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Purification of melibiose-binding lectins from two cultivars of Chinese black soybeans

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A dimeric 50 kDa melibiose-binding lectin was isolated from the seeds of the cultivar of soybean (*Glycine max*), called the small glossy black soybean. The isolation procedure comprised ion exchange chromatography on Q Sepharose, SP Sepharose and Mono Q followed by gel filtration on Superdex 75. The lectin was adsorbed on all three ion exchangers, and it exhibited an N-terminal sequence identical to that of soybean lectin. Of all the sugars tested, melibiose most potently inhibited the hemagglutinating activity of the lectin, which was stable between pH 3–12 and 0–70 ºC. The lectin evoked maximal mitogenic response at about the same molar concentration as Con A. However, the response was much weaker. The soybean lectin inhibited the activity of HIV-1 reverse transcriptase as well as the proliferation of breast cancer MCF7 cells and hepatoma HepG2 cells with an IC_{50} of 2.82 μM, 2.6 μM and 4.1 μM, respectively. There was no antifungal activity. Another lectin was isolated from a different cultivar of soybean called little black soybean. The lectin was essentially similar to small glossy black soybean lectin except for a larger subunit molecular mass (31 kDa), a more potent mitogenic activity and lower thermostability. The results indicate that different cultivars of soybean produce lectins that are not identical in every aspect.

Keywords purification; melibiose-binding lectin; Chinese black soybean

Lectins are proteins that exhibit carbohydrate-binding specificity. They can be divided into groups, such as mannose-binding, glucose/mannose-binding, N-acetylglucosamine-binding, galactose-binding, N-acetylgalactosamine-binding, fucose-binding, and sialic acid-binding, according to their carbohydrate-binding specificity. Lectins have been isolated from a diversity of organisms including flowering plants [1], animals [2,3], fungi [4], and bacteria [5]. Many biological activities, including anti-proliferative, anti-tumor, immunopotentiating, anti-insect, antifungal, antiviral and antibacterial activities, have been found in lectins [6–11]. As a result, lectins have captured the attention of many investigators.

Soybean is an agricultural crop of great importance. From it, many important proteins and non-proteinaceous compounds have been isolated. They comprise trypsin inhibitors [12], lectins [13], antifungal proteins [14], and phytoestrogens [15]. Phytoestrogens are reported to be protective against osteoporosis and cardiovascular disease [15,16]. Soybean trypsin inhibitor has an anti-tumor action [17]. Soybeans have been made into a variety of food products including drinks and bean curds. As stated in *Ben Cao Gang Mu* by Li Shizhen published in 1590, the black soybean is used in traditional Chinese medicine for treating backaches, combating debility and preventing aging. According to current popular literature, the black soybean has more protein, potassium and vitamins than the yellow soybean [18]. The former has chlorophyll and black pigment while the latter has carotenoids.

Different cultivars of the same species may produce different proteins; for example, the miraculin-like antifungal protein sativin and the ribosome-inactivating protein pisavin, which has similar sequence to miraculin, are produced by different cultivars of *Pisum sativum*, the sugar snap and the garden pea, respectively [19,20]. Different cultivars of soybean exist, including the yellow soybean, Chinese black soybean and Japanese black soybean. The yellow soybean produces trypsin inhibitors of the Bowman-Birk type and also the Kunitz type [12]. The Chinese dull black soybean produces only the Kunitz-type trypsin inhibitor [21], whereas the Japanese black soybean produces only the Bowman-Birk type [22]. Thus different cultivars may have dissimilar protein products.

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Lectin from yellow soybeans has been isolated and characterized. However, it is not known if the Chinese black soybean produces the same lectin. The Chinese black soybean is a special cultivar of soybean that differs from the common yellow soybean in pharmacological activities in traditional Chinese medicine. Thus, it was deemed worthwhile to isolate a lectin from each of the two cultivars of Chinese black soybean, namely the Chinese small glossy black soybean and the Chinese little black soybean, to compare them with the lectin from common yellow soybean, and to explore other potentially exploitable activities of the lectins.

Materials and Methods

Materials

Small glossy black soybeans and little black soybeans (*Glycine max*) from China were purchased from a local vendor. They were authenticated by Prof. Shiuying Hu, Honorary Professor of Chinese Medicine, the Chinese University of Hong Kong (Hong Kong, China).

Protein purification

The same isolation procedure and assay were employed for both cultivars. The beans were homogenized in distilled water. Tris-HCl buffer (pH 7.4) was added to the dialyzed supernatant until the concentration of Tris attained 10 mM. The supernatant was then loaded on a 5 cm×20 cm column of Q Sepharose (GE Healthcare, Hong Kong, China) in 10 mM Tris-HCl buffer (pH 7.4). After removing the unadsorbed fraction, adsorbed fractions (Q1, Q2 and Q3) were eluted with starting buffer containing 0. 2, 0.5 and 1 M NaCl, respectively. After examining the hemagglutinating effect of fractions Q1–3, the lectin-enriched fraction Q1 was dialyzed against 10 mM NH_4OAc buffer (pH 4.5) at 4 ºC, and then chromatographed on a 2.5 cm×20 cm column of SP Sepharose (GE Healthcare), which had previously been equilibrated with and was then eluted with 10 mM NH_4OAc buffer (pH 4.5). Unadsorbed proteins were eluted with the buffer, and then adsorbed proteins were eluted stepwise with the same buffer containing 0.2 M NaCl and 1 M NaCl, respectively. The fraction eluted with 0.2 M NaCl was then applied on a 1 ml Mono Q (GE Healthcare) column in 10 mM NH_4HCO_3 buffer (pH 9). Following elution of unadsorbed materials, adsorbed proteins (fraction S1, S2 and S3), were desorbed with two NaCl concentration gradients (0–0.3 M and adsorbed proteins 0.3–1 M) in 10 mM NH_4HCO_3 buffer (pH 9). The adsorbed fraction (S2), which was eluted by approximately 0.2 M NaCl, was the lectin-enriched fraction. It was dialyzed, lyophilized and subjected to gel filtration on a Superdex 75 HR 10/30 column (GE Healthcare) using an AKTA Purifier (GE Healthcare) in 10 mM NH_4HCO_3 buffer (pH 9.4). The first eluted absorbance peak constituted purified lectin.

Molecular mass determination by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and by Fast Protein Liquid Chromatography (FPLC)-gel filtration

SDS-PAGE was carried out in accordance with the procedure used by Nielsen and Reynolds [23], using a 12% resolving gel and a 5% stacking gel. The gel was cast on the miniprotein Set II (Bio-Rad, Shanghai, China). Samples were diluted with sample loading buffer (10% glycerol, 0.4% SDS, 0.005% bromophenol blue and 20 mM EDTA in 0.5 M Tris-HCl, pH 7.5) followed by addition of 5% β-mercaptoethanol. The samples were boiled in a water bath for 10 min. Electrophoresis was performed at a constant current of 10 mA at room temperature. At the end of electrophoresis, the gel was stained with Coomassie brilliant blue. FPLC-gel filtration was carried out using a Superdex 200 HR 10/30 column (GE Healthcare) that had been calibrated with molecular-mass standards (GE Healthcare).

Analysis of the N-terminal amino acid sequence

Amino acid sequence analysis was carried out using an HP G1000A Edman degradation unit and an HP 1000 HPLC system from Hewlett/Packard (Palo Alto, USA)[20]

Assay of hemagglutinating activity

In the assay for lectin (hemagglutinating) activity, a serial two-fold dilutions of the lectin solution in microtiter U-plates (50 μl) were mixed with 50 μl of 2% suspension of rabbit red blood cells in phosphate-buffered saline (pH 7. 2) at 20 °C. The results were recorded after about 1 h, when the blank containing only red cells had fully sedimented and appeared as a dot at the bottom of the well. The hemagglutination titer, defined as the reciprocal of the highest dilution exhibiting hemagglutination, is defined as one hemagglutination unit. Specific activity is the number of hemagglutination units per mg protein [11].

Inhibition of lectin-induced hemagglutination by carbohydrates

The hemagglutinating inhibition tests to investigate inhibition of lectin-induced hemagglutination by various carbohydrates for 30 min at room temperature were performed in a manner analogous to the hemagglutination test [11]. Serial two-fold dilutions of sugar samples were prepared

in phosphate buffered saline. All of the dilutions were mixed with an equal volume (25 μl) of a solution of the lectin with 32 hemagglutination units. The mixture was allowed to stand for 30 min at room temperature, and then mixed with 50 μl of 2% rabbit erythrocyte suspension. The minimum concentration of the sugar in the final reaction mixture, which completely inhibited 32 hemagglutination units of the lectin preparation, was calculated.

Effect of temperature and pH on lectin-induced hemagglutination

The effects of temperature and pH on hemagglutinating activity of the lectin were examined [11]. A solution of lectin was incubated at various temperatures (0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 °C) for 30 min. The tubes were then put on ice and assay of hemagglutinating activity was then carried out. A solution of lectin was incubated at various pH values (pH 1–14) for 30 min. The reaction mixtures in the tubes were neutralized and assay of hemagglutinating activity was then carried out.

Assay of mitogenic activity

Incubation of splenocytes from BALB/c mice (20–25 g) was carried out at 37 ºC in a humidified atmosphere of 5% CO_2 in the presence or absence of the lectin for 24 h in a 96-well culture plate. We added 10 μl (methyl-^{3}H) thymidine (0.25 μCi; GE Healthcare) before the splenocytes were incubated for another 6 h under the same conditions. The splenocytes were then harvested onto a glass fiber filter, and the radioactivity was measured [24–26].

Assay of antiproliferative activity on tumor cell lines

Tumor cells, MCF7 and HepG2, in their exponential growth phase were seeded into a culture plate and incubated for 3 h before addition of the lectin. Incubation was carried out for another 48 h. Radioactive precursor, 1 μCi of (methyl-^{3}H)thymidine, was then added to each well and incubated for 6 h. The cultures were then harvested, and the incorporated radioactivity was determined [25].

Assay of HIV reverse transcriptase inhibitory activity

The assay was carried out by using an enzyme-linked immunosorbent assay kit as described by Collins *et al* [16]. The assay for HIV reverse transcriptase inhibitory activity was carried out in accordance with instructions supplied with the assay kit from Boehringer Mannheim (Berlin, Germany) since some antifungal proteins possess this activity. The assay takes advantage of the ability of reverse transcriptase to synthesize DNA, starting from the template/primer hybrid poly(A) oligo(dT)15. The digoxigenin- and biotin-labeled nucleotides in an optimized ratio are incorporated into one of the same DNA molecule, which is freshly synthesized by the reverse transcriptase (RT). The detection and quantification of synthesized DNA as a parameter for RT activity follows a Sandwich ELISA protocol. Biotin-labeled DNA binds to the surface of microtiter plate modules that have been pre-coated with streptavidin. In the next step, an antibody to digoxigenin, conjugated to peroxidase, binds to the digoxigenin-labeled DNA. In the final step, the peroxidase substrate is added. The peroxidase enzyme catalyzes the cleavage of the substrate, producing a colored reaction product. The absorbance of the sample at 405 nm can be determined using a microtiter plate reader and is directly correlated to the level of RT activity. A fixed amount (4–6 ng) of recombinant HIV-1 reverse transcriptase was used. The inhibitory activity of the lectin was calculated as percent inhibition as compared to a control without the lectin [27].

Assay of HIV-I integrase and Severe acute respiratory syndrome (SARS) proteinase activities

The assay of HIV-I integrase and Severe acute respiratory syndrome (SARS) proteinase activities was conducted as previously reported [28].

Expression and purification of recombinant HIV-1 integrase The plasmid that expressed His-tagged wild-type HIV-1 integrase, pT7-7-His (Y|TX)-HIV-1-IN, was a generous gift from Professor S. A. Chow (School of Medicine, UCLA). To express the protein, a 1-liter culture of *E. coli* BL21(DE3) cells containing the expression plasmid was grown at 37 ºC until OD_{600} reached 0.7–0.8. Cells were induced by addition of 0.8 mM isopropyl-β-*D*-thiogalactopyranoside (IPTG) and harvested, after 4 h of incubation, by centrifugation at 6000 *g* for 10 min at 4 °C. Cells were suspended at a concentration of 10 ml/g wet cell paste in 20 mM Tris-HCl buffer (pH 8.0) containing 0.1 mM EDTA, 2 mM β-mercaptoethanol, 0.5 M NaCl and 5 mM imidazole. Lysozyme was added to a concentration of 0.2 mg/ml. After incubation at 4 ºC for 1 h, the lysate was sonicated and centrifuged at 40,000 *g* at 4 ºC for 20 min. The pellet was homogenized in 50 ml buffer A (20 mM Tris-HCl, pH 8.0, 2 M NaCl, 2 mM β-mercaptoethanol) containing 5 mM imidazole. The suspension was rotated at 4 ºC for 1 h, and cleared by centrifugation at 40,000 *g* at 4 ºC for 20 min. The supernatant was loaded onto a 1-ml chelating Sepharose

(GE Healthcare) column charged with 50 mM imidazole. The column was washed with five column volumes of buffer A containing 5 mM imidazole, and the protein was eluted with three column volumes of buffer A containing 200 mM and 400 mM imidazole, respectively. Protein-containing fractions were pooled, and EDTA was added to a final concentration of 5 mM. The protein was dialyzed against buffer B (20 mM HEPES, pH 7.5, 1 mM EDTA, 1 M NaCl, 20% glycerol) containing 2 mM β-mercaptoethanol, and then against buffer B containing 1 mM dithiothreitol. Aliquots of the protein were stored at –70 ºC.

HIV-1 integrase assay A non-radioactive ELISA-based HIV-1 integrase assay was performed according to the DNA-coated plate method. In this study, 1 μg of *Sma*I-linearized pBluescript SK was coated onto each well in the presence of 2 M NaCl as target DNA. The donor DNA was prepared by annealing VU5BR (5′-biotin-GTGTGGAAAATCTCTAGCAGT-3′) and VU5 (5′-ACTGCTAGAGATTTTCCACAC-3′) in solution containing 10 mM Tris-HC1, pH 8.0, 1 mM EDTA and 0.1 M NaCl at 80 ºC followed by 30 min at room temperature. Integrase reaction was performed in 20 mM HEPES (pH 7.5) containing 10 mM $MnCl_2$, 30 mM NaCl, 10 mM dithiothreitol and 0.05% Nonidet-P40 (Sigma, St. Louis, USA). After the integrase reaction, the biotinylated DNA immobilized on the wells was detected by incubation with streptavidin-conjugated alkaline phosphatase (Boehringer Mannheim), followed by colorimetric detection with 1 mg/ml *p*-nitrophenyl phosphate in 10% diethanolamine buffer (pH 9.8) containing 0.5 mM $MgCl_2$. The absorbance of each well due to the alkaline phosphatase reaction was measured at 415 nm.

Screening for inhibitory effect on SARS Coronavirus (CoV) protease The activity of SARS CoV protease was indicated by a cleavage of designed substrate which was composed of two proteins linked by a cleavage site for SARS CoV protease. The reaction was performed in a mixture containing 5 μM SARS CoV protease, 5 μM sample, 20 μM substrate and buffer (20 mM Tris-HCl, pH 7.5, 20 mM NaCl and 10 mM beta-mercaptoethanol) for 40 min at 37 ºC. After 40 min, the reaction was stopped by heating at 100 ºC for 2 min. Then the reaction mixture was analyzed by SDS-PAGE. If SARS CoV protease is inhibited by the test sample, there is only one band, which is the intact substrate, shown in SDS-PAGE.

Results

Isolation of small glossy black soybean lectin and little black soybean lectin

The crude extract of small glossy black soybeans was fractionated on a Q Sepharose column into an unadsorbed fraction and three adsorbed fractions (Q1, Q2 and Q3) were obtained by stepwise elution with 0.2, 0.5 and 1 M NaCl in Tris-HCl buffer [**Fig. 1(A)**]. Hemagglutinating activity was confined to fraction Q1. This fraction was resolved on an SP Sepharose column into an unadsorbed fraction and two adsorbed fractions (SP1 and SP2) respectively when eluted with 0.2 M NaCl and 1 M NaCl in the NH_4OAc buffer [**Fig. 1(B)**]. Hemagglutinating activity resided in the smaller fraction SP1. Fraction SP1 was further resolved by ion exchange chromatography on an FPLC-Mono Q column into a tiny unadsorbed fraction and several adsorbed fractions [**Fig. 1(C)**]. Hemagglutinating activity was recovered only in fraction S2, which was subsequently separated on a Superdex 75 column into a sharp larger fraction and a smaller fraction. Activity was detected only in the larger fraction [**Fig. 1 (D)**].

From Chinese little black soybeans, a lectin was isolated using the same procedure except for the omission of ion exchange chromatography on Mono Q (**Fig. 2**).

Determination of molecular weight

The small glossy black soybean lectin product appeared as a single band with a molecular mass of 25 kDa in SDS-PAGE (**Fig. 3**), and exhibited a single peak with a molecular mass of 50.2 kDa in gel filtration on Superdex 75 [**Fig. 1(D)**].

The little black soybean lectin appeared as a single band with a molecular mass of 31 kDa in SDS-PAGE (**Fig. 3**), and exhibited a single peak with a molecular mass of 62 kDa in gel filtration on Superdex 75 [**Fig. 2(C)**].

Determination of N-terminal sequence

The small glossy black soybean lectin was obtained with a specific activity of 7168 U/mg and a purification fold of 29 (**Table 1**). The N-terminal sequence of the lectin was ^{1}AETVSFSWNKFVPKQ^{15}, which was identical to lectin from yellow soybean and similar to partial sequences near the N-terminals of *Medicago sativa* (^{27}AETTSFS-ITKFVPDQ^{41}) and *Robinia pseudoacacia* lectins (^{35}ESVSFSFTKFVP^{46}). There was only one peak in every sequencing cycle, indicating that the lectin preparation was homogeneous. It appears that soybean lectins are similar in N-terminal sequence to only some members of the pea family Fabaceae.

The N-terminal sequence of little black soybean lectin

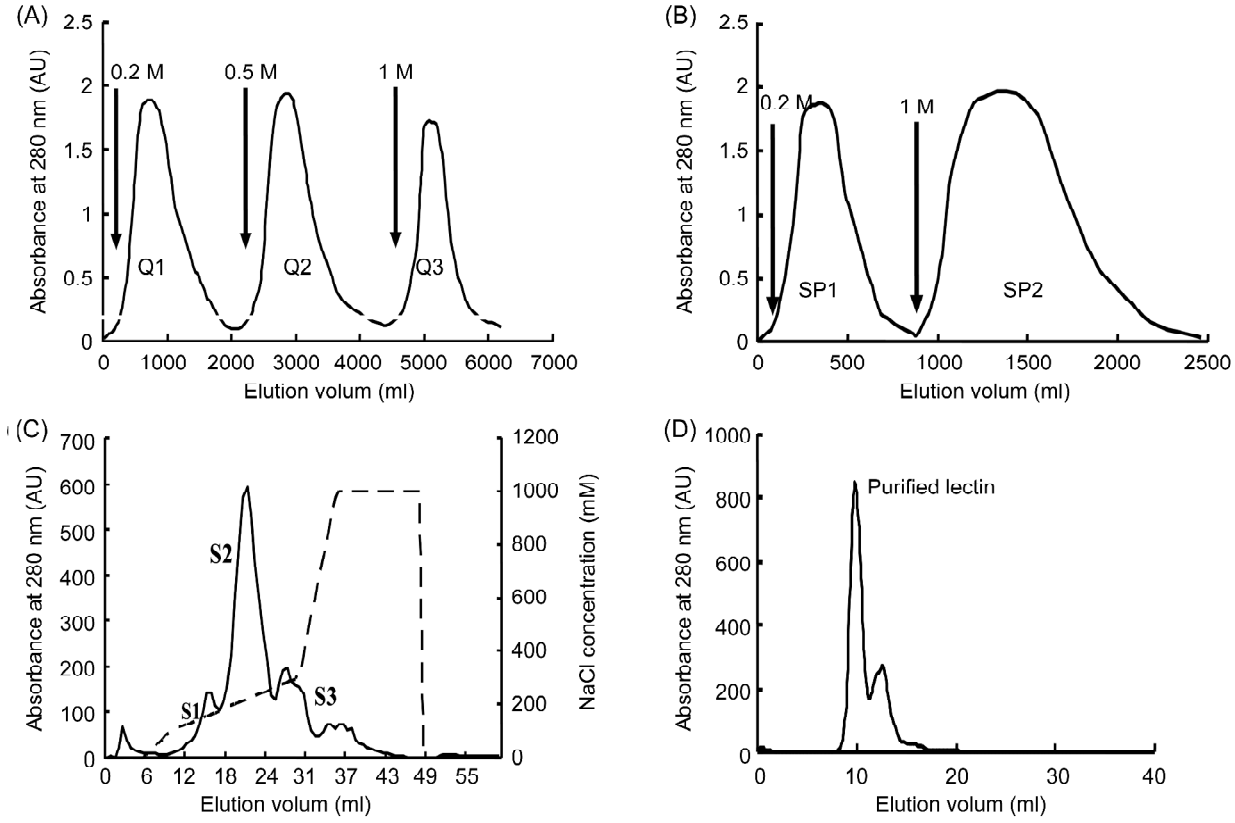

Fig. 1 Purification of small glossy black soybean lectin by chromatography (A) The black soybean extract was applied on a Q Sepharose column (5 cm×20 cm). After elution of unadsorbed proteins with 10 mM Tris-HCl buffer (pH 7.4), the column was eluted stepwise with 0.2 M NaCl, 0.5 M NaCl, and 1 M NaCl added to the Tris-HCl buffer as indicated by the arrows. (B) Fraction Q1 from the Q Sepharose column was dialyzed and applied on an SP Sepharose column (2.5 cm×20 cm) in 10 mM NH_4OAc buffer (pH 4.5). After elution of unadsorbed proteins, the column was eluted stepwise with 0.2 M NaCl and then with 1 M NaCl added to the buffer as indicated by the arrows. (C) Fraction SP1 from the SP Sepharose column was loaded on a 1 ml Mono Q column. Following elution of unadsorbed proteins with 10 mM NH_4HCO_3 buffer (pH 9), adsorbed proteins were eluted sequentially, first with a 0–0.3 M NaCl gradient and then with a 0.3–1 M NaCl gradient. (D) Fraction S2 from the Mono Q column was subjected to gel filtration on a Superdex 75 HR 10/30 column in 10 mM NH_4HCO_3 buffer (pH 9.4).

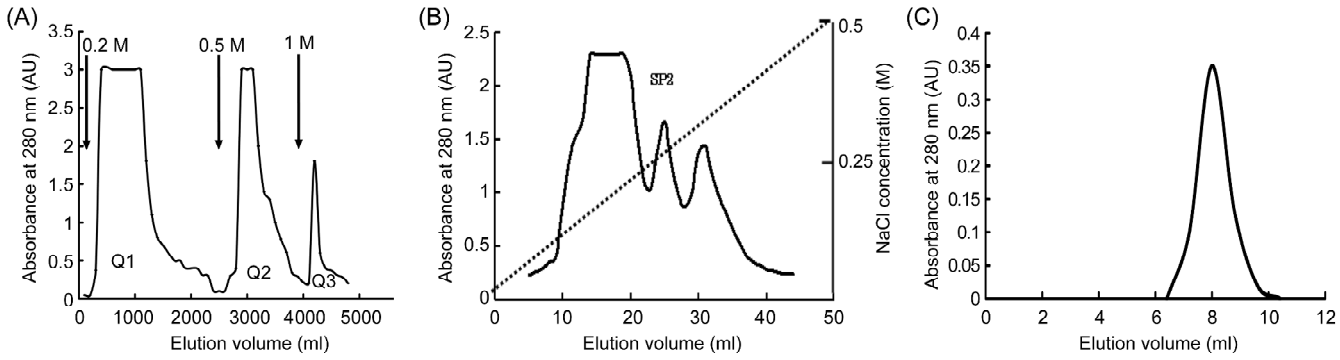

Fig. 2 Purification of little black soybean lectin by chromatography on (A) Little black soybean extract was applied on a Q Sepharose column (5 cm×20 cm). After elution of unadsorbed proteins with 10 mM Tris-HCl buffer (pH 7.4), the column was eluted stepwise with 0.2 M NaCl, 0.5 M NaCl, and 1 M NaCl added to the Tris-HCl buffer as indicated by the arrows. (B) Fraction Q1 from the Q Sepharose column was dialyzed and applied on an SP Sepharose column (2.5 cm×20 cm) in 10 mM NH_4OAc buffer (pH 4.5). After elution of unadsorbed proteins, adsorbed proteins were eluted with a linear NaCl concentration gradient (0–0.5 M) in 100 mM NH_4OAc buffer (pH 4.5) as indicated by the slanting line across the chromatogram. Lectin activity was observed only in fraction SP2. (C) Fraction SP2 from the SP Sepharose column was subjected to gel filtration on a Superdex 75 HR 10/30 column in 10 mM NH_4HCO_3 buffer (pH 9.4).

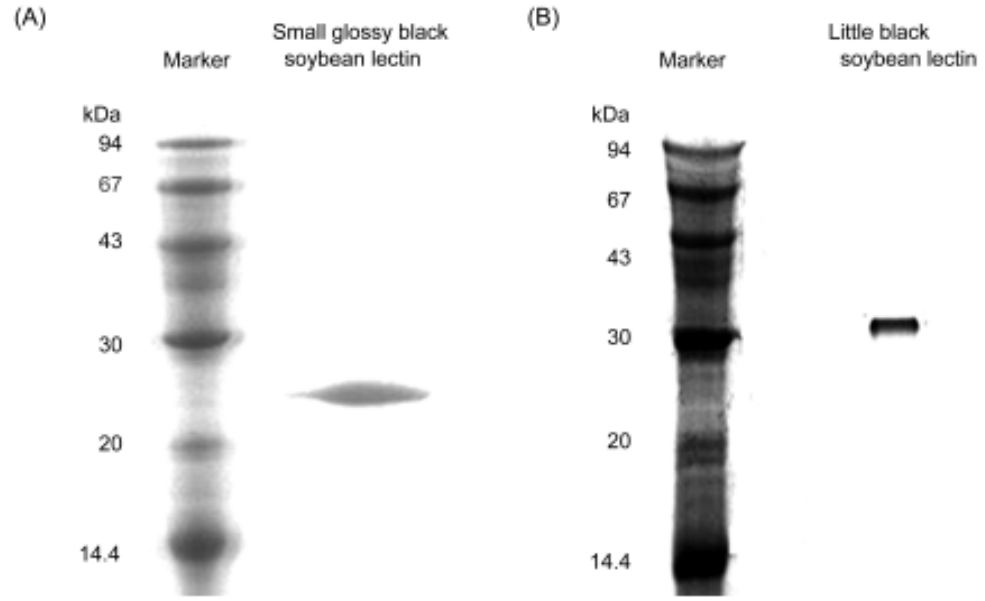

Fig. 3 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis results (A) Left lane, molecular mass (MW) marker proteins including phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14.4 kDa); right lane, purified small glossy black soybean lectin [first fraction from Superdex 75 as shown in **Fig. 1(D)**]. Molecular mass of the purified lectin is 50.2 kDa and it is a dimer. (B) Left lane, molecular mass marker proteins; right lane, purified little black soybean lectin [first fraction from Superdex 75 as shown in **Fig. 2(C)**]. Molecular mass of the purified lectin is 62 kDa and it is a dimer.

Table 1 Summary of purification of small glossy black soybean lectin from 100 g seeds

Chromatographic fraction with hemagglutinating activity	Total protein (mg)	Purification fold	Total activity (titer×10^3)	Specific activity (titer/mg)	Recovery of activity (%)
Crude extract	17240	1.0	4138	240	100
Q1 (after Q Sepharose)	6130	2.1	2979	486	72
SP1 (after SP Sepharos)	970	8.5	1986	2047	48
S2 (after Mono Q)	350	17.7	1490	4256	36
Purified lectin (after Superdex 75)	127	29.0	910	7168	22

was identical to that of small glossy black soybean lectin (**Table 2**).

Determination of sugar specificity, pH stability and thermostability of hemagglutinating activity

Melibiose, arabinose, raffinose and galactose at 40 mM concentration inhibited the hemagglutinating activity of the small glossy black soybean lectin. However, glucose, fucose, rhamnose, mannose, lactose, xylose, glucuronic acid, polygalacturonic acid, and mannosamine had no effect (**Table 2**). The hemagglutinating activity was stable after exposure for 30 min to the pH range 3–12 and the temperature range 0–70 ºC.

The carbohydrate specificity of little black soybean lectin was identical to that of small glossy black soybean lectin. The hemagglutinating activity of little black soybean lectin was stable only up to 40 ºC for 20 min, but the small glossy black soybean lectin was stable up to 70 ºC for 30 min.

Other biological activities

The small glossy black soybean lectin inhibited proliferation of HepG2 cells and MCF7 cells with an IC_{50} of 4.1 μM and 2.6 μM, respectively (**Fig. 4**) and the activity of HIV-1 reverse transcriptase with an IC_{50} of 2.82 μM (**Fig. 5**).

Table 2 Comparison of sugar specificity of lectins from different cultivars of soybean

Sugar (40 mM)	Yellow soybean lectin	Chinese small glossy black soybean lectin	Chinese little black soybean lectin
Melibiose	–	+	+
Galactose	–	+	+
Arabinose		+	+
Raffinose		+	+
N-acetylgalactosamine	+	–	–

Glucose, fucose, rhamnose, mannose, lactose, xylose, glucuronic acid, polygalacturonic acid, and mannosamine were all inactive when tested in 500 mM. +, inhibition of hemagglutinating activity of lectin; –, no inhibition of hemagglutinating activity of lectin.

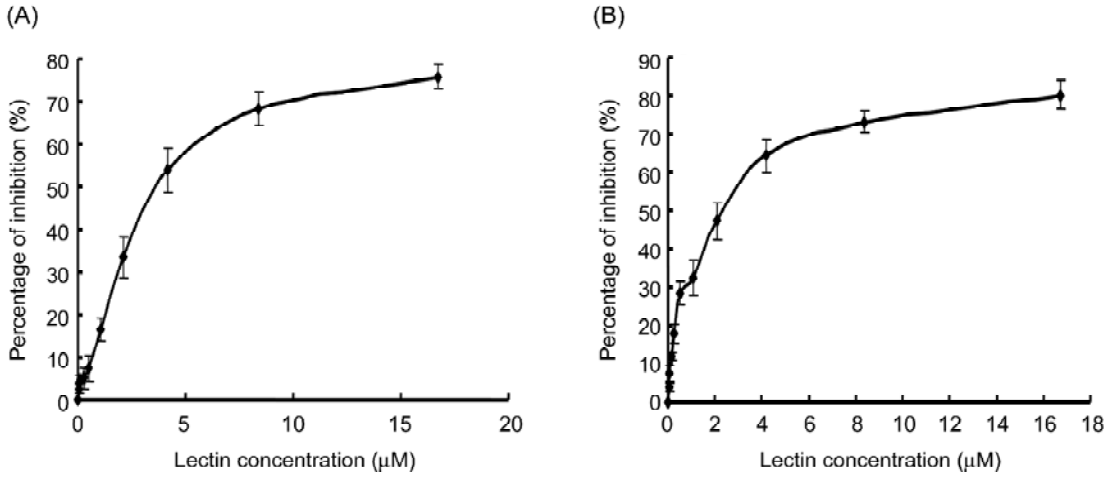

Fig. 4 Antiproliferative activity of small glossy black soybean lectin on (A) HepG2 hepatoma cells and (B) MCF7 breast cancer cells These cells were treated with the lectin for 72 h at 37 °C in an atmosphere of 95% CO_2 and 5% O_2. Results are presented as the mean±SD (n=3). IC_{50} values for HepG2 cells and MCF cells were 4.1 μM and 2.6 μM, respectively.

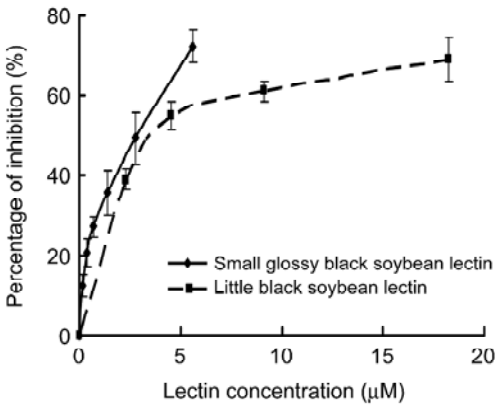

Fig. 5 HIV-1 reverse transcriptase inhibition by small glossy black soybean lectin and little black soybean lectin Results are presented as the mean±SD (n=3). IC_{50} of small glossy black soybean was 2.82 μM. IC_{50} of little black soybean lectin was 3.54 μM.

The mitogenic activity of the lectin on mouse splenocytes is shown in **Fig. 6**. Both the lectin and Con A stimulated maximal mitogenic response at similar concentrations. However, the maximal response achieved by small glossy black soybean lectin was weaker than that evoked by Con A (**Fig. 6**). Small glossy black soybean lectin did not inhibit HIV integrase or SARS proteinase when tested up to 20 μM (data not shown).

The little black soybean lectin inhibited HIV-1 reverse transcriptase with an IC_{50} of 3.54 μM (**Fig. 5**). The maximal mitogenic response that both the small glossy black soybean lectin and the little black soybean lectin elicited was about 20% of the maximal response to Con A. The maximal mitogenic response was achieved by similar concentrations of small glossy black soybean lectin, little black soybean lectin and Con A (**Fig 6**).

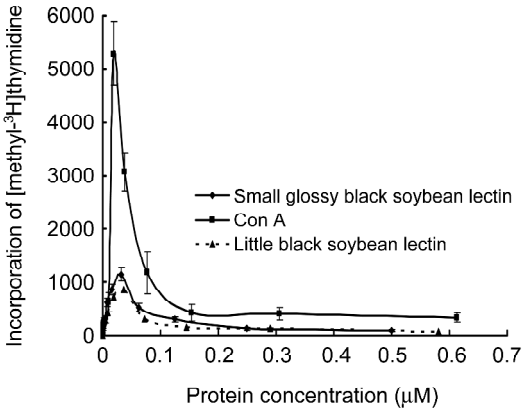

Fig. 6 Mitogenic response of small glossy black soybean lectin and little black soybean lectin from mouse spleen cells The lectins started to evoke a mitogenic response at a lower concentration than Con A although the maximal response was much smaller in magnitude.

Comparison of two black soybean lectins

A comparison of the characteristics of the two black soybean lectins isolated in this study with those of soybean lectins is presented in **Table 3**.

Discussion

The two Chinese small glossy black soybean lectins differ somewhat from common yellow soybean lectin (hereinafter referred to as soybean lectin) in carbohydrate specificity. Soybean lectin is galactose-specific [13], whereas Chinese black soybean lectins are melibiose-specific although the latter two are also galactose-specific. The two Chinese black soybean lectins potently inhibit HIV-1 reverse transcriptase (IC_{50} about 2.8 μM). It is known that some lectins exhibit HIV-1 reverse transcriptase inhibitory activity and inhibit HIV replication [27, 29,30]. However, just like the French bean defensin [28], the two black soybean lectins are devoid of HIV-1 integrase inhibitory and SARS proteinase inhibitory activity. The antiproliferative and anti-tumor activities of lectins are well documented [25,27,31–33]. Chinese small glossy black soybean lectin exerts potent antiproliferative activity toward HepG2 and MCF7 cells, with an IC_{50} of 4.1 μM and 2.6 μM, respectively. Both black soybean lectins manifest weaker mitogenic activity than Con A toward splenocytes. This is in line with findings on other lectins [24,25].

The mitogenic activity of soybean lectin toward human and murine lymphocytes is enhanced after polymerization by physical or chemical means [34]. Soybean lectin has immunomodulatory and anti-tumor actions [34]. Natural suppressor cells from the spleen and cyclophosphamide-generated suppressor cells react specifically with soybean lectin and can thus be isolated by agglutination from the bone marrow [35,36]. Soybean lectin is employed for purging the marrow of T cells during treatment of acute lymphoblastic leukemia in order to decrease the risk of graft-versus-host disease [37]; soybean lectin reacts with and eliminates cancer cells from the marrow [38]. This investigation's findings on the two black soybean lectins' mitogenic activity towards splenocytes and their antiproliferative activity toward tumor cells are consistent with previous observations [34,38]. An anti-tumor action mechanism of soybean lectins has been proposed involving the action of the lectins on tumor cell membranes, the reduction of tumor cell proliferation, the induction of tumor-specific cytotoxicity of macrophages, and apoptosis. Thus, tumor cells are more susceptible to attack by macrophages after treatment with lectins. Furthermore, lectins exert an immunomodulatory effect on altering interleukins production [39].

Soybean lectin reportedly reacts preferentially with some rumen fungi [40], but an antifungal action has not been demonstrated. Likewise, the two black soybean lectins are devoid of antifungal activity (data not shown).

Table 3 Comparison of lectins from different cultivars of soybean

	Molecular weight (kDa)	Number of subunit	pH stability	Thermostability	Anti-tumor activity	Mitogenic activity
Yellow soybean lectin	122	4	pH 2–13	Up to 50 °C	Existed	Existed
Chinese small glossy black soybean lectin	50	2	pH 2–13	Up to 70 °C	HepG2 IC_{50}: 4.1 μM; MCF7 IC_{50}: 2.6 μM	Maximal response about 20% of Con A
Chinese little black soybean lectin	62	2	pH 3–13	Up to 40 °C	Existed	Maximal response about 20% of Con A

In fact, to date, only a handful of lectins have been reported with antifungal activity [41,42].

Small glossy black soybean lectin has fair pH stability and thermostability. Its hemagglutinating activity is preserved in the pH range 3–12 and in the temperature range 0–70 ºC. Little black soybean lectin is stable in the pH range 3–12 and in the temperature range 0–40 ºC. Yellow soybean lectin is active in the pH range 3–12 and in the temperature range 0–50 ºC. French bean lectin is stable in pH range 4–10 and in the temperature range 0–90 ºC [30]. Hence the French bean lectin and small glossy black soybean lectin appear to be similar in stability.

The protocol employed in the present investigation for purifying the two black soybean lectins entailed successive ion exchange chromatography on Q Sepharose, SP Sepharose and Mono Q (with the exception of little black soybean lectin), and gel filtration on Superdex 75. French bean lectin was isolated using a similar procedure involving chromatography on SP Sepharose, Affi-gel blue gel, Q Sepharose and Superdex 200 [28]. The two black soybean lectins were adsorbed on SP Sepharose and Q Sepharose. Thus, the three leguminous lectins appear to have similar chromatographic behavior. A 29-fold purification was obtained in the present study for small glossy black soybean lectin compared to 10-fold purification in the case of French bean lectin [28]. Thus lectins from the two cultivars of Chinese black soybean appear to differ in subunit molecular mass, thermostability and mitogenic activity, although they share the same N-terminal amino acid sequence and carbohydrate specificity and have similar HIV-reverse transcriptase inhibitory activity. This finding is reminiscent of the observation that lectins from different cultivars of the bean *Phaseolus vulgaris*, such as the pinto bean [24], haricot bean [25], red kidney bean [30], and dark red kidney bean [26], have similar but not identical N-terminal sequences. Their other characteristics, including carbohydrate specificity and antiproliferative activity toward tumor cells, may not be all alike [24–26,30].

The results of the present investigation indicate that different soybean cultivars produce lectins that are not identical. The lectins isolated in the present study are characterized by potent antiproliferative activity toward cancer cells and inhibitory activity against HIV-1 reverse transcriptase, as well as moderate pH stability and thermostability. Its antiproliferative activity against cancer cells of mammalian origin and HIV-1 reverse transcriptase inhibitory activities indicate that it is a defense protein. This is in line with the defense function of lectins in plants [43]. As such, the biological activities of black soybean lectins are potentially exploitable in medicine.

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