Purification and Characterization of a New Galactoside Specific Lectin from *Trichosanthes kirilowii* Root

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Abstract: A new lectin, named TRA, was purified from Trichosanthes kirilowii root by acid-treated Sepharose 6B, Mono-Q, and TSK-gel 3000SW column sequential chromatography. The lectin appeared homogeneous by native gel electrophoresis at pH 4.3 and gave two protein bands of Mr=31 and 28 kDa by SDS-PAGE. The N-terminal amino acid sequences of the polypeptides of TRA have not been reported in amino acid sequences of the lectins. TRA lectin formed a precipitate with asialofetuin, neuraminidase-treated fetuin. A sugar inhibition assay indicated that N-acetyl-D-galactosamine, among the monosaccharides tested, was the most potent inhibitor of TRA-induced hemagglutination. Asialofetuin showed a 260-times stronger inhibitory activity than N-acetyl-D-galactosamine. TRA lectin also showed agglutination with normal leukocytes and lymphoma cells, but not with premature hemopoietic cells. These results suggest that TRA is a novel plant lectin.
Key words: Lectin, TRA, asialofetuin, hemagglutination, hemopoietic cell.

Plant lectins are known as a heterogenous group of proteins and glycoproteins that share a common ability to recognize and bind specific sugar residues. Since the unique biological properties of lectins were exploited in the investigation of numerous biochemical and cellular phenomena (Sharon, 1977), efforts have been made to isolate lectins that have unique and unusual sugar-binding specificities. Lectins are useful for: 1) the separation and characterization of glycoproteins and the study of glycolipids; 2) study of changes that occur on cell surfaces during the physiological and pathological processes of cell differentiation to cancer; 3) histochemical studies of cells and tissues; 4) typing blood cells and bacteria; and 5) study of fractionation of lymphocytes and of bone marrow cells for bone marrow transplantation (Fischer, 1985; Kolb *et al.*, 1978; Lis and Sharon, 1984; Goldstein and Poretz, 1986). Lectins are also used to stimulate lymphocytes to assess the immune state of patients, and for chromosomal analysis in human cytogenetics, as well as for the production of cytokines (Sharon and Lis, 1989; Sharon and Lis, 1993). In addition, a lectin isolated from mistletoe (*Viscum album* L.) received approval in Europe for clinical use in the theraphy of tumors and allergy (Luther *et al.*, 1980; Hajto, 1986). Recently, a lectin (TRL) was purified from Trichosanthis radix, a root of *Trichosanthes kirilowii* known as a medicinal herb in the orient. This lectin is a homodimer with a subunit molecular weight of 29 kDa (Park and Lee, 1992; Kwon *et al.*, 1993). Inhibition studies have revealed that lactose shows the most potent inhibitory effect on TRL-induced hemagglutination. In this study another type of lectin (TRA) from *Trichosanthes kirilowii* root was purified using different methods. TRA lectin shows β-galactoside specificity and agglutinates human A, B, and O type erythrocytes and various leukemia cells. This new lectin is distinct from the TRL reported by Park and Lee (1992) in molecular weight, heterogeneous subunits, and sugar specificities. Herein, purification steps using affinity chromatography are described, along with the characterization of this new TRA lectin.

Materials and Methods

Materials

Trichosanthes kirilowii root (Trichosanthis radix) was purchased from a medicinal herb market in Pusan, Korea. Sepharose 6B, fetuin, asialofetuin, and various sugars were obtained from Sigma Chemical Co. (St. Louis, MO). Protein molecular weight standards for

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SDS-PAGE and gel filtration matrix were products of Gibco BRL (Gaithersburg, MD). Centricon and ultrafiltration membrane were purchased from Amicon Products (Bevery, MA).

Purification of T. radix (TRA) Lectin

Trichosanthes kirilowii root meal was extracted three times overnight with 1% NaCl at 4℃. Ten to ninty percent (10~90%) saturation of crude extracts was made with ammonium sulfate. The precipitate was collected by centrifugation (12,000 g x 20 min) and dialyzed against buffer A (50 mM Tris-HCl, pH 7.4, 10 mM $CaCl_2$). After centrifugation to remove insoluble material, the dialyzate was applied to an acid-treated Sepharose 6B column (4×25 cm) prepared using the method of Allen and Johnson (1976). The unbound materials were extensively washed from the column with the same buffer, then the adsorbed lectins were eluted with buffer A containing 10 mM lactose. Fractions containing galactoside binding proteins were pooled, concentrated using on Amicon ultrafiltration kit, and dialyzed against buffer B (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, and 0.1 mM DTT). The dialyzates were loaded onto a Mono-Q HPLC column (0.5×5 cm) pre-equilibrated with buffer B at a flow rate of 1 ml/min. After washing the column with buffer B the bound proteins were eluted with a linear salt gradient from 0 to 0.5 M KCl.

Agglutination and inhibition assay

Agglutination assays were performed by a serial two-fold dilution method using a microtiter plate. Red blood cells were washed and resuspended in saline to a 2% suspension. Jurkat cells (human T cell lymphoma), Daudi cells (human B cell lymphoma), SP2 cells (mouse myeloma), HL60 cells (human promyelocytic leukemia), U937 cells (monoblast leukemia), and K562 cells (human chronic myelogenous leukemia) were cultured in RPMI 1640 medium containing fetal bovine serum (FBS). Cells were harvested, washed, and resuspended in phosphate buffered saline solution (PBS). To measure the inhibition of agglutination by sugar or sugar derivatives, various concentrations of sugars in saline (using a serial 2-fold dilution method) were added to 20 μl of TRA (3 μl). After incubation of sugar and TRA for 30 min at room temperature, type B human red blood cells (3% suspension) were added to the incubation mixture. The degree of agglutination was observed under a light microscope.

Precipitin reactions

Precipitin reactions were conducted by a slightly modified method of So and Goldstein (1967). Various amounts of glycoconjugate, fetuin or asialofetuin, were incubated with 100 μg of TRA in a total volume of 250 μg. After incubation for 1 h at 37℃, reaction mixtures were stored for 48 h at 4℃. Precipitates were centrifuged (15,000 g×30 min), washed with cold PBS, then dissolved in 0.05 M NaOH.

Determination of molecular weight

Polyacrylamide gel electrophoresis: Polyacrylamide gel electrophoresis was carried out according to Laemmli (1972) in the presence of 0.1% SDS using a 12% slab gel. An SDS-lectin complex, incubated either with or without β-mercaptoethanol for 5 min at 95℃, was used for the sample. Native gel electrophoresis using a 10% slab gel was performed at pH 4.3, according to the method of Reisfeld *et al.* (1962). Protein bands on the gel were stained with Coomassie Brilliant Blue R250. The method of Grossman (1971) was used for staining.

Gel filtration chromatography: Native molecular weight was determined by gel filtration using a TSK G3000SW HPLC column (0.75×60 cm) pre-equilibrated with 50 mM Tris-HCl, pH 7.4. The flow rate was 0.5 ml per min.

Determination of the N-terminal amino acid sequence

N-terminal amino acid sequence analysis was performed according to the method of Matsudaira (1989). Separated TRA subunits which appeared on the 12% SDS-PAGE gel were transferred electrophoretically onto a PVDF (polyvinylidene difluoride) membrane for 3 h at 400 mA. The protein bands on the PVDF membrane were visualized with Coomassie Blue, and the N-terminal amino acid sequence was determined using an ABI 471A Protein Sequencer (Foster City, CA. USA) according to the manufacturer's procedure.

Other methods

Protein concentration was determined according to the method of Bradford (1976) with bovine serum albumin (BSA) as a standard. The total neutral sugar content of the lectin was measured colorimetrically by the phenol-sulfuric acid method (Debois *et al.*, 1956) using glucose as a standard. Total amounts of hexosamine were determined using Ehrlich reagent in alkaline acetylacetone (Ludowieg and Benmaman, 1967) with glucosamine as a standard.

Results

Purification of TRA lectin

Extracts were prepared from 500 g (dry weight) of *T. kirilowii* root with 1% NaCl and precipitated using

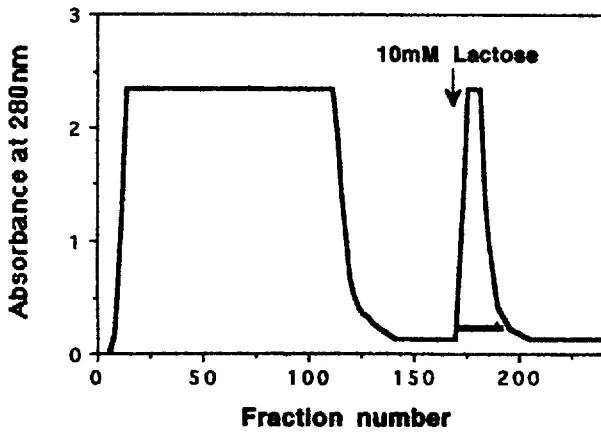


Fig. 1. Affinity chromatography on Sepharose 6B column. Ammonium sulfate precipitate proteins from Trichosanthis radix extract was applied in 50 mM Tris-HCl, pH 7.4, containing 10 mM $CaCl_2$ as described under "Materials and Methods". Bounded galactoside-specific proteins were eluted with 10 mM lactose in above buffer. The absorbance at 280 nm was determined for each 10 ml fraction and protein peak fractions were pooled.

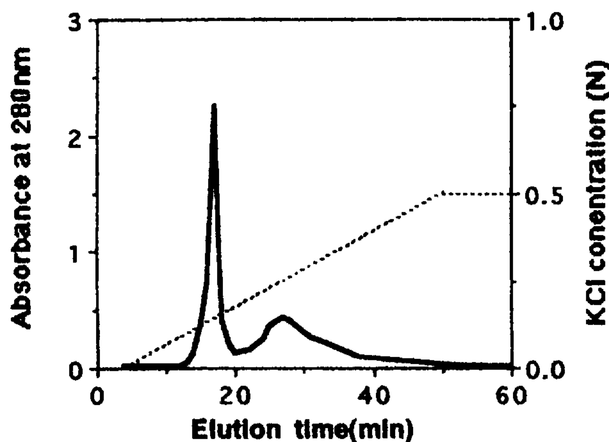


Fig. 2. Anion exchange chromatography on Mono-Q. Pooled fractions from Sepharose 6B chromatography were applied to a Mono-Q HPLC column and bounded proteins were eluted with a linear gradient from 0 to 0.5 M KCl as described under "Materials and Methods"; ——, absorbance at 280 nm (protein) and ······, concentration of KCl.

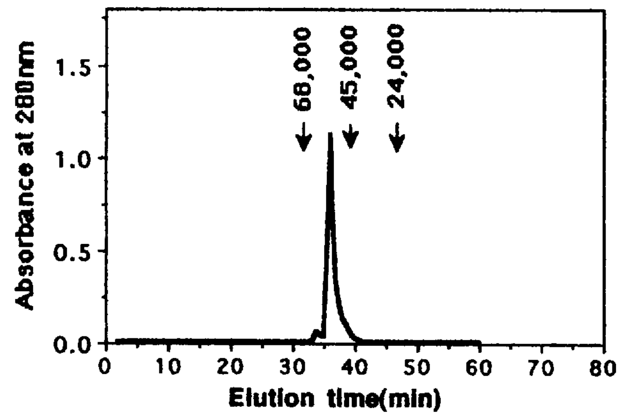


Fig. 3. HPLC gel filtration chromatography. Peak I fraction from Mono-Q HPLC column was applied to TSK G3000SW HPLC gel filtration column. Fraction were collected at a flow rate of 0.5 ml/min. Arrows indicate molecular weight markers used as standards; bovine serum albumin (68,000), ovalbumin (45,000) and chymotrypsinogen (24,000).

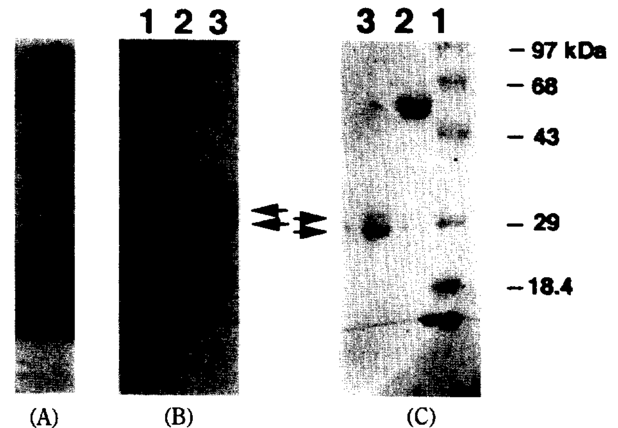


Fig. 4. Polyacrylamide gel electrophoresis. Final fraction from gel filtration was electrophoresed on 10% native polyacrylamide gel (A) and 12% SDS-polyacrylamide gel electrophoresis and visualized with Coomassie Brilliant blue staining (B) or PAS-staining (C). The lanes were loaded as follows: protein molecular weight markers, from top to bottom: phosphorylase b, 97 kDa; bovine serum albumin, 68 kDa; ovalbimin, 43 kDa; carbonic anhydrase, 29 kDa; β-lactoglobulin, 18.4 kDa as indicated on the righ side (lane 1); TRA lectins heated without (lane 2) or with (lane 3) 5% β-mercaptoethanol in sample buffer containing 2% SDS. Arrows indicate positions of the dissociated subunits of TRA.

ammonium sulfate. The resulting precipitates were collected and subjected to chromatography on an acid-treated Sepharose 6B column. As shown in Fig. 1, the lectins bound tightly to this matrix were eluted with 10 mM lactose. The peak fractions were pooled, concentrated, and applied to a Mono-Q anion exchange column. The column was then eluted with a KCl gradient (0 to 0.5 M). As shown in Fig. 2, proteins were separated into two peaks (peak I and peak II) between 0.08 M to 0.13 M, and between 0.2 to 0.35 M KCl, respectively. Both peaks showed hemaggulutinating activities for human A, B, and O erythrocytes. A further agglutination inhibition assay revealed that peak II had a specific binding ability to lactose and was slightly inhibited by N-acetyl-D-galactosamine, whereas peak I was more strongly inhibited by N-acetyl-D-galactosamine than lactose (data not shown). This result suggests that peak II may contain a lectin (TRL) which was purified previously by Park and Lee (1992). Because peak I exhibited novel characteristics, it was further purified by chromatography on a TSK G3000SW HPLC gel filtration column. A protein was eluted at the fraction

corresponding to a molecular weight of 58 kDa (Fig. 3). This protein was designated TRA.

Characterization of TRA lectin

Polyacrylamide gel analysis: The TRA lectins obtained from the gel filtration HPLC column were analyzed on native-and SDS-polyacrylamide gels. As shown in Fig. 4A, purified TRA lectin gave a single protein band on 10% native gel electrophoresis at pH 4.3. When analyzed on 12% SDS-polyacrylamide gel electrophoresis under nonreducing conditions (absence of β-mercatoethanol), purified TRA exhibited a single protein band corresponding to a molecular weight of 60

Table 1. N-terminal partial amino acid sequences of TRA lectin

	N-terminal amino acid sequence
Larger chain	Val-Ala-Asn-Asn-Ile-Glu-Thr-Ser-Thr-Val-Arg
Smaller chain	Asn-Leu-Ser-Val-Ile-Phe-Thr-Ile

Table 2. Agglutination of blood cells and cultured cells by purified TRA

Cell types	TRA concentration required for agglutination
Human erythrocytes	μM
Type A	5.0
B	2.5
AB	2.5
O	5.0
Human leukocyte	2.5
Rabbit erythrocytes	Not agglutination
Jurkat cell	0.078
Daudi cell	0.078
SP2 cell	0.078
HL 60 cell[a]	Not agglutination
U937 cell[a]	Not agglutination
K562 cell[a]	Not agglutination

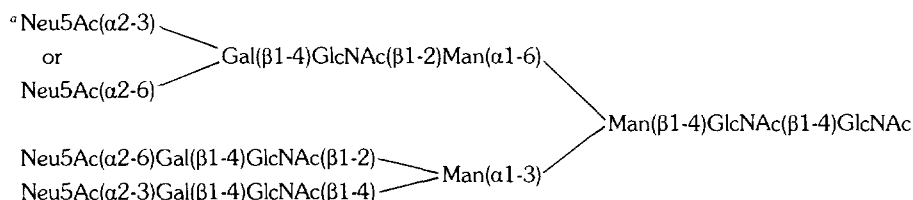
[a] HL 60, U937 and K562 cells were agglutinated slightly after incubating 60 min with 5 mM of TRA.

Table 3. Minimum concentration of various sugars and *p*-nitrophenyl glycosides necessary to completely inhibit hemagglutination induced by TRA

Sugars	Minimum concentration (mM)
Sugars	
L-Arabinose	50
D-Galactose	6.25
D-Galactosamine	12.5
N-Acetyl-D-galactosamine	1.25
Lactose, Gal(β1-4)Glc	2.5
Melibiose, Gal(α1-6)Glc	6.25
Raffinose, Gal(α1-6)Glc(α1-2)Fru	25
N-Acetylneuramin-lactose, Neu5Ac(2-3) and (2-6)Gal(β1-4)Glu	No inhibition at 17 mM
Methlyl or p-nitrophenyl glycosides	
2-O-methyl-Gal(β1-4)Glc	1.25
p-Nitrophenyl β-D-Galactoside	2.5
p-Nitrophenyl α-D-Galactoside	5.1
Glycoproteins	
Asialofetuin	0.0048
Fetuin[a]	No inhibition at 5 mM

The following sugars were not inhibitory at concentration of 200 mM

D-Glucose, D-mannose, D-ribose, L-fucose, N-acetylneuraminic acid, D-glucosamine, N-acetyl-D-glucosamine, D-mannosamine, N-acetyl-D-mannosamine, 1-O-methyl-D-mannosamine, 1-O-methyl-D-mannose

[a]


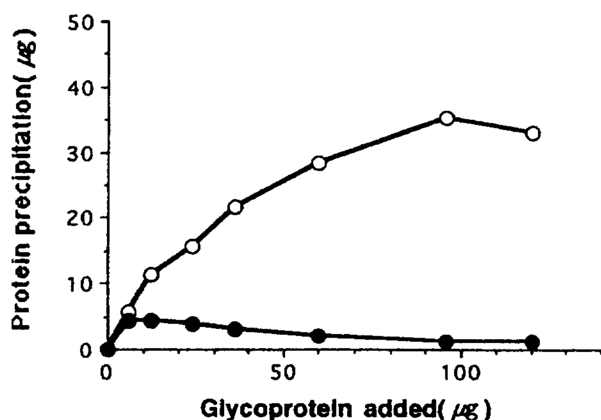


Fig. 5. Quantitative precipitation of glycoprotein by TRA. Various amounts of glycoprotein were mixed with 100 μg of TRA and incubated for 2 days at 4℃. Precipitates were centrifuged, washed and analyzed for the protein concentration as described under "Materials and Methods". ○-○: asialofetuin; ●-●: fetuin.

kDa (Fig. 4B and 4C, Lane 2). This estimated molecular weight was coincident with the result of gel filtration chromatography (Fig. 3). When the same set of purified TRAs was treated with the reducing agent β-mercaptoethanol (5%), two protein bands with molecular weights of 31 and 28 kDa were detected by both Coomassie Brilliant blue staining and PAS staining (Fig. 4B and 4C, Lane 3). These results suggest that TRA is a glycoprotein made up of two heterogeneous subunits.

Carbohydrate analysis: Carbohydrate analysis of TRA lectin was performed colorimetrically. TRA lectin contained approximately 0.9% and 4.0% neutral sugar and hexosamine, respectively. The carbohydrate content in the 28 kDa subunit of TRA was more than the content in the 31 kDa subunit (Fig. 4C, Lane 3).

N-terminal amino acid sequence of TRA: The subunits of TRA were separated on 12% SDS-PAGE and transferred to PVDF membrane and the N-terminal amino acid sequence of each peptide was analyzed. The partial amino acid sequence of the TRA subunits is shown in Table 1. The N-termini of the 31 and 28 kDa peptides were valine and asparagine, respectively.

Agglutination properties: TRA lectin agglutinated human A, B, and O type red blood cells equally well, but not rabbit red blood cells. TRA lectin also agglutinated human T cells and lymphoma cells immediately, but did not react with other premature hemopoietic cells (Table 2). To determine the binding specificities of TRA lectin to carbohydrates, inhibition studies were conducted using human B red blood cells (Table 3). D-Galactose, N-acetyl-D-galactosamine, and various oligosaccharides containing these sugar residues at the nonreducing termini were inhibitory in the range of 1.25 to 25 mM. Oligosaccharides and glycosides containing β-D-galactosyl or β-D-galactosaminyl termini showed greater inhibitory ability than the corresponding β-anomers (melibiose and raffinose). The weak inhibition by L-arabinose can be explained by the fact that it has the same arrangement of hydroxyl groups at the C-3 and C-4 positions as D-galactose. N-acetylneuraminic acid, N-acetylneuramin-lactose, and fetuin were not inhibitory, but neuraminidase-treated fetuin (asialofetuin) was the most potent inhibitor among the tested sugars.

Precipitation of glycoproteins with TRA: TRA is a β-galactoside-specific lectin, based on sugar inhibition tests. TRA also agglutinates human red blood cells which contain sialic acid at the nonreducing terminal. When TRA lectin reacts with fetuin a small amount of precipitate is produced. However, when desialization is performed the degree of precipitation increases substantially (Fig. 5).

Discussion

In this report the purification and characterization of a novel lectin referred to as TRA is described. The lectin was purified to near homogeneity from Trichosanthis radix root. The molecular weight of the purified TRA, determined by gel filtration chromatography and native gel electrophoresis, was estimated to be 58 and 63 kDa, respectively (Fig. 3 and 4B). When analyzed on SDS-polyacrylamide gel electrophoresis in reducing conditions (in the presence of 5% β-mercaptoethanol), purified TRA exhibited two heterogeneous subunits corresponding to molecular weights of 31 and 28 kDa (Fig. 4B, and Table 1). TRA has an extended carbohydrate-binding site that is specific for Gal/GalNAc(β1-4) Glc/GlcNAc (Table 3), whereas the TRL purified by Park and Lee (1992) from the same source (Trichosantis radix) has a much higher affinity to lactose than galactosamine or N-acetyl-D-galactosamine. These characteristics suggest that purified TRA may be a previously unknown structurally distinct lectin.

The binding specificity of TRA is similar to the affinity of peanut agglutinin (PNA) in that it recognizes carbohydrates containing galactose residues. However, TRA agglutinates native human type A, B, and O red blood cells which are neuraminylated (sialylated) at the site of the nonreducing carbohydrate terminal (Table 2), while PNA agglutinates only neuraminidase-treated red blood cells (Lotan *et al.*, 1975; Gillespie *et al.*, 1993). In an agglutination inhibition assay, asialofetuin showed a 260 times greater inhibition than N-acetylgalactosamine, whereas fetuin was not inhibitory. These observations suggest that PNA recognizes the terminal galactose residue as the primary binding ligand, whe-

reas TRA recognizes the internal Gal/GalNAc residue as its primary binding ligand (Rinderle *et al.*, 1989).

Recently, it has been shown that purified TRA stimulates the phosphorylation of phospholipase C-γ1 (PLC-γ1) on tyrosine residues, and increases phosphoinositide hydrolysis in T lymphoma Jurkat cells (Kim *et al.*, 1993). The activation of PLC has long been recognized as one of the important events that stimulates the effector function of T-cells after the binding of antigens to the T cell antigen receptors. The mechanism of PLC activation in T cells appears to be complex. However, there is increasing evidence that the primary means of regulating PLC activity in T-cells after the binding of the antigen is the activation of some tyrosine kinases, such as *fyn* and *lck*, members of the *src* family (Samelson *et al.*, 1990). TRA also agglutinates some lymphoma cell lines, including human Burkitt's lymphoma Daudi and murine myeloma SP2 (Table 2). Therefore, it is of interest to determine whether TRA activates tyrosine kinase activity in these cell lines. Further research regarding the detailed binding specificity of other glycoconjugates is under way.

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