarily related proteins. The significance of this lectin distributed in various parts of plant tissues with different quantities is not clear apart from the structural and defensive function (*8*). Nevertheless, it may be useful in medical research and agricultural biotechnology. In medical research, the antiviral effect to human viruses is the general property of those mannose-binding proteins of monocots (*8*, *15*). It is worth mentioning that the Cyanovirin-N and Scytovirin from cyanobacterium, two potent anti-HIV agents, are also mannose-binding proteins, which have high affinity for high-mannose oligosaccharides (*16*−*18*). In agricultural biotechnology, transgenic plants with monocot mannose-binding lectin genes, such as the GNA gene, enhanced the insecticidal effect to sucking insects (such as aphids and leafhoppers), thus achieving the plant's better self-protection ability (*9*, *19*). Some other useful applications were also found for plant mannose-binding protein, such as using lectin from *Galanthus nivalis* as a ligand to purify IgM from murine species (*20*) and low-density lipoprotein (LDL) receptors (α-2 macroglobulin) in human serum (*21*). Recently the preserving capability for hematopoietic progenitors in suspension culture by the mannose-binding lectin from *Dolichos lablab* was reported (*22*). It is hoped that the significant usefulness of this mannose-binding lectin (SGM2) may be further elucidated.

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