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Isolation and Characterization of Lectins from Stem and Leaves of Korean Mistletoe (*Viscum album* var. *coloratum*) by Affinity Chromatography

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We attempted to isolate and characterize the lectins from stem and leaves of Korean mistletoe (*Viscum album* var. *coloratum*) by affinity chromatography. Lectin I was isolated only from stem. Lectin II was not isolated from Korean mistletoe, whereas lectin III was isolated from the stem and leaves. The hemagglutinating activity of lectin I was 16HU and inhibited by D-galactose, lactose, and N-acetyl-D-galactosamine. The lectin I has molecular weight of 60,000D being composed of two basic subunits with molecular weights of 32,000D and 28,000D which are linked by a disufide bond. The lectin III from stem has molecular weight of 66,000D being two basic subunits which have molecular weights of 34,000D and 29,000D and are linked by a disufide bond. The activity of lectin I was stable at the pH range of 4.00~8.50 and at a wide range of temperature (0~42°C). The lectin I showed more potent mitogenic activity to murine lymphocytes than concanavalin A.

Key words : Hemagglutinating activity, Lectin, Korean mistletoe, Mitogenic, Affinity chromatography

INTRODUCTION

Mistletoe is a common semiparasistic plant which grows on deciduous trees all over the world. Since the early twenties, an aqueous extract of European mistletoe (*Viscum album,* L) has been used for the treatment of cancer under the trade name of Iscador (Evans *et al.*, 1973; Ribereau-Gayon *et al.*, 1986, Jung *et al.*, 1990). The anticancer activities of mistletoe have been ascribed to a combination of cytotoxic and immunological effects. Mistletoe treatments, unlike cytotoxic drugs, are not immunosuppressive (Bloksma *et al.*, 1979; Khwaja *et al.*, 1981). Other work also suggests that lectins from mistletoe have not only cytotoxic properties but also immunostimulant properties in low concentrations. A possible correlation between immunomodulation and antitumor activity has been discussed and it has been the subject of clinical studies (Pfüller *et al.*, 1993).

It is well established that mistletoe contains a toxic lectin which is structurally and functionally related to abrin and ricin. Evidence has been presented that lectins from mistletoe, like other plant toxins, consist of two polypeptide chains, the "A chain and B chain", linked by disufides. The B chain has a sugar binding affinity to galactose and N-acetylgalactosamine groups. The A chain which is the toxic principle blocks protein synthesis by inactivating the 60S subunit of ribosome (Stirpe *et al.*, 1980; Franz, 1986).

It is reported that European mistletoe (*Viscum album*) contains three toxic lectins, ML I (mistletoe lectin I), ML II and ML III (Franz *et al.*, 1986; Dietrich, *et al.*, 1992; Jäggy, *et al.*, 1995). ML I is mainly specific for D-galactose, ML III for N-acetylgalactosamine, and ML II for both sugars. ML I, also called viscumin, increases the production of tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1) and IL-6 by mononuclear cells. It has been proposed that the biological activity of mistletoe preparations is due to ML I. The mechanism of the toxic effect of ML I is identical with that of ricin. First, ML I binds to the cell surface through the interaction with its B chain and sugars which leads to endocytosis of the lectin into the cell, and enzymatic inhibition of ribosomes by the A chain occurs (Metzner, *et al.*, 1985).

While a number of investigations concerning the European mistletoe (*Viscum album*) have been reported, few studies of Koeran mistletoe (*Viscum album* var. *coloratum*) have been performed (Manjikian *et al.*, 1986; Khwaja *et al.*, 1986; Park *et al.*, 1994a, 1994b, 1995). In this study, we attempted to isolate the lectin components from leaves and stem of Korean mistletoe by affinity chromatography and characterize their properties.

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MATERIALS AND METHODS

Crude extract preparation

For the isolation of lectins Korean mistletoe (*Viscum album* var. *coloratum*) grown on oaks was harvested in winter and identified by professor Sanghak Doh in Dong-Duck Women's University. The sample was stored at -20°C until use. The sample 100 g (frozen leaves and stem respectively) was crushed and pressed between two rollers going in opposite directions by vegetable juicer miller adding 500 ml of 50 mM borax buffer (pH 8.0). The juice was precipitated with saturated $(NH_4)_2SO_4$ followed by centrifugation at 8000 rpm for 20 min. Precipitate was collected and dialyzed against 50 mM borax buffer (pH 8.0) and centrifuged at 8000 rpm for 20 min. The supernatant was concentrated using Amicon ultrafiltration kit and lyophilized. The protein content of the sample was estimated by BCA assay.

Isolation of lectins I, II and III

The isolation procedure of lectins I, II, and III is shown in Scheme 1. Crude extract (0.2 g) was solubilized with 0.15 M NaCl and used as a starting material. Lectin I was isolated by eluting 0.2 M D-galactose using acid-treated Sepharose 4B as carrier (Ziska *et al.*, 1978; Franz *et al.*, 1981). The non-adsorbed material was applied to a column (2.6×30 cm) of immunoglobulin-Sepharose 4B and the column was washed with 0.15 M NaCl. The affinity adsorbent had been prepared by coupling human immunoglobulin G to CNBr-activated Sepharose 4B (20 mg/ml of gel). Lectin II was eluted with 0.2 M-D-galactose in 0.15 M NaCl. The fractions containing the hemagglutinating protein were pooled, dialyzed against water. After the lectin II had been displaced from the column, lectin III was eluted with 0.2 M glycine/HCl buffer at pH 2.6. The fractions containing active materials were pooled and neutralized with Na_2CO_3.

Mistletoe (100 g) ground and homogenized
with 500 ml of 50 mM borax buffer, pH 8.0
↓ → Centrifuge and discard precipitate
Supernatant
↓ → Precipitate with sat. $(NH_4)_2SO_4$ and centrifuge
Precipitate
↓ → Dialyze against 50 mM borax buffer,
pH 8.0 and centrifuge
Supernatant
↓ → Freeze-dried
Crude extract dialyzed against 0.15 M NaCl
↓
Acid-treated Sepharose 4B column washed
with 0.15 M NaCl
↓ → Elute with 0.2 M D-galactose → Lectin I
Effluent
↓
Immunoglobulin-Sepharose 4B column washed
with 0.15 M NaCl
↓ → Elute with 0.2 M D-galactose → Lectin II
Elute with 0.2 M glycine/HCl, pH 2.6
↓
Neutralized
↓
Lectin III

Scheme 1. Flow sheet for the isolation of mistletoe lectins I, II, and III.

Hemagglutination test

The lectin solution was dialyzed to remove remaining sugar and hemagglutination test was performed by a serial two-fold dilution method using a microtiter plate. Red blood cells were washed and resuspended in saline to a 3% suspension. 50 μl of red blood cell suspension was added to the 50 μl of lectin solutions in a serial dilution and the plate was incubated at 37°C for 1 h. The definition of hemagglutination unit (HU) is the maximum number of a serial two-fold dilution of lectin solution showing hemagglutinating activity.

To measure the inhibition of hemagglutination by sugars or sugar derivatives, various concentrations of sugars in saline (using a two-fold dilution method) were added to the 50 μl of lectin solution showing hemaggluting activity of 2 units. After incubation of the sugar and lectin solution for 1 h at 4°C, human red blood cells (3% suspension) were added to the incubation mixture and incubated for 1 h at 37°C.

To measure the effects of pH on the hemagglutinating activities of lectin, the pH of lectin solutions showing 2 HU was adjusted to 2.10~10.20 and incubated for 2.5 h at 4°C. The effects of temperature on the hemagglutinating activities of lectin were also measured by incubating the lectin showing 2HU at 0~80°C for 30 min. The residual hemagglutinating activities were examined.

SDS-PAGE and molecular weight determination

Purity check and molecular weight determination were performed by discontinuous system, 8% polyacrylamide gels were used as separating gel and 5% as stacking gel. The samples were dialyzed to eliminate residual amounts of sugar and treated with 1% SDS and 1% β-mercaptoethanol (Weber and Osborn, 1969) when necessary. Staining was performed with Coomassie brilliant blue R-250. Silver staining method was also performed when necessary. The results were analyzed by Bio-1D program on image analysis system (Vilber-Lourmat).

Amino acid analysis

Concentrated 2 ml of sample was hydrolyzed with 2

ml of 6N HCl in nitrogen-filled tubes for 24h at 110°C and analyzed with amino acid analyzer (Beckmann 6300 system).

Lymphocyte stimulating activity test

Preparation of mouse lymphocyte was carried out as described (Hunt, 1987). The spleens of ICR mouse were removed and put in a petri dish containing a few ml of Dulbecco's medium (PBS containing 2% bovine serum). The spleen were cut into fragments and gently disrupted. The suspension was filtered through a very loose pledget of absorbant cotton and the absorbant was washed through with a few ml of fresh medium. Erythrocytes were removed by 0.83% (w/v) ammonium chloride treatment. Most of macrophages and granulocytes were also removed by drawing the cell suspension into a syringe which had been loosely prepared with glass wool. Dye exclusion tests (Trypan blue, 0.5%) for viable test were routinely performed.

Lymphocyte stimulating activity test was carried out as described (Pandolfino *et al.*, 1983). The lectin solution was filtered through a sterilized filter paper (Millipore, 0.22 m). The medium was prepared with RPMI 1640 (pH 8.0) containing 25 mM HEPES and 1.4 μl/ml gentamicin. The lymphocyte was incubated in duplicate in the U-shaped microtiter plate. The prepared lymphocytes (100 μl, 2.4×10^6 cells/ml) and fetal bovine serum (100 μl) were added to the lectin solution (100 μl) in two-fold serial dilution. After incubation in CO_2 incubator (95% air:5% CO_2 at 37°C) for 48 hr and MTT and SRB assay were performed.

RESULTS AND DISCUSSION

Isolation and purification of lectin

It is reported that three lectins, ML I, ML II and ML III were isolated mainly from leaves of European mistletoe by using affinity chromatography with different carriers. ML I was isolated by eluting with 0.2M D-galactose using acid-treated Sepharose 4B. And ML II was isolated by eluting with 0.2M D-galactose using immunoglobulin G-Sepharose 4B. After the ML II had been displaced from the column, ML III was eluted with 0.2M glycine/HCl buffer (pH 2.6). We also attempted to isolate three different lectins (lectin I, II and III) from the crude extracts of sample (Korean mistletoe) by eluting with same buffer using same carrier. Lectin I was able to be isolated from the stem of the sample (Fig. 1), but was not able to be isolated from the leaves. Lectin III was isolated from both of the stem and leaves (Fig. 2, 3). Lectin II was not isolated from the sample.

Compared with European mistletoe, where considerable amount of three lectins was purified from leaves, it is assumed that Korean mistletoe has different lectin distribution from those of European mistletoe.

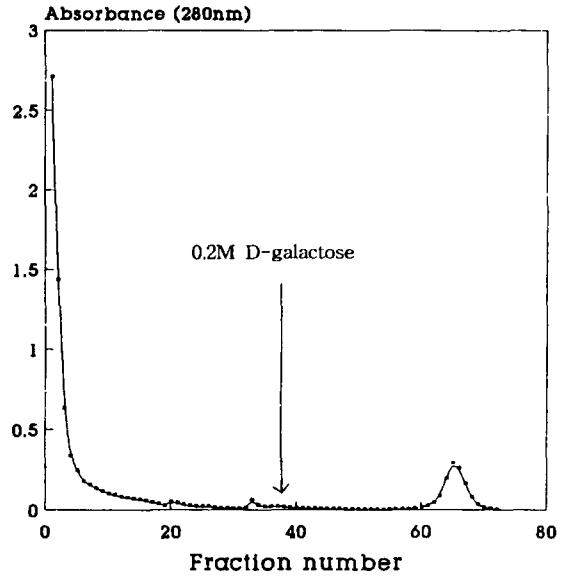

Fig. 1. Affinity chromatogram of lectin I from mistletoe stem by eluting 0.2M D-galactose on acid-treated Sepharose 4B.

The protein content was measured by BCA assay. The protein content was 566 μg/ml in lectin I, 78 μg/ml in lectin III from the stem and 65 μg/ml in lectin III from the leaves.

The isolated lectins had no blood group specificities of human blood type of A, B, O and AB. The hemagglutinating activity of lectin I was 16 HU and the activities of lectin III from leaves and stem were 2

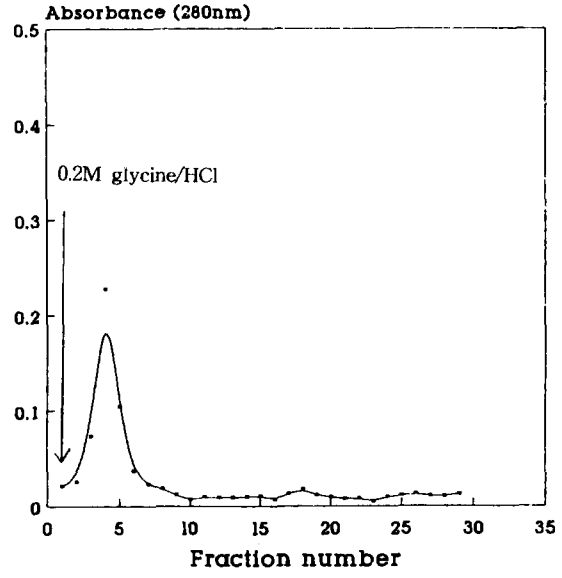

Fig. 2. Affinity chromatogram of lectin III from mistletoe stem by eluting 0.2M glycine/HCl on immunoglobulin-Sepharose 4B.

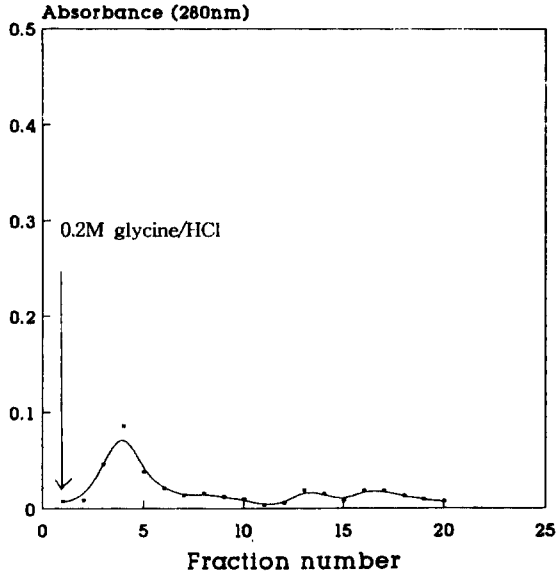

Fig. 3. Affinity chromatogram of lectin III from mistletoe leaves by eluting 0.2M glycine/HCl on immunoglobulin-Sepharose 4B.

SDS-PAGE showed a resemblance between lectin I from Korean mistletoe and ML II from European mistletoe. Lectin III has N-acetyl-D-galactosamine specificity, which was identical with that of ML III from European mistletoe.

SDS-PAGE and molecular weight determination

Investigations using SDS-PAGE (sodium dodecyl sulfate/polyacrylamide gel electrophoresis) in the absence and presence of reducing agent (2-mercaptoethanol) showed that the lectin I from the stem of Korean mistletoe consists of two different chains connected by disulfide bonds (Fig. 4). In the absence of reducing agent, a major band corresponding to M_r=60,000 and two minor bands corresponding to M_r=30,000 and 26,000 were found (Fig. 4, lane 7). In the presence of 2-mecaptoethanol (lane 6), only two bands corresponding to M_r=32,000 and M_r=26,000 were found. Comparison of the panels shows that lighter chain migrated at the same rate in the absence and presence of reducing agent. In the reduced sample there was no band migrating corresponding to M_r=30,000. Since no new bands with a more rapid migration rate appeared in the gel and the densities of two bands corresponding to M_r=32,000 and M_r=26,000 measured by image analysis system have increased, it is most likely that material migrating corresponding to M_r=30,000 in the unreduced sample was present in the band migrating corresponding to M_r=32,000 in the reduced sample. Such a decrease in the migration rate could be due to reduction of one or more intrachain disufide bridges and a corresponding unfolding of the polypeptide chain. We reported that the molecular weight of mistletoe

Table I. Inhibition of hemagglutinating activities (concentration, M/mL) of lectin I from stem, lectin III_a from leaves, and lectin III^b from stem of Korean mistletoe by sugars. The inhibition concentrations are expressed as M of carbohydrate/ml needed for complete inhibition of 2HU

Sugars	Lectin I	Lectin III_a	Lectin III_b
D-galactose	12.5	-	-
N-acetyl-D-galactosamine	3.2	6.3	6.3
D-galactosamine	-	-	-
D-glucose	-	-	-
D-(+)-mannose	-	-	-
Lactose	6.3	-	-
L-(+)-arabinose	-	-	-

HU respectively.

Sugar specificities

Hemagglutinating activity of lectin I from the stem of Korean mistletoe was inhibited by D-galactose, N-acetyl-D-galactosamine and lactose, whereas lectin I from European mistletoe has been reported to be inhibited only by D-galactose (Franz *et al.* 1981). The most effective inhibitor for lectin I was N-acetyl-D-galactosamine (Table I). From these results, it is assumed that the lectin I from Korean mistletoe has specificities for D-galactose configuration. But another assumption related to acetyl group can be deduced from the result that the hemagglutinating activity of lectin I from Korean mistletoe was not inhibited by D-galactosamine. It is quite interesting that the sugar specificity of lectin I from Korean mistletoe is similar to those of ML II from European mistletoe which has D-galactose and N-acetyl-D-galactosamine specificities. It is noteworthy that we failed to isolate lectin II, and

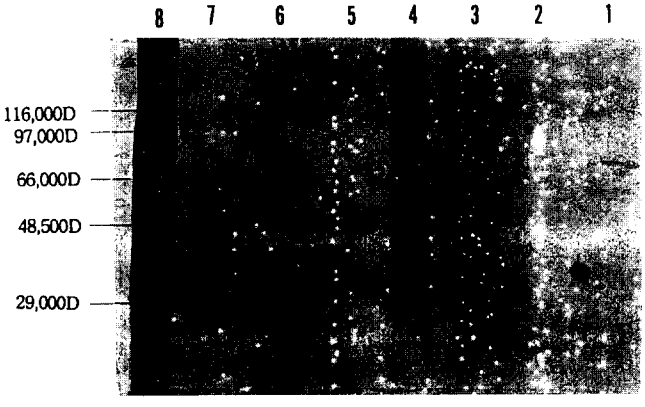

Fig. 4. SDS-PAGE patterns of mistletoe lectins. lane 1: lectin III from mistletoe leaves treated with SDS and 2-mercaptoethanol, lane 2: lectin III from mistletoe leaves treated with SDS, lane 3: lectin III from mistletoe stem treated with SDS and 2-mercaptoethanol, lane 4: lectin III from mistletoe stem treated with SDS, lane 6: lectin I from mistletoe stem treated with SDS and 2-mercaptoethanol, lane 7: lectin I from mistletoe stem treated with SDS, lane 8: molecular weight marker

lectin purified by gel filtration chromatography was 60,000 daltons and it was composed of two sununits (M_r=33,000 and M^r=28,000) (Park *et al.* 1994a), but it is not clear whether the lectin isolated by gel filtration is identical with the lectin I isolated in this study.

It is reported that ML I from European mistletoe at higher concentrations forms a dimer (molecular weight 115,000 daltons) and that the dimerization may be due to hydrophobic interactions of the chains (Franz *et al.*, 1986). The monomer of ML I at lower concentrations is composed of two chains linked by disulfide bond and the molecular weights of each chain of ML I were 34,000 and 29,000 daltons. ML II (molecular weight 60,000 daltons) is composed of two subunits which molecular weights were 32,000 and 27,000 daltons respectively. It is noteworthy that the molecular weights of lectin I from Korean mistletoe and ML II from European mistletoe are identical and that the result is in agreement with those of sugar specificities.

It should be noted that in all cases the bands of the lectin III from stem somewhat diffused, possibly indicating microheterogeneity. In the absence of 2-mercaptoethanol, there were a diffused band corresponding to M_r=66,000 and two weak bands migrating corresponding to M^r=34,000 and 29,000 (Fig. 4, lane 4), whereas after treatment with reducing agent, more distinct two bands (M_r=34,000 and 29,000) and bands with high molecular weight (M_r=100,000, 66,000 and 53,500) were found (lane 3). It is not certain that these results come from the imputrity of the lectin III isolated. The lectin III from leaves shows two bands migrating corresponding to M_r=79,000 and M_r=62,000 (lane 1) in the presense of reducing agent, whereas the bands of the lectin III from leaves are too indistinct to detect in the absence of the reducing agent. The ML III (molecular weight 50,000 daltons) from European mistletoe consist of two different chains connected by disulfide bonds and the molecular weights of each chain are 30,000 and 25,000 daltons, which are quite different from the molecular weights of lectin III from Korean mistletoe.

Effects of pH and temperature on the hemagglutinating activity

Fig. 5 shows the effects of pH on the hemagglutinating activity of lectin I. The activity of lectin I was stable at the pH range of 4.00~8.50 (the erythrocyte was unstable below pH 3.90) and decreased to 50% at pH 9.50 and disappeared completely at pH 10.25.

The hemagglutinating activity of lectin I was stable at an wide range of temperature (0~42°C) (Fig. 6). Half of the activity was maintained at 45°C, but the activity disappeared over 50°C. The results of effects of pH and temperature are in accordance with the results obtained by gel and ion exchange chromatography (Park *et al ., 1994a).*

The effects of pH and temperature on the hemagglutinating activity of lectin III are similar to those of lectin I (data are not shown).

Amino acid composition

Table II shows the amino acid composition of lectin I. The major amino acids were aspartic acid, glutamic acid, leucine, and arginine. The histidine, which

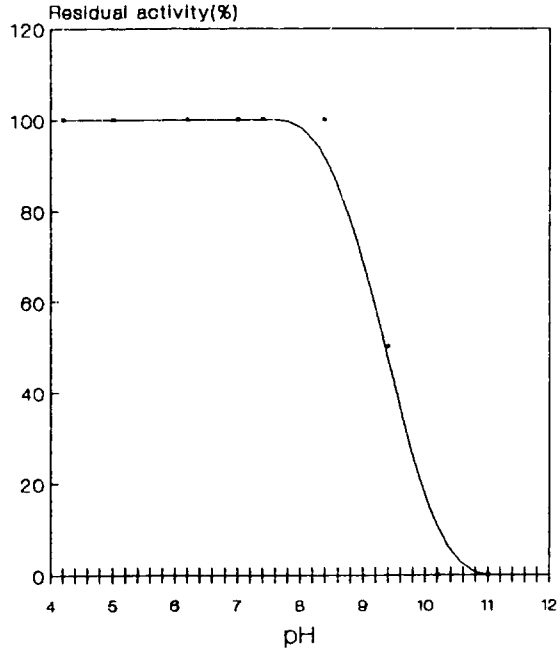

Fig. 5. Effect of pH on hemagglutinating activity of mistletoe lectin I.

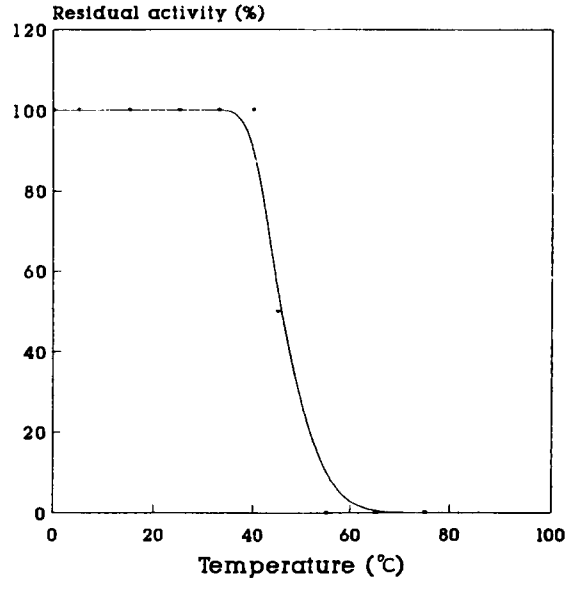

Fig. 6. Effect of temperature on hemagglutinating activity of mistletoe lectin I.

Table II. Amino acid composition (g/g%) of lectin I from mistletoe stem

Amino acids	Percentages
Aspartic acid	13.82
Threonine	7.33
Serine	8.49
Glutamic acid	14.15
Proline	4.87
Glycine	6.64
Alanine	5.48
Cystine	-
Valine	5.41
Methionine	-
Isoleucine	7.06
Leucine	11.10
Tyrosine	-
Phenyl alanine	2.29
Histidine	1.07
Lysine	2.14
Arginine	10.05

Table III. Lymphocyte stimulating activity of lectin I from mistletoe stem (unit : OD)

Mitogen	MTT assay	SRB assay
Con A	0.201	2.235
Lectin I	0.238	2.395

was the most abundant amino acid in the results reported (Park *et al.*, 1994a), was minor amino acid. We did not attempt to analyze the amino acid composition of lectin III because of possibility of their impurity.

Lymphocyte stimulating activity

Since mitogenic activity of phytohemagglutinin (PHA, the lectin from *Phaseolus vulgaris*, red kidney bean) has been reported (Nowell, 1960), a number of mitogenic lectins have been reported. Many of them have been obtained in purified form and their various properties were clarified (Sharon, 1984). Various biological effects of lectins on subpopulations (T and B cells) of lymphcytes were reported (Liener *et al.*, 1986, Pfuller *et al.*, 1993). The mitogenic activity of lectin is also significant in using the lymphokines such as interleukin-2 (IL-2) and γ-interferon produced during the mitogenic process (Liener *et al.*, 1986).

The lectin I was more mitogenic to murine lymphocytes than concanavalin A (Con A) (Table III).

REFERENCES CITED

Bloksma, N., S van Dijk, H., Korst, P. and Willers, J. M., Cellular and humoral adjuvant activity of a mistletoe extract. *Immunobiology*, 156, 309-319 (1979).

Dietrich, J. B., Ribereau-Gayon, G., Jung, M. L., Franz, H., Beck, J. P. and Anton, R., Identity of the mistletoe (*Viscum album* L.) lectins : Homology with ricin-like plant toxins and single-chain ribosome-inhibiting proteins. *Anticancer Drugs*, 3, 507-511 (1992).

Evans, M. R. and Preece, A. W., *Viscum album*-a possible treatment for cancer? *Bristol Med. Chir. J.* 88, 17-20 (1973).

Franz, H., Ziska P. and Kindt A., Isolation and properties of three lectins from mistletoe (*Viscum album*). *Biochem. J.*, 195, 481-484 (1981).

Franz, H., Mistletoe lectins and their A and B chains. *Oncology*, 43 (suppl. 1), 23-34 (1986).

Hunt. S., Preparation of lymphocytes and accessory cells. in Hunt S.(Ed.), *Lymphocytes*. IRL Press, 1987, pp. 16-18.

Jäggy, C., Urech, M. K. and Schaller G., Quantitative determination of lectins in mistletoe preparations. *Drug Res.*, 45, 905-909 (1995).

Jung, M. L., Baudino, S. and Beck J-P., Charaterization of cytotoxic proteins from mistletoe (*Viscum album*). *Cancer Lett.*, 51, 103-108 (1990).

Khwaja T. A., Dias C. B., Papoian, T. and Pentecost, S., Studies on cytotoxic and immunological effects of *Viscum album* (mistletoe). *Proc. Am. Assoc. Cancer Res.*, 22, 253 (1981).

Khwaja, T. A., Dias C. B. and Pentecost S., Recent studies on the anticancer activities of mistletoe (*Viscum album*) and its alkaloids. *Oncology*, 43 (suppl. 1), 42-50 (1986).

Liner, I. E., Sharon, N. and Goldstein, I. J., *The Lectins*, Academic Press. N.Y., 1986.

Manjikian, S., Pentecost, S. and Khwaja T. A., Isolation of cytotoxic proteins from *Viscum album coloratum*. *Proc. Am. Assoc. Cancer Res.*, 27 (1986).

Metzner, G., Franz, H., Kindt, A., Fahlbush, B. and S ss, J., The in vitro activity of lectin I from mistletoe (ML I) and its isolated A- and B- chains on functions of macrophages and polymorphonuclear cells. *Immunology*, 169, 461-471 (1985).

Pandolfino, E. R., Namen, A. E., Munske G. R. and Magnuson, J. A., A comparison of the mitogenic and nonmitogenic lectins from lima beans. *J. Biol. Chem.* 258, 9203-9207 (1983).

Park, W. B. and Kim, H. S., Isolation and characterization of lectin from *Viscum coloratum*. *Yakhak Hoeji*, 38, 418-424 (1994a).

Park, W. B. and Kim, H. S., Changes of lectin from *Vscum coloratum* by fermentation with *Lactobacillus plantarum*-Isolation and purification. *Yakhak Hoeji*, 38, 687-695 (1994b).

Park, W. B., Kim, H. S., Na, H. B. and Ham S. S., Changes of lectin from *Viscum coloratum* by fermentation with *Lactobacillus plantarum*-Effects of pH and temperature, sugar specificity and lymphocyte stimulating activity. *Yakhak Hoeji*, 39, 24-30 (1995).

Püfller, U., Kopp, J., Körner, Zwanzig, M., Göc keritz, W. and Franz, H., Immunotoxins with mistletoe lectin 1 A- chain directed against interleukin-2 receptor of human lymphocytes: comparison of efficiency and specificity. *Biology, Biochemistry, and Clinical Biochemistry,* 8, 34-40 (1993).

Ribereau-Gayon, G., Jung, M. L., Scala, D. D. and Beck J. P., Comparison of the effects of fermented and unfermented mistletoe preparations of cultured tumor cell. *Oncology,* 43 (suppl. 1), 35-41 (1986).

Sharon, N., *Mitogens in Immunology,* Academic Press, N.Y., 1989.

Stirpe, F., Legg, R. F., Onyon, L. J., Ziska, P. and Franz, H., Inhibition of protein synthesis by a toxic lectin from *Viscum album Biochem. J.,* 190, 843-845 (1980).