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Isolation and Characterization of β-Galactoside Specific Lectin from Korean Mistletoe (*Viscum album* var. *coloratum*) with Lactose-BSA-Sepharose 4B and Changes of Lectin Conformation

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Lectins and its A- and B-chains from Korean mistletoe (*Viscum album* var. *coloratum*) were isolated by affinity chromatography on the Sepharose 4B modified by lactose-BSA conjugate synthesized by reductive amination of ligand (lactose) to ε-amino groups of lysine residues of spacer (BSA) after reduction by $NaCNBH_3$. The lactose-BSA conjugate was coupled to Sepharose 4B activated by cyanogen bromide. The molecular weight determined by SDS-PAGE were a 31 kD of A-chain and a 35kD of B-chain. Amino acid analysis and *N*-terminal sequencing were performed. The effects of pH, temperature and guanidine chloride on the conformation of the lectin were investigated by measuring its intrinsic fluorescence and compared with its hemagglutinating activities. Blue shift was detected on the acidic pH and there was a close relationship between activities and conformation of the lectin. Under denaturing conditions, the tryptophan emission profile of lectin showed typical denaturational red shift which also correspond to the conformations and activity of lectin.

Key words : Lectin, Mistletoe, *Viscum album* var. *coloratum*, Affinity chromatography , Conformation

INTRODUCTION

Aqueous European mistletoe (*Viscum album* L., VAL) preparation has been used in adjuvant cancer therapy with both immunostimulatory at low concentrations and cytostatic/cytotoxic properties at higher concentrations (Büssing *et al.*, 1996, Evans *et al.*, 1973, Jung *et al.*, 1990). VAL has been reported to stimulate the immune system nonspecifically, as it increases the number and activity of natural killer cells and neutrophils (Hajto *et al.*, 1989, 1990, Schultze *et al.*, 1991), induces cytokines such as tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), interleukin-1 (IL-1) and IL-6 (Hajto *et al.*, 1990, Schultze *et al.*, 1991, Müller *et al.*, 1990) and possess cytostatic and cytotoxic activities on both cultured tumor cells and human lymphocytes (Büssing *et al.*). It has been also reported that another effect of VAL could be a DNA stabilizing property which seems to be restricted to the mononuclear cells of the peripheral blood at low concentrations. And even after treatment with the alkylating substance cyclophosphamide, VAL was protective against the effects of this drug *in vitro* (Büssing *et al.*, 1995).

VAL contains various toxic and nontoxic proteins, alkaloids, flavonoids and polysaccharides (Pfüller *et al.*, 1993, Ribéreau-Gayon, *et al.*, 1986, 1993). One major fraction of proteins in mistletoe extracts consists of three structually similar lectins (ML I, ML II and ML III) having molecular masses ranging from 50 to 60 kD (Franz *et al.*, 1986, Dietrich *et al.*, 1992 a,b). Each of them consists of two subunits (A and B chains) covalently linked by disulfide bridges. Chain B has carbohydrate-binding properties whereas chain A can inhibit protein synthesis in cells via irreversible inactivation of the 60S ribosomal subunits as structually similar β-galactose-specific plant lectins do (Stirpe *et al.*, 1980, Franz 1986). ML I has a specificity for β-galactosyl-residues. ML III is specific for N-acetyl-D-galactosamine residues, whereas ML II interacts with both N-acetyl-D-galactosamine- and β-galactosyl-residues. 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. 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 inhibi-

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tion 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 Korean mistletoe (*Viscum album* var. *coloratum*) have been performed (Manjikian *et al.*, 1986; Khwaja *et al.*, 1981, 1986; Park *et al.*, 1994a, 1994b, 1995, 1997). Although several methods have been proven useful for lectin isolation, affinity chromatography provides a powerful tool for isolation of carbohydrate-binding proteins. However, the use of different affinity ligands for purification of lectins has shown that such changes can yield qualitative and quantitative differences. Thus rational selection of the affinity ligands is a prerequisite for optimal lectin recovery. To establish methods for isolation of Korean mistletoe lectins, affinity matrices were prepared by combination of ligands and spacers and compared with their ability to purify lectins, and A-chain and B-chain of lectin were isolated and characterized. The conformational changes of mistletoe lectin were also investigated (under denaturating conditions).

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 the sample was stored at -20°C until use. The sample (100 g) was crushed and pressed between two rollers going in opposite directions by vegetable juicer miller adding 500 ml of 0.15 M NaCl solution. The juice was precipitated with saturated $(NH_4)_2SO_4$ followed by centrifugation at 8,000 rpm for 20 min. The precipitate was collected and dialyzed against 0.15 M NaCl solution and centrifugated at 4000 rpm for 10 min. The supernatant was used as crude extract.

Preparation of lactose-BSA-Sepharose 4B

The preparation procedure of lactose-BSA-Sepahrose 4B is shown in Fig. 1 and 2. BSA (bovine serum albumin, 68 μg), lactose (100 mg), and sodium cyanoborohydride (100 mg) were dissolved in 5.0 ml of 0.2 M potassium phosphate buffer (pH 7.0) and incubated for 10 days at 37°C. The reaction mixture was applied to the Sephadex G-25 column (2.5×45 cm) in 0.1 M potassium phosphate buffer (pH 7.0) containing 0.02% sodium azide. Fractions containing lactose-BSA conjugate were detected by absorbance at 280 nm, pooled and dialyzed extensively against distilled water. These fractions were concentrated using Amicon ultrafiltration kit. The protein content was estimated by the BCA (bicinchoninic acid) assay.

The required amount of freeze-dried CNBr-activated Sepharose 4B powder was suspended in 1 mM of ice-cold HCl. The swollen gel was washed on a sintered glass filter with the same solution. And the gel was transferred to a 0.1M bicarbonated coupling buffer solution (in 0.5 M NaCl, pH 8.3) containing BSA-lactose conjugates. The mixture containing BSA-lactose conjugates and swollen gel were rotated gently overnight at 4°C. To block excess active group on the gel after coupling, the gel was transferred to the buffer with blocking agent, 0.2 M glycine (in 0.5 M NaCl, pH 8.0) and incubated overnight at 4°C. To remove the excess uncoupled lactose-BSA conjugate remained after coupling, the adsorbent was washed alternately with high and low pH buffer solutions, 0.1 M acetate buffer (pH 4.0) and coupling buffer (pH 8.3) containing 0.5 M NaCl several times.

Isolation of lectin and its A- and B-chain

Crude extract (0.2 g) was loaded on the lactose-BSA-Sepharose 4B and washed with 0.15 M NaCl. The column bound lectin was isolated by eluting 0.2 M lactose or was reduced to A- and B-chain by incubating with 5% 2-mercaptoethanol at room temperature overnight. A-chain was eluted with 0.15 M NaCl and the effluent was loaded to a second column of the lactose-BSA-Sepharose 4B to eliminate residual B-chain. B-chain bound to the column was eluted with 0.2 M lactose. The fractions were concentrated and purified by ultrafiltration (Amicon, MW cut off =10,000). The protein contents of the samples were measured by the BCA assay.

SDS-PAGE and molecular weight determination

Purity check and molecular weight determination were performed by discontinuous system, 8% polyacrylamide gel was used as separating gel and 5% as stacking gel.

The samples were treated with 1% 2-mercaptoethanol when necessary. Staining was performed with Coomassie brilliant blue R-250. The results were analyzed by Bio-1D program on image analysis system.

Carbohydrate assay

The carbohydrate contents of A- and B-chain of lectin were measured by anthrone assay and the ratio of carbohydrate content to the protein content was calculated.

Amino acid analysis

Concentrated 2 ml of the samples were hydrolyzed with 2 ml of 6N HCl in nitrogen-filled tubes for 24 hours at 110°C and centrifugated to remove precipitate. The supernatant was dried for 15 hours at 80°C. These samples were dissolved with 1 ml of 0.08 M so-

dium citrate buffer (in 0.2 N HCl) and analyzed with amino acid analyzer.

N-Terminal amino acid sequencing

The B-chain of lectin was separated by SDS-PAGE under reducing conditions and then electroblotted onto polyvinylidene difluoride membranes. Picomole quantities of mistletoe lectin B-chain were then sequenced in a protein sequencer (Applied Biosystems Procise Sequencer).

Hemagglutination

Hemagglutinating 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 the red blood cell suspension was added to the 50 μl of crude extract solution in a serial dilution and the plate was incubated at 37°C for 1 hour. The definition of hemagglutinating unit (HU) is the maximum number of a serial two-fold dilution of lectin solution showing hemagglutinating activity. The same method was performed with lectins and their A- and B-chain respectively.

Sugar specificity

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 crude extract solution showing hemagglutinating activity of 4HU. After incubation of the sugar and crude extract solution for 1 hour at 4°C, human red blood cells (3% suspension) were added to the incubation mixture and incubated for 1 hour at 37°C.

Fluorescence

Fluorescence was measured with Hitachi F-2000 spectrophotometer and was excited by light from a xenon lamp at 280 nm with a standard quartz cuvette (1×1cm). The absolute values of the fluorescence quantum yield were determined by assuming that the quantum yield of tryptophan in neutral water solutions is equal to 0.2 at 25°C.

RESULTS AND DISCUSSION

Sugar specificities of crude extract

The crude extract of the sample had no blood group specificities of human blood type of A, B, O and AB and the hemagglutinating activity of the crude extract was 516HU.

Sugar specificities of the crude extract preparation were investigated to prepare affinity chromatography. Hemagglutinating activity of the crude extract was inhibited by D-galactose, N-acetyl-D-galactosamine, lactose and L-(+)-arabinose (Table I). The most effective inhibitor was N-acetyl-D-galactosamine which was similar to lectin I isolated from Korean mistletoe by affinity chromatography on the acid-treated Sepharose 4B (Park *et al.* 1997). It is assumed that the major lectin in Korean mistletoe has specificities for D-galactose configuration. But another assumption related to acetyl group can be deduced from the results that the hemagglutinating activity of the crude extract was not inhibited by D-galactosamine.

Table I. The inhibition of hemagglutinating activity (concentration, μM) of crude extract from Korean mistletoe by sugars. The inhibition concentration was expressed as μM carbohydrate needed for complete inhibition of 4HU

Sugars	crude extract of mistletoe (unit : μM)
D-galactose	25
N-acetyl-D-galactosamine	3.1
D-galactosamine	-
D-glucose	-
D-(+)-mannose	-
Lactose	12.5
L-(+)-arabinose	50

Preparation of lactose-BSA-Sepharose 4B and isolation and identification of lectin and its A-and B-chain

Lectin from Korean mistletoe had so high affinity to *N*-acetyl-D-galactosamine that it was too difficult to isolate lectin from the column modified by *N*-acetyl-D-galactosamine. As a consequence lactose was chosen as a ligand of affinity chromatography column. Lactose-BSA was synthesized by reductive amination of ligand (lactose) to ε-amino groups of lysine residues of BSA (spacer) after reduction by $NaBH_3CN$ and it was purified by chromatography on Sephadex G-25 (chromatogram is not shown). And Lactose-BSA (neoglycoprotein) was coupled to CNBr-activated Sepharose 4B.

Lectin was isolated by eluting with 0.2 M lactose using lactose-BSA- Sepharose 4B (Fig. 3). The A- and B-chains of lectin were separated by using the same column, A-chain passed through the column together with 2-mercaptoethanol and the remaining B-chain was eluted with 0.2 M lactose (Fig. 4, 5). 22.6 μg/g of lectin, 6.9 μg/g of A-chain and 3.1 μg/g of B-chain were isolated from the sample. The carbohydrate in the samples of B-chain was not detected by anthrone test. The hemaglutinating activities of lectin and B-chain were 32HU and 8HU respectively. A-chain did not show any hemaglutinating activity. From these results, we assumed that the carbohydrate moiety of

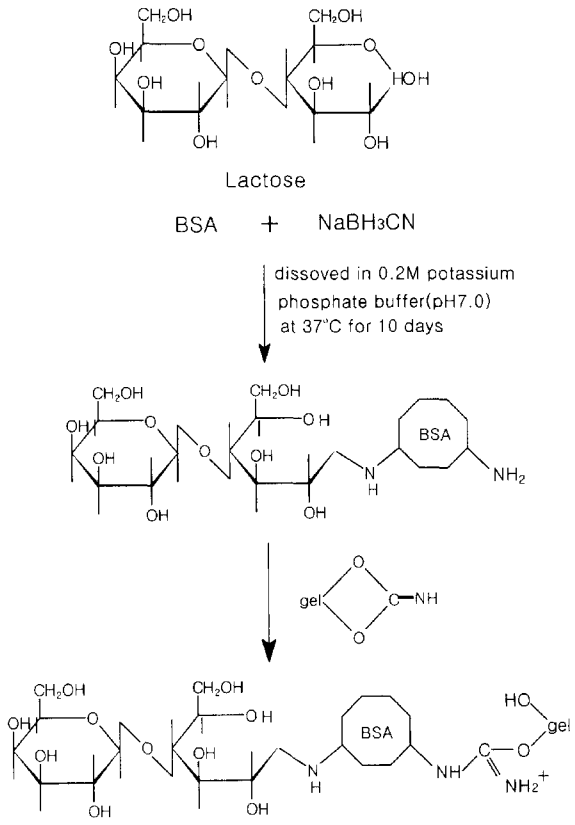

Fig. 1. Conjugation of lactose-BSA by reductive amination and coupling of lactose-BSA conjugate to CNBr-activated Sepharose 4B.

Lactose (100 mg), BSA (68 μg), $NaBH_3CN$ (100 mg)

dissoved in pot. phosphate buffer (pH 7.0)
incubated at 37°C for 10 days
gel filtration on Sephadex G-25
eluted with pot. phosphate buffer (pH 7.0)
dialyzed in distilled water for 2 days
concentrated
dissolved in 0.1 M $NaHCO_3$/0.5 M NaCl
coupling buffer (pH 8.3)

Lactose-BSA conjugates

added to CNBr-activated Sepharose 4B
rotated gently at 4°C, overnight
transferred to 0.2 M glycine/0.5 M NaCl (pH 8.0)
incubated at 4°C, overnight
washed alternately with 0.1 M acetate/0.5 M NaCl buffer (pH 4.0) and coupling buffer (pH 8.3)

Lactose-BSA-CNBr-activated Sepharose 4B

Fig. 2. Procedure of conjugation of lactose-BSA by reductive amination and coupling of lactose-BSA conjugate to CNBr-activated Sepharose 4B.

lectin was bound to B-chain and that A-chain did not have a sugar binding affinity.

SDS-PAGE and molecular weight determination

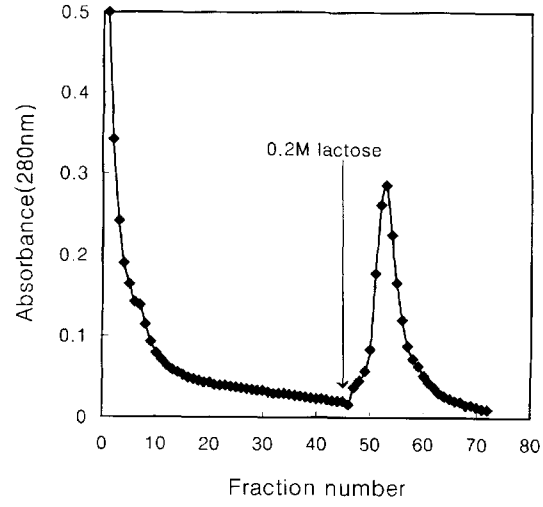

Fig. 3. Affinity chromatogram of lection by eluting with 0.2 M lactose on lactose-BSA-Sepharose 4B.

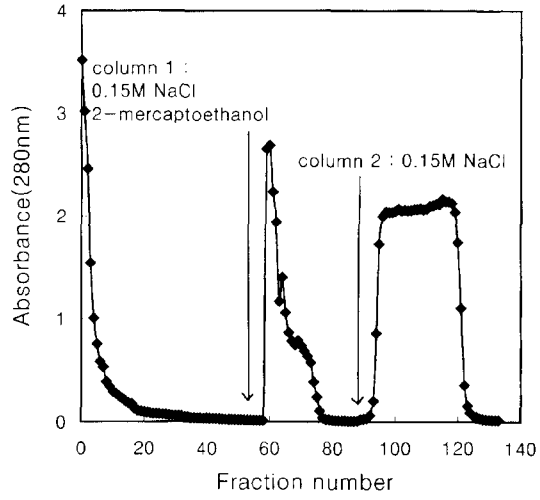

Fig. 4. Affinity chromatogram of A-chain eluted with 0.15 M NaCl on lactose-BSA-Sepharose 4B. The effluent containing A-chain was loaded to a second column of latose-BSA-Sepharose 4B to eliminate residual B-chain.

Investigations using SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) in the absence and presence of a reducing agent (2-mercaptoethanol) showed that the lectin from Korean mistletoe consisted of two different chains connected by disulfide bonds (Fig. 6). In the absence of the reducing agent, a major band corresponding to Mr=60,000 and two minor bands corresponding to Mr=35,000 and 31,000 were found (Fig. 6, lane 2). In the presence of 2-mercaptoethanol (Fig. 6, lane 3), two major bands corresponding to Mr=35,000 and 31,000 and an indistinct band corresponding to Mr=60,000, which was caused by uncompleted reaction of 2-mercaptoethanol were found. The SDS-PAGE showed that A-chain and B-chain of lectin were isolated and purified (Fig. 6, lanes 4, 6). Comparision of the panels showed that the lighter

chain was A-chain corresponding to Mr=31,000 and the heavy chain was B-chain corresponding to Mr= 35,000.

Amino acid contents and N-terminal amino acid sequencing of B-chain

Aspartic acid was the major amino acid of lectin and B-chain, whereas glutamic acid was the major amino acid of lectin and A-chain. Isoleucine and threonine were significantly high in A-chain and in B-chain respectively. It is noticed that samples of A-chain and B-chain reduced by 2-mercaptoethanol contained cysteine, whereas lectin sample did not contain cysteine (Table II).

The *N*-terminal amino acid sequencing of B-chain was identified as Tyr-Glu-Arg-Leu-Lys-Leu-Arg-Val-Thr-His. Sequencing of A-chain was not successful and needs further investigation.

Effects of pH, temperature and guanidine hydrochloride on the conformation and hemagglutinating activity of lectin

Fig. 7 and Fig. 8 show the effects of pH on the hemagglutinating activity and fluorescence parameters of lectin. The activity was stable at the pH range of 4.00~8.00 and decreased up to 50% at pH 9.00 and disappeared completely at pH 10.00. It is noticed that the activity was enhanced to 200% at the pH range of 4.00~6.00. The enhancing of lectin activity induced by pH changes in fluorescence position of spectral maximum evidently indicated the significant structural transition in the environment of tryptophan residues. Blue shift was detected on the acidic pH, which suggested unfolding of the protein structure near tryptophan residues. Similar changes also occurred at the alkaline pH range of 8.00~10.00. These changes corresponded to the decrease of activity in the alkaline pH. From these results, it is assumed that high activity of lectin at pH 4.00~6.00 was caused by more suitable folding structure of lectin. The hemagglutinating activity of lectin was stable at a wide range of temperature (0~45°C) (Fig. 9). Half of the activity was maintained at 55°C, but the activity disappeared over 65°C. And Fig. 10 showed that the increase in temperature resulted in a typical denaturational shift of the protein spectra towards a position characteristic of free tryptophan (353 nm). From these results, it was assumed that there was a strong relationship between activity and conformation of lectin.

In denaturing conditions, such as high concentration of guanidine hydrochloride, tryptophan emission profile of lectin showed typical denaturational red

Table II. Amino acid contents in lectin and its A- and B-chain (unit : g/g%)

Amino acid	Lectin	A-chain	B-chain
Aspartic acid	12.44	12.95	15.02
Threonine	8.50	9.38	15.30
Serine	6.09	5.77	5.93
Glutamic acid	12.62	14.36	12.06
Proline	3.98	4.30	4.01
Glycine	5.95	4.92	6.32
Alanine	4.88	6.11	4.83
Cysteine	-	0.23	0.77
Valine	5.59	4.86	4.56
Methionine	-	-	0.57
Isoleucine	4.60	13.96	3.38
Leucine	8.99	9.95	8.23
Tryptophan	9.44	3.28	7.05
Phenyl alanine	4.43	4.92	0.94
Histidine	1.12	3.17	1.08
Lysine	3.18	4.41	4.08
Arginine	8.91	7.46	6.05

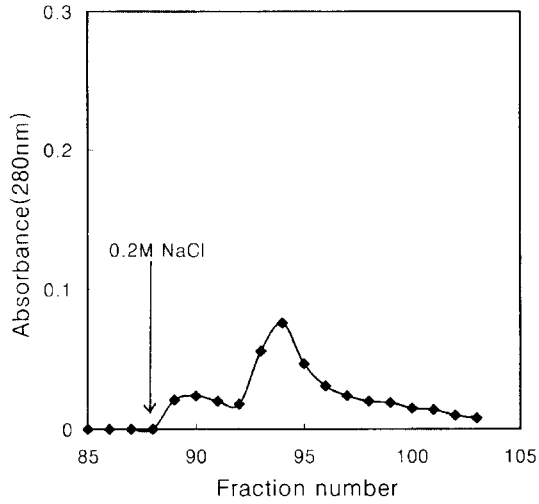

Fig. 5. Affinity chromatogram of B-chain eluted with 0.2 M lactose on lactose-BSA-Sepharose 4B.

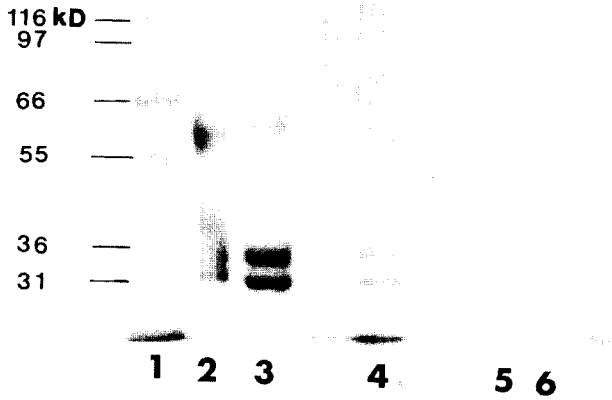

Fig. 6. SDS-PAGE patterns of fresh mistletoe lectins. lane 1: Molecular weight marker, lane 2: Lectin from mistletoe treated with SDS, lane 3: Lectin from mistletoe treated with SDS and 2-mercaptoethanol, lane 4: B-chain of mistletoe isolated and purified, lane 6: A-chain of mistletoe isolated and purified.

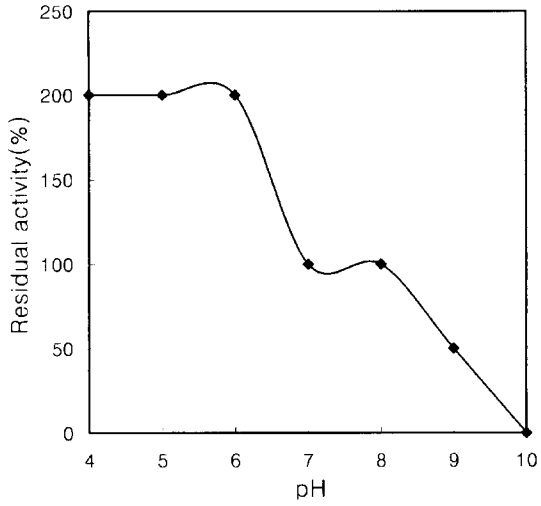

Fig. 7. Effect of pH on hemagglutinating activity of mistletoe lection.

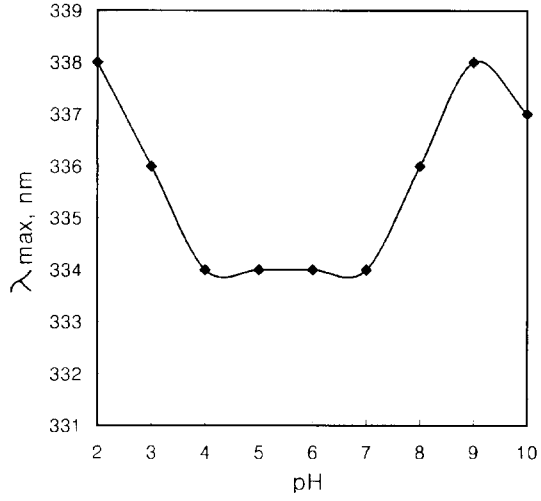

Fig. 8. Effects of pH on the fluorescence of mistletoe lection.

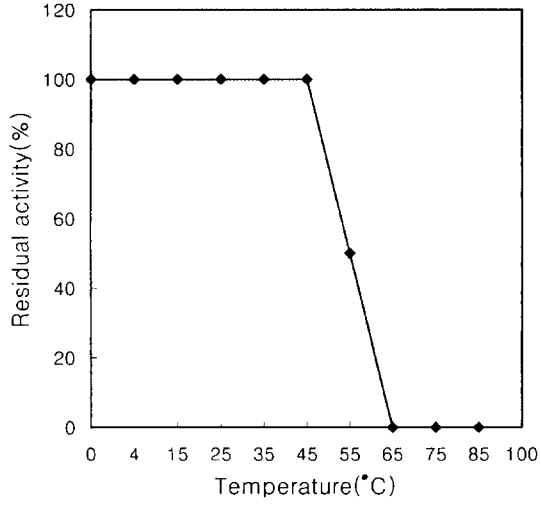

Fig. 9. Effects of temperature on hemagglutinating activity of mistletoe lectin.

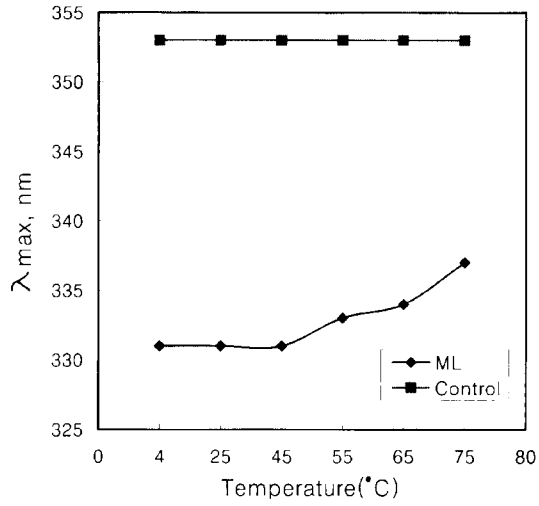

Fig. 10. Effects of temperature on the fluorescence of mistletoe lectin and control (tryptophan).

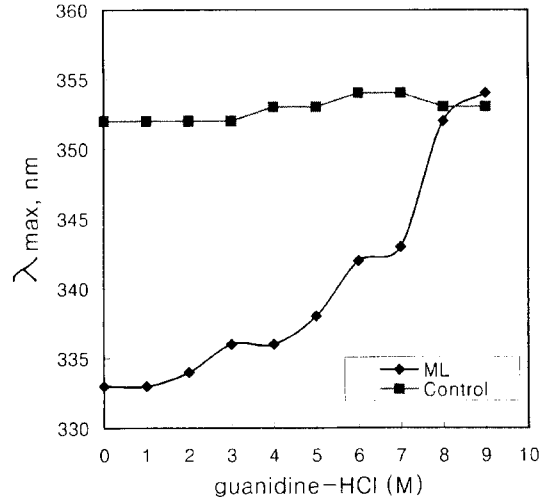

Fig. 11. Effects of guanidine hydrochloride on the fluorescence of mistletoe lectin and control (tryptophan).

shift, which also corresponded to the conformation and activity of lectin (Fig. 11).

ACKNOWLEDGEMENTS

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