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Research Article

Isolation and characterization of biologically active lectin from Korean mistletoe, *Viscum album* var. *Coloratum*

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Abstract. A mistletoe lectin was isolated from water extracts of Korean mistletoe, a subspecies of *Viscum album*, grown on *Quercus mongolica* using CM-Sepharose chromatography followed by an affinity chromatography on a concanavalin A-Sepharose column. The compound proved to be a mistletoe lectin II with D-galactose and *N*-acetyl-D-galactosamine specificity. Matrix-assisted laser desorption time-of-flight mass spectroscopy showed it to have an average molecular mass of 62.7 kDa and to consist of two subunits of 30.6 kDa and 32.5 kDa. It was a basic protein with isoelectric points of 9.4 and 9.6 by capillary isoelectric focusing and was cytotoxic to Molt4 cell.

Key words. Korean mistletoe; mistletoe lectin II (ML II); concanavalin A chromatography; MALDI-TOF.

Mistletoe is a half-parasitic plant that grows on deciduous trees all over the world. Extracts from mistletoe have been used therapeutically against various diseases, including cancer, arthrosis and cardiovascular illness. Mistletoe extracts are widely used in adjuvant cancer therapy, and seven different types of European mistletoe extracts are commercially available [1–4].

The anticancer activities of European mistletoe (*Viscum album*) might have been ascribed to high molecular weight compounds including viscotoxins [5–8], lectins [8–15] and polysaccharides [16]. Mistletoe lectins (MLs) are glycoproteins that consist of two disulfide-linked polypeptide chains and are classified according to their sugar specificities: ML I (galactose-specific), ML II (galactose- and *N*-acetylgalactosamine-specific) and ML III (*N*-acetylgalactosamine-specific) [8–15]. The composition of MLs varies depending on the host tree, the subspecies of mistletoe, the parts used and the time of harvest [15, 17–19].

Khwaja et al. [17, 20] reported that there was no ML-like cytotoxic protein in Korean mistletoe, a different subspecies of *V. album* from European mistletoe, and that the anticancer activity of Korean mistletoe might be attributable to cytotoxic alkaloids. Recently, there have been a few reports on the biological activity of Korean mistletoe [17, 20, 21]. No detailed study has been made of the isolation and characterization of ML from Korean mistletoe. Thus the purpose of this study was to isolate ML from Korean mistletoe and characterize its biochemical properties.

Materials and methods

Purification of ML II from Korean mistletoe. Korean mistletoe (*Viscum album* var. *Coloratum*) grown on *Quercus mongolica* was harvested in February 1997 from Duk Yoo Mountain, located in Muju, Chollabuk-

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do and stored at -70 °C until use. It was inspected by Professor Moo Yeol Kim, Department of Biology, Chunbook University.

The stems of Korean mistletoe were chopped into slices of approximately 5 mm and homogenized with four volumes of 10 mM phosphate buffer (pH 6.5) containing 100 mM lactose in a blender mixer. The green homogenate was filtered through cheesecloth, and the filtrate was stirred with a magnetic stirrer at 4 °C overnight. The suspension was centrifuged at 10,000*g* for 20 min, and the pellet was discarded. The supernatant was extracted three times with the same volume of ether to remove lipid. The aqueous layer was applied to a column of CM-Sepharose (1.5 cm I.D. × 20 cm, Pharmacia, Sweden) equilibrated with 10 mM phosphate buffer (pH 6.5). After washing with 10 mM phosphate buffer (pH 6.5) and 100 mM NaCl in the same buffer, a peak eluted with 500 mM NaCl in the same buffer was dialyzed with phosphate buffered saline (PBS, pH 7.4). The fractions containing hemagglutinating protein were applied to a column of concanavalin A-Sepharose (1.5 cm I.D. × 20 cm, Sigma, USA) equilibrated with PBS (pH 7.4). The column was washed with PBS (pH 7.4) and eluted with 300 mM glucose in the same buffer. Fractions containing 60-kDa protein in SDS-polyacrylamide gel electrophoresis (PAGE) were pooled, dialyzed against water and freeze-dried.

PAGE. Electrophoresis in the presence of SDS was carried out in 12% polyacrylamide gel slabs. Protein samples were treated either with or without 2-mercaptoethanol for 3 min at 95 °C, and electrophoresis was performed under a constant current of 30 mA until the marker dye, bromophenol blue, reached the bottom of the gel. Protein bands on the gel were stained with Coomassie Brilliant Blue (ICN, USA).

Matrix-assisted laser desorption time-of-flight (MALDI-TOF). The mass spectral data were acquired using a MALDI-TOF PerSeptive Voyager mass analyzer. The mass spectral samples were prepared by adding 0.2 μl aliquots of ML II samples into 1 μl of a matrix solution containing sinapinic acid at 10 mg/ml in acetonitrile/0.1% aqueous trifluoroacetic acid (7:3, v/v). The samples were irradiated with a pulsed nitrogen laser operated in the positive ion mode with a 25 kV accelerating voltage.

Capillary isoelectric focusing (CIEF). CIEF was performed on a Bio-Rad BioFocus 3000 capillary electrophoresis system using a fused-silica capillary (24 cm × 25 μm, internal diameter, I.D.) coated with linear polyacrylamide. The purified ML was mixed with Bio-Lyte pH 3/10 ampholytes at a final ampholyte concentration of 2% and centrifuged for 10 s at 5000*g*. BioMark isoelectric point (pI) markers ranging from pI 10.4 to 5.3 were used as internal standards. The sample was injected by applying pressure for 60 s at 100 psi. Focusing was performed at 15 kV constant voltage for 4 min using 20 mM phosphoric acid and 40 mM sodium hydroxide as the anolyte and catholyte, respectively. Chemical mobilization was performed at 15 kV constant voltage for another 21 min. Sample and capillary temperature were maintained at 20 and 27 °C, respectively.

Hemagglutination inhibition test. Human erythrocytes were washed twice in physiological saline solution and diluted to 10^8 erythrocytes/ml. To each well of 96-well plate, 50 μl of twofold serial dilutions of sugars were mixed with 25 μl of ML (100 μg/ml) with a hemagglutinating activity, and 25 μl of human erythrocyte suspension was added. After incubation at 37 °C for 1 h, the lowest concentration of added sugar inhibiting hemagglutination was determined.

In vitro cytotoxicity test. Molt4 cells in 96-well microtiter plates of 1×10^4 cells/well were treated with various concentrations of ML II in complete RPMI 1640 medium supplemented with 10% heat-inactivated fetal

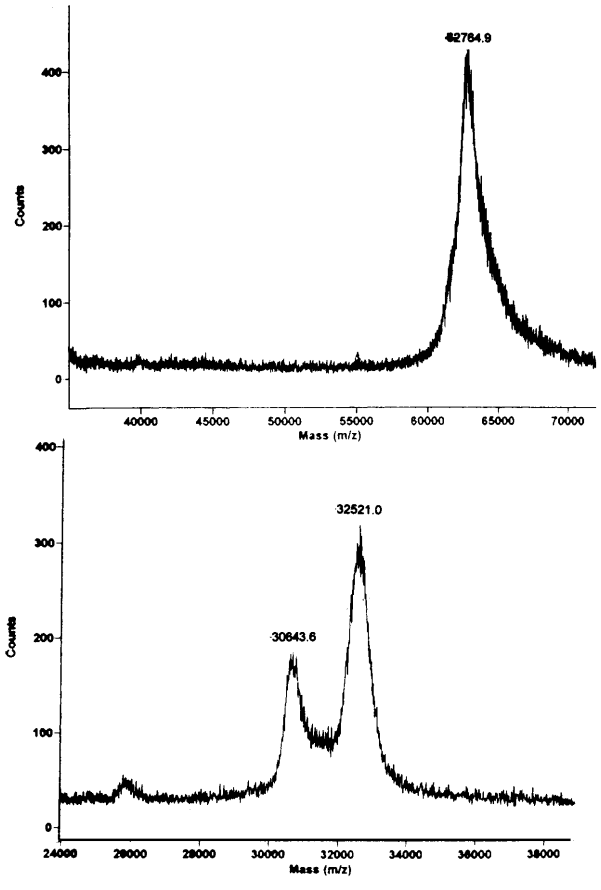

Figure 1. MALDI-TOF mass spectra of purified Korean mistletoe lectin II (*A*) and its 2-mercaptoethanol-treated fraction (*B*).

Table 1. Hemagglutination inhibition test of purified Korean mistletoe lectin II.

Sugar	Minimum sugar concentratio for inhibition (μmol/ml)
D-Galactose	10
N-Acetylgalactosamine	1.25
D-Galactosamine	–
D-Glucosamine	–
D-Glucose	–
D-Lactose	2.5

–, no inhibition.

calf serum (Gibco, USA) for 12 h at 37 °C in humidified atmospheric 5% CO_2. The number of viable cells was determined by 3-4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide (MTT) assay as previously described [22].

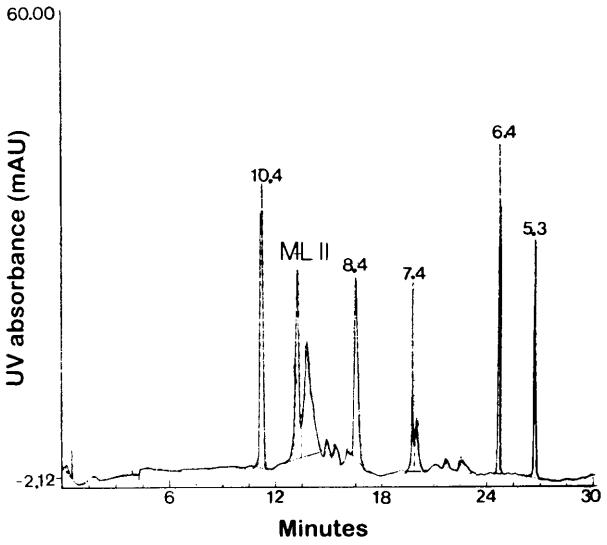

Figure 2. Capillary isoelectric focusing chromatogram of purified Korean mistletoe lectin II with pI markers.

Results and discussion

The crude extract of Korean mistletoe was first passed through a column of CM-Sepharose to which several proteins including ML were bound. The hemagglutinating fractions eluted with 500 mM NaCl in 10 mM phosphate buffer (pH 6.5) were conformed as ML-containing fractions by SDS-PAGE and purified using affinity chromatography on a concanavalin A column. Most of the protein was eluted with PBS (pH 7.4), and a peak eluted with 300 mM glucose in PBS (pH 7.4) was identified as an ML peak by SDS-PAGE under non-reducing and reducing conditions. The ML fraction isolated from Korean mistletoe showed no affinity on Sepharose 4B and very little affinity on asialofetuin column, which was not enough for separation of ML from mistletoe. D-galactose-specific ML I has predominantly been obtained from European mistletoe using affinity chromatography on Sepharose 4B [10–13] but Korean mistletoe did not contain Sepharose 4B-binding ML I apart from European mistletoe.

Hemagglutination activity of ML was < 25 μg/ml using human blood group O erythrocytes. From hemagglutination inhibition of ML by different sugars (table 1), ML purified from Korean mistletoe was specific for D-galactose and *N*-acetyl-D-galactosamine and could be regarded as ML II.

The MALDI-TOF analysis of the isolated ML II fraction and its 2-mercaptoethanol-treated fraction showed that ML II had an average m/z of 62764 (fig. 1A) and consisted of two subunits, 30643 and 32521 (fig. 1B). From SDS-PAGE analysis, the molecular weights (MWs) of ML II and two subunits were estimated as 60 kDa, 30 kDa and 34 kDa, respectively. According to Franz et al. [11], the MW of European ML II was 60 kDa, and two subunits were 32 and 27 kDa. In this study, the MW of ML II and its two subunits was correctly analyzed by MALDI-TOF for the first time.

The pIs of Korean ML II were estimated at pH 9.4 and 9.6 by CIEF (fig. 2), not in accordance with the finding of Wagner et al. [18], who reported on proteins with pI 4.4–6.8 but with the report of Vester et al. [14] on basic proteins with pI of 8–11.

The in vitro cytotoxicity of ML II on human leukemia Molt4 cells was measured by MTT assay. A 50% inhibition of cell viability was obtained with 1.66×10^{-13} M, comparable to the findings of Dietrich et al. [23].

From these results, anticancer activity of Korean mistletoe, a different subspecies of *Viscum album*, might be ascribed to ML in the same way as European mistletoe, which contradicts Khwaja et al. [17, 20], who found no ML-like cytotoxic protein. A new purification method for the isolation of ML II was investigated using cation-exchange chromatography followed by affinity chromatography with concanavalin A column.

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