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A Novel Mannose-binding Tuber Lectin from *Typhonium divaricatum* (L.) Decne (family Araceae) with Antiviral Activity Against HSV-II and Anti-proliferative Effect on Human Cancer Cell Lines

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A novel mannose-binding tuber lectin with *in vitro* anti-proliferative activity towards human cancer cell lines and antiviral activity against HSV-II was isolated from fresh tubers of a traditional Chinese medicinal herb, *Typhonium divaricatum* (L.) Decne by a combined procedure involving extraction, ammonium sulfate precipitation, ion exchange chromatography on DEAE-SEPHAROSE, CM-SEPHAROSE and gel-filtration on sephacryl S-200. The apparent molecular mass of the purified *Typhonium divaricatum* lectin (TDL) was 48 kDa. TDL exhibits hemagglutinating activity toward rabbit erythrocytes at 0.95 μg/ml, and its activity could be strongly inhibited by mannan, ovomucoid, asialofetuin and thyroglobulin. TDL showed anti-proliferative activity towards some well established human cancer cell lines, e.g. Pro-01 (56.7 ± 6.8), Bre-04 (41.5 ± 4.8), and Lu-04 (11.4 ± 0.3). The anti-HSV-II activity of TDL was elucidated by testing its HSV-II infection inhibitory activity in Vero cells with TC_{50} and EC_{50} of 5.176 mg/ml and 3.054 μg/ml respectively. The full-length cDNA sequence of TDL was 1145 bp and contained an 813-bp open reading frame (ORF) encoding a 271 amino acid precursor of 29-kDa. Homology analysis showed that TDL had high homology with many other mannose-binding lectins. Secondary and three-dimensional structures analyses showed that TDL is heterotetramer and similar with lectins from mannose-binding lectin superfamily, especially those from family Araceae.

Keywords: Antiviral activity, Anti-proliferative activity, Molecular cloning, Purification and characterization, Sequence analysis, *Typhonium divaricatum* (L.) Decne lectin

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Introduction

Lectins, possessing at least one non-catalytic domain, are capable of recognizing and binding to polysaccharides or glycoproteins expressed on cell surfaces (Goldstein *et al*., 1980). The interaction of lectins with certain carbohydrate is as specific as enzyme-substrate or antigen-antibody interactions. Nowadays they are being widely used in studies of biochemistry, cell biology, immunology, glycobiology and have widespread applications in biomedical researches (Sharon and Lis, 1989), being one of the major forces driving the progress of glycoscience. Accumulating evidence indicated that lectins can bind to varieties of Gram-positive/negative bacteria, yeasts, viruses, protozoa, plant cells and animal cells, detecting subtle differences in complex carbohydrate structures.

Owing to the fine specificity, most plant lectins have been employed for various applications including cancer therapy and virus research. Varieties of alterations in carbohydrate structure have been observed in cancer cells, including increased sialylation and branching of complex carbohydrates, or occasional emergence of some novel structures (Kaur *et al*., 2006). In this endeavor, lectins are excellent candidates in cancer research and therapy for their usefulness in recognition of the alterations in malignant cells, for reducing the cell tumorigenicity, and for prognosis of immune status of patient. Moreover, many plant lectins possess inhibitory activity towards the infection by herpes simplex, including *Canavalia ensiformis* agglutinin, *Concanavalin* A (ConA) (Okada and Kim, 1972), Soybean agglutinin (SBA), *Wisteria floribunda* agglutinin (WFA), *Narcissus pseudonarcissus* agglutinin (NPA), *Bauhinia purpurea* agglutinin (BPA) and *Eranthis hyemalis* agglutinins (EHA) (Marchetti *et al*., 1995) at concentrations below their cytotoxicity threshold, indicating excellent implication in anti-HSV research.

During the last decade, lectins with interesting properties

have been isolated and characterized (Van Damme *et al*., 1998). Interestingly, most of these plant lectins belong to a single monocot mannose-binding superfamily as revealed by their molecular evolution, physiological roles, sequence homologies, and exclusive specificity for mannose or mannoside. However, unlike aforementioned agglutinins, lectins from family *Araceae* exhibit either very weak or no affinity to mono-, di- or oligosaccharides, whereas they bind with great avidity to glycans or glycoproteins (Van Damme *et al*., 1995a). *Typhonium divaricatum* (L.) Decne, belonging to the family *Araceae*, is a traditional Chinese medicine herb endowing with detumescence, anti-inflammation and anti-virus bioactivities. In this article, we are now poised to present our investigation aiming to reveal the structure and biological activity of a novel mannose-binding monocot araceous lectin extracted from bulbs of this plant. The lectin was found to possess various *in vitro* anti-proliferative activities towards human cancer cell lines and potent antiviral response against HSV-II at low concentration far below the cytotoxicity threshold.

Materials and Methods

Chemicals and reagents. N-Bromosuccinimide (NBS), urea, guanidine-HCl, bovine serum albumin (BSA), Acyclovir, dimethylsulfoxide (DMSO), isopropanol, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), most monosaccharides, oligosaccharides, their derivatives, polysaccharides and glycoproteins were purchased from Sigma (St. Louis, USA). Standard molecular weight markers (gel-filtration and SDS-PAGE protein markers) and ampholine of pH range 3.0-10.0 were procured from Amersham Pharmacia (New Jersey, USA). PUC18-T vector, RNA Extraction Kit and 3' and 5' RACE kit were purchased from Takara Biotechnology, Dalian, China. All the other reagents were of analytical grade.

Plant material. Tubers of *Typhonium divaricatum* (L.) Decne were collected from the campus of Sichuan University, Chengdu, China, during the month of August, and were instantly frozen in liquid nitrogen and stored at −70°C before use.

Cell lines and virus. The various human cancer cell lines employed i.e., Pro-01 (prostate), Lu-04 (lung), Bre-04 (breast), HepG2 (liver) and Hela (cervix), were procured from Di'ao Group, Chengdu, China. Cell lines were maintained in RPMI 1640 medium with 10% FCS, 10 U/ml penicillin and 100 μg/ml streptomycin at 37°C, in humidified atmosphere (90% air and 10% CO_2) in CO_2 incubator (Heraeus, Heracell). African green monkey kidney cells (Vero) and herpes simplex virus type 2 (HSV-II strain 333) were obtained from West China Medical School of Sichuan University. Vero cells, in which HSV-II was propagated, were grown in RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and penicillin, streptomycin and fungizone (100 U/ml, 100 and 2.5 μg/ml, respectively). The virus was stored at −70°C until used.

Protein determination. Protein concentration was determined as described by Lowry *et al*. (1951) using crystalline bovine serum albumin (BSA) as standard.

Purification of *Typhonium divaricatum* Lectin. Fresh tubers of *T. divaricatum* (100 g) were homogenized in 0.15 M NaCl using a Waring blender and soaked overnight at 4°C before filtered through muslin cloth. The slurry was centrifuged at 9,000 g for 20 min and the supernatant was collected and precipitated with 80% saturation of ammonium sulfate. It was thereafter dissolved in 20 mM Tris-HCl, pH 8.0 (buffer A). The dialysate was loaded on a DEAE-SEPHAROSE equilibrated with buffer A and eluted with 0-0.5 M NaCl gradient. Active fractions were pooled and dialysed against 20 mM acetate buffer, pH 4.3 (buffer B) before applied to a CM-SEPHAROSE column. Then active fractions were concentrated to 3 mg/ml and loaded onto a Sephacryl S-200 column equilibrated with 20 mM, PBS, pH 7.0 (buffer C). The purified lectin, designated as TDL, was obtained by elution from the column with buffer C, followed by dialysis against distilled water and then lyophilization.

Determination of molecular mass of TDL by gel-filtration and SDS-PAGE. The molecular mass of TDL was estimated by gel-filtration chromatograph on the same Sephacryl S-200 column as mentioned previously following the procedure of P. Andrews (1964). The column was calibrated with soybean trypsin inhibitor (30.2 kDa), bovine serum albumin (68 kDa), ovalbumin (45 kDa), chymotrypsinogen A (25 kDa) and cytochrome C (12.5 kDa). Gel-filtration purified lectin was subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), pH 8.3, using 11% (w/v) acrylamide slab gel for subunit molecular mass determination as described by Laemmli (1970).

Hemagglutinating activity assays. Hemagglutinating activity of TDL was assayed in 96-well microtiter U plates according to the serial double dilution method using rabbit erythrocytes (Oda and Minami, 1986). Agglutinating activity of the lectin sample was expressed as units, namely the reciprocal of the highest dilution giving a positive result. The specific hemagglutinating activity was defined as unit/mg protein (Wang *et al*., 2000).

Carbohydrates-binding specificity. For hemagglutinating inhibition tests, serial two-fold dilutions of sugar samples were prepared in phosphate-buffered saline. All dilutions were mixed with 25 μl of TDL with two units. The mixture was allowed to stand for 30 min at 20°C and mixed with an equal volume of 2% rabbit erythrocyte suspension. Sugars or their derivatives were tested at concentration of 200 mM while polysaccharides and glycoproteins at concentration of 4 mg/ml. The minimal inhibitory sugar concentration (MIC) of the final reaction mixture, which completely inhibited the lectin preparation, was calculated.

Effect of temperature, pH and denaturants. To determine the thermal stability, TDL was dissolved in phosphate-buffered saline (PBS, 0.15 M, pH 7.0) at 50 mg/L and treated at 10-100°C in 10°C increments for 1 h. The samples were then cooled down to room temperature immediately in ice water.

The effect of pH on activity of TDL was performed by dissolving the lectin to 50 mg/L in a buffer of desired pH and tested at 4°C. The erythrocytes suspension used for the hemagglutination assay was also prepared in the same buffer. The buffers used were as follows: acetate for pH 2.0, 4.0 and 4.9, phosphate for pH 5.6 and 7.0, Tris-HCl for pH 8.6, carbonate for pH 10.0 and KCl/NaOH for pH 12.0 (all 100 mM).

The effect of two denaturing agents i.e., urea and guanidine-HCl, at a concentration range of 0-8.0 M, was tested on lectin activity by incubating 2 ml of each denaturant solution (prepared in 0.02 M PBS buffer, pH 7.0) with equal volume of TDL at 4°C.

Anti-HSV II assay

Virus titration (Cytopathogenic effect). Quadruplicate confluent monolayers in 96-well plates were overlaid with equal volume of serial appropriately diluted virus suspension. After 1 h of adsorption, the unabsorbed virus was washed by PBS and fresh culture medium was added to each well. The morphology of Vero cells was inspected for microscopically detectable alterations, i.e. loss of monolayer, rounding, shrinking of cells, granulation, and vacuolization in the cytoplasm. After 72 h of incubation at 37°C in a 5% CO_2 incubator, the value of $TCID_{50}$ (tissue culture infection dose) was calculated (Monks *et al*., 1991).

Cytotoxicity assay. Quadruplicate confluent monolayers in 96-well plates were overlaid with 0.2 ml of serial twofold test sample. After 72 h of incubation, 20 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/ml in PBS) was added to each well, and the monolayers were incubated for 4 h at 37°C. The resulting formazan precipitate was dissolved in isopropanol with the addition of DMSO and the extinctions were determined by absorbance ($OD_{570-630}$). TC_{50} (toxic concentration) was the concentration required to reduce the absorbance (OD) by 50% (Kodama *et al*., 1996).

Antiviral assay. The antiviral activity of TDL against HSV-II was also assessed using MTT method (Kodama *et al*., 1996). In short, quadruplicate confluent monolayers in 96-well plates were pre-incubated with 20 µl of 100 times diluted virus suspension (10^4 $TCID_{50}$/ml) for 1 h at 37°C. Then 0.2 ml of maintenance medium containing appropriate serially diluted concentrations of the test sample was added. Acyclovir was used as the positive control for HSV-II. Cell control and virus control were run simultaneously. After 3 days of incubation, the absorbance $OD_{530-630}$ was determined by Anthos Microplate Reader. The percentage of viable treated cells was calculated in relation to untreated controls and was defined as 50% effective concentration (EC_{50}). The therapeutic index (TI) was calculated from the ratio TC_{50}/EC_{50} (Kodama *et al*., 1996).

Assay of *in vitro* anti-proliferative potential on human cancer cell lines. The *in vitro* anticancer potential of TDL against five human cancer cell lines including Pro-01 (prostate), Lu-04 (lung), Bre-04 (breast), HepG2 (liver) and Hela (cervix), was tested according to the method of Monks *et al*. (1991), known as SRB assay. Cells were seeded at 10^4 cells/well in 100 µl RPMI medium containing 10% FCS in 96-well tissue culture plate. These cell lines were suspended as single cells in the above-mentioned medium and incubated for 24 h in CO_2 incubator. Subsequently, 100 µl of lectin solution (prepared in RPMI 1640 medium) at concentrations ranging from 0-100 µg/ml, was added to cells and incubated for 48 h. After the incubation period, adherent cell cultures were mixed in situ by adding 50 µl of 50% (v/v) trichloroacetic acid and incubated for 1 h at 4°C. The supernatant was discarded and plates were washed five times with deionized water and dried. 50 µl sulforhodamine B (SRB, 0.4% w/v in acetic acid) was added to each well and the cultures were incubated for 15 min at 4°C. The unbound SRB was removed by washing with 1% acetic acid and plates were air-dried. The dye bound to basic amino acids of the cell membrane was solubilized with 200 µl Tris buffer (10 mM, pH 10.5) and the absorbance was measured at 540 nm using ELISA reader to determine the relative cell growth viability in the treated and untreated cells.

RNA extraction and full-length cDNA cloning. Total RNA was extracted from *T. divaricatum* bulbs using an RNA Extraction Kit (Takara Biotechnology, Dalian, China) and reversely transcribed with a cDNA synthesis primer AP (5'-GGCCACGCGTCGACTAG $TAC(T)_{21}$-3'). The 3' sequence of *T. divaricatum* lectin was obtained by rapid amplification of cDNA ends (RACE) with a 3'-full RACE kit (TaKaRa Biotechnology) with primer1 (5'-GACTGCAATCTG GTCTTGTACAA-3') and primer 2 (5'-GGCCACGCGTCGACTA GTAC-3'), designed according to the conserved mannose-binding amino acid sequence QDNVY present in conservative mannose-binding sites of mannose-binding lectins. PCR was performed under the following conditions: cDNA was denatured at 94°C for 30 s, followed by 30 cycles of amplification (94°C for 30 s, 55°C for 30 s and 72°C for 2 min) and 7 min at 72°C. Specific primers 5'-ACTTCGCATTCACCTTCTCCG-3' (primer 3), 5'-ATCATCATCT CCTCTGCAGCG-3' (primer 4), and 5'-CTTCGAGCCGGTCGAA AAGATG-3' (primer 5) were designed to amplify the 5' end of TDL. The RNA was reverse transcribed followed by tailing cDNA with primer 3. The first round of PCR was performed with primer 4 and Abridged Anchor Primer (AAP). PCR was carried out as previous steps. The PCR product was diluted 50-fold for the third round of amplification with primer 5 and Abridged Universal Anchor Primer (AUAP). The PCR product was purified and cloned into a pUC18-T vector (Takara Biotechnology) for sequencing. Forward primer 6 (5'-ATGGCCTCCAAGCACCTCCTC-3') and reverse primer 7 (5'-TTACTTCGCATTCACCTTCTCC-3') were designed for the amplification of full-length cDNA of TDL. The thermal cycling program was the same as that utilized for 3' and 5' RACE.

Bioinformatic analysis of the sequence and structure of TDL. The DNA sequence and associated molecular information of TDL were analyzed using DNA Tools 6.0, Vector NTI 9.0 (invitrogen, Carlsdad, USA), HCAdraw 2 (Doriane, Paris, France) and Swiss-Model (http://swiss-model.expasy.org//SWISS-MODEL.html).

Results and Discussion

Purification of *Typhonium divaricatum* lectin. Preliminary experiments with crude extracts from tubers of *Typhonium divaricatum* (L.) Decne indicated the presence of a lectin that

strongly agglutinated rabbit erythrocytes but was inactive with human erythrocytes irrespective of blood group. This was in agreement with other araceous lectins being all capable of agglutinating rabbit, guinea pig, rat and sheep erythrocytes but not human erythrocytes (Sandu *et al*., 1990). Thus erythrocytes from rabbit were employed for monitoring hemagglutinating activity during purification steps.

TDL was purified by a combination of ammonium sulphate fractionation, ion exchange and gel chromatography. After DEAE-SEPHAROSE and CM-SEPHAROSE chromatography, fraction A_3 (Fig. 1A) and B_2 (Fig. 1B) displayed highest activity after elution with NaCl linear gradient. Upon gel-filtration on Sephacryl S-200, the activity was observed on C_2 which was fractionated from B_2 (Fig. 1C). As shown in Table 1, the overall yield was approximately 1 mg/g fresh weight, corresponding to 12% of the total soluble tuber protein. This data, as well as the low purification fold, indicated high lectin content of the total extractable proteins present in storage tissues (Van Damme *et al*., 1995a). This is in consonance with earlier findings from family Araceae and compares with 1 mg/g in *Arum maculatum* bulbs (Allen *et al*., 1995), 3.2 mg/g in *Arisaema flavum* bulbs (Singh *et al*., 2004), 3.0 mg/g in *Sauromatum venosum* bulbs (Bains *et al*., 2005), and 1.1 mg/g in *Arisaema helleborifolium Schott* bulbs (Shangary *et al*., 1995).

Determination of molecular mass of TDL by gel-filtration and SDS-PAGE. Upon SDS-PAGE in the presence and absence of 2-mercaptoethanol, TDL migrated as an apparent single band of about 12 kDa (Fig. 2), indicating the homogeneity of the lectin. Gel-filtration chromatography of the lectin gave a symmetrical single peak corresponding to an apparent molecular mass of 48 kDa (Fig. 3). These results suggested the tetrameric nature of TDL in which subunits are not held by disulphide linkages but joined together by noncovalent bonds. This finding is in good agreement with most of araceous lectins which have no disulphide linkage between subunits (Anthony *et al*., 1995; Shangary *et al*., 1995; Singh *et al*., 2004; Singh *et al*., 2005).

Hemagglutinating activity and carbohydrates-binding specificity. Hemagglutinating test showed strong interaction ability of the lectin, for TDL maintained its activity even at concentration of 0.95 μg/ml. The inhibitory concentrations of sugars and glycoproteins are given in Table 2. Unlike most lectins from other families, TDL differs distinctly in carbohydrate binding specificity, in that it readily formed precipitins with complex saccharides and various glycoproteins, such as mannan (yeast mannan extracted from *Saccharomyces cerevisiae*), asialofetuin, ovomucoid and thyroglobulin in a concentration-dependent manner, while other saccharides tested were devoid of any inhibitory effect at all. This observation was quite similar with other araceous lectins, including those from *A. maculatum*, *C. esculenta* and *Xanthosoma sagittifolium*, which were also precipitated by both asialoglycoproteins and yeast mannan, partially

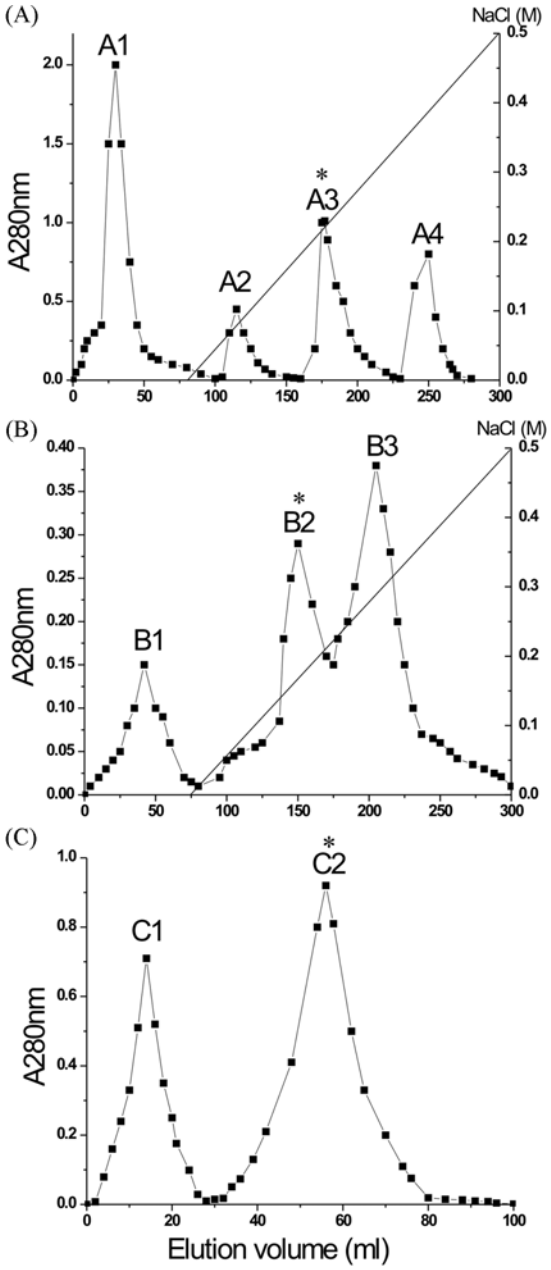

Fig. 1. (A) Anion exchange chromatography of proteins derived from the ammonium sulfate precipitate of *Typhonium divaricatum* (L.) Decne tuber extracts on DEAE-SEPHAROSE column pre-equilibrated with buffer A (pH 8.0). The bound protein was eluted with 0-0.5 mM NaCl gradient at a flow rate of 1 ml/min. (B) Anion exchange chromatography of fraction A_3 on CM-SEPHAROSE column pre-equilibrated with buffer B (pH 4.3) and eluted with 0-0.5 mM NaCl gradient at a flow rate of 2 ml/min. (C) Gel-filtration of fracion B_2 on Sephacryl S-200 column pre-equilibrated with buffer C (pH 7.0) at a flow rate of 45 ml/h. The elution profiles were monitored at 280 nm.

supporting the idea proposed by Mo *et al.* (1999) that the presence of two distinct types of binding sites might be a general characteristic of araceous lectins. Just like other

Table 1. Hemagglutinating activities of *Typhonium divaricatum* (L.) Decne lectin chromatographic fractions against rabbit erythrocytes

Steps	Yield (mg)	Specific hemagglutinating activity (HU/mg)	Total hemagglutinating activity (HU)[a,b]	Recovery of activity (%)	Folds of purification
Crude extracts	830	24	19920	100	1
DEAE-SEPHAROSE Chromatography	275	64	17600	87.5	2.7
CM-SEPHAROSE Chromatography	141	99	14000	70.0	4.1
Gel-chromatography	100	132	13200	66.3	5.5

The calculations represent the data for 100 g of tubers.
[a]Total hemagglutinating units and two percent of rabbit erythrocytes were used for hemagglutinating activity.
[b]Calculated as inverse of the minimum concentration producing a positive reaction in the hemagglutination assay.

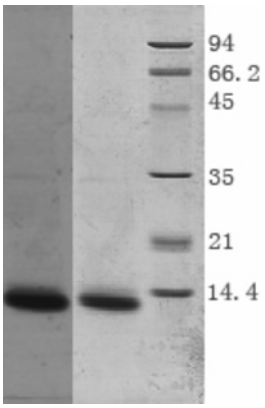

Fig. 2. SDS-PAGE, pH 8.3, patterns of purified TDL using 11% gel in the presence (lane left) and without (Lane in the middle) 2-mercaptoethanol (running time 3 h at a constant 100 V). The amount of purified lectin loaded is 50 g. Lane right, Mr markers (from top to bottom): Phosphorylase b (94 kDa); albumin bovine (66.2 kDa); ovalbumin (45 kDa); carbonic anhydrase (35 kDa); trypsin inhibitor (21 kDa); and alactalbumin (14.4 kDa). The gels were stained with Coomassie brilliant blue.

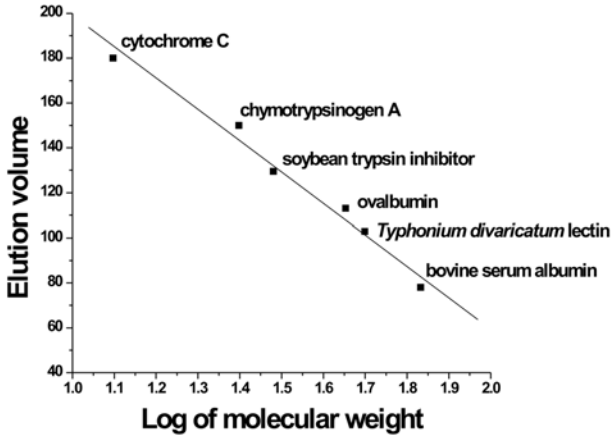

Fig. 3. Native molecular mass estimation by standard plot on gel-filtration chromatography column. Standards used for gel-filtration analysis were soybean trypsin inhibitor (30.2 kDa), bovine serum albumin (68 kDa), ovalbumin (45 kDa), chymotrypsinogen A (25 kDa) and cytochrome C (12.5 kDa).

tetrameric lectins showing potent anti-tumor, insecticidal and antiviral properties, TDL might be of similar bioactivities due to the multi-valency and its capability to interact with great avidity towards complex glycol-conjugates.

Effect of temperature, pH and denaturants. When submitted to heat treatment, TDL is thermally stable over a wide range of temperatures from 20 to 60°C. However, the activity starts to decline very rapidly afterwards, and is completely abolished after 10 min exposure at 90°C and 100°C. TDL was quite stable from pH 5.6 to 8.6, and still retains 50% and 60% agglutination activity at pH 2 and 12 respectively, suggesting the pH stability of the protein. Urea at 3.0 M and guanidine-HCl at 5.0 M reduced the lectin activity by 50%. The denaturation by these two agents indicates the globular nature of lectins, stabilized mainly by hydrophobic interactions (Kaur *et al*., 2006).

Anti HSV-II assay. The $TCID_{50}$ of HSV-II against Vero cells, determined by cytopathogenic effect (CPE), was 10^{-4} in the virus titration assay. The amount of surviving cells after incubation with sample solutions at different concentrations was estimated by cytotoxicity assay. The toxicity of most formulations tested was relatively low and there were no significant differences between them (Table 3). The TC_{50} obtained was at 5.176 mg/ml, indicating low cytotoxicity of the lectin towards Vero cells. As for antiviral assay, 43% and 60.4% inhibition was observed when the concentration of TDL was at 2 μg/ml and 4 μg/ml respectively for HSV-II, and 90.9% inhibition was observed at 8 μg/ml (Table 4). The 50% effective concentration (EC_{50}) obtained was comparatively low at 3.054 μg/ml, and the therapeutic index (TI), calculated from the ratio TC_{50}/EC_{50}, was 1695, suggesting excellent therapeutic security of the lectin. All these data pointed to the feasibility that TDL may be a good candidate for the treatment of human HSV-II and probably endowed with potential

Table 2. Carbohydrate-binding specificity

Sugar/glycoprotein	Minimum inhibitory sugar concentration (MIC) in the presence of TDL
Mannan	100 μg/ml
Asialofetuin	75 μg/ml
Ovomucoid	125 μg/ml
Thyroglobulin	12.5 μg/ml

The carbohydrate-binding specificity was determined by hemagglutinating inhibition tests using a series of simple sugars and some glycoproteins. The following sugars were not inhibitory at a final concentration of 200 mM: Sialic acid, Arabinose, N-acetyl-D-glucosamine, D-Mannose, 2-methyl-D-glucoside, N-acetygalactosamine, N-acetylactosamine, D-galactose, Maltose, D+ galactosamine, D-fructose, Lactose, D-Glucosamine Hydrochloride, Fetuin, Sucrose, Chitin, Sialofetuin. The Minimal inhibitory sugar concentration (MIC) required for complete inhibition was calculated and given bellow.

application in clinical therapy for this desease. Though the mechanism of the antiviral effect of the lectin was not clear at present, we extrapolate that TDL may bind the high-mannose glycans on the surface of the virus particles and directly interfered the binding of virus-cell membrane.

Assay of *in vitro* anti-proliferative potential on human cancer cell lines. The *in vitro* anti-proliferative activity of TDL was evaluated against five human cancer cell lines representing different organs and tissues namely Pro-01 (prostate), Lu-04 (lung), Bre-04 (breast), HepG2 (liver) and Hela (cervix). Table 5 shows the dose-dependent effect of TDL with a concentration range of 0.01-100 μg/ml, after a treatment period of 48 hours. Of the five cancer cell lines, TDL possesses the most deleterious effect towards Pro-01 (56.7 ± 6.8%), relatively milder toxicity towards Lu-04 (41.5 ± 4.8%), and only minor inhibitory towards Lu-04 (11.4 ± 0.3%), while the effect on the viability of HepG2 and Hela cell lines was undetectable. Some araceous lectins having potent anti-proliferative activities have also been reported earlier (Singh *et al*., 2004; Bains *et al*., 2005). However, the exact molecular mechanism of these action is poorly understood, but evidence is now emerging that lectins are dynamic contributors to tumor cell recognition, cell adhesion and localization, signal transduction across membranes, mitogenic stimulation, host immune defense augmentation, cytotoxicity and apoptosis (Karasaki *et al*., 2001). Saccharide determinants or glycoconjugates present on tumor cell surface serve as important binding sites for specific lectins. Differences in terminal sugars in various tumor cell lines can be one of the reasons for the variation of proliferation inhibition. TDL may interact with branches of complex carbohydrates located on the cell membrane of these cancer cell lines.

Full-length cDNA cloning of TDL. Based on the primers designed according to the conserved amino acid sequence of most monocot mannose-binding lectins, a 550-bp fragment of 3' end and a 800-bp fragment of 5' end of TDL cDNA were obtained. The full-length cDNA sequence of TDL, obtained using an RT-PCR reaction, was 1145 bp and contained an 813-bp open reading frame (ORF) encoding a 271 amino acid protein (Fig. 4). The new nucleotide sequence data reported in the present study have been submitted to GenBank and are available under the accession number EF194099. According

Table 3. Cytotoxicity of TDL toward Vero cell line

Lectin concentration (mg/ml)	8	4	2	1	0.5	0.25	0.125	0.0625	control	TC_{50}[a] (mg/ml)
OD	0.438	1.182	1.213	1.249	1.335	1.434	1.479	1.499	1.520	5.176
±s[b]	0.02	0.01	0.04	0.03	0.01	0.04	0.02	0.02	0.02	
% inhibition[c]	28.3	77.8	79.8	82.3	87.8	94.3	97.3	98.6		

[a]50% cytotoxic concentration.
[b]Data represent mean values of OD for four independent experiments (mean value ± S.D.).
[c]The percentage of inhibition was calculated in relation to untreated controls.

Table 4. Anti-HSV-II activity of TDL

Lectin concentration (μg/ml)	8	4	2	1	0.5	0.25	control (virus)	control (cell)	EC_{50}[a] (μg/ml)
OD	1.376	0.914	0.651	0.332	0.207	0.211	0.124	1.514	3.054
±s[b]	0.27	0.18	0.23	0.14	0.11	0.13	0.07	0.03	
% inhibition[c]	90.9	60.4	43.0	21.9	13.7	13.9			

[a]50% cytotoxic concentration.
[b]Data represent mean values of OD for four independent experiments (mean value ± S.D.).
[c]The percentage of viable treated cells was calculated in relation to untreated controls.

Table 5. *In vitro* anti-proliferative effect of TDL

	Human cell lines cancer	Lectin dose (μg/ml)					
		Control (0)	0.01	0.1	1.0	10	100
Percentage of Inhibition (%)	Pro-01	0	0	0	9.2 ± 0.4[a]	10.0 ± 0.5	56.7 ± 6.8
	Lu-04	0	0	0.2 ± 0.1	3.8 ± 0.4	7.4 ± 0.5	11.4 ± 0.3
	Bre-04	0	0	6.7 ± 0.5	8.4 ± 1.3	10.0 ± 1.1	41.5 ± 4.8
	HepG2	0	ND[b]	ND	ND	ND	ND
	Hela	0	ND	ND	ND	ND	ND

The anti-proliferative activity of TDL was tested on five human cancer cell lines: Pro-01, Lu-04, Bre-04, HepG2 and Hela. In the control wells, cells were cultured with medium alone (no lectins). The growth/inhibition of the cells was measured by ELISA reader.
[a]Data represent means ± S.E.M. (n = 3)
[b]Effect undetectable.

to the rules of predicting lectin signal peptide (Von Heijine, 1986), a 24 amino acid signal peptide with the signal peptide cleavage site between T_{24} and V_{25} was identified from the full-length cDNA sequence, which is in good agreement with

```
   1 tttgatagggttttgcactcagtagccaggtctagcaagccccggttccttcctcatggcc
                                                           M  A
  61 tccaagcacctcctcctcctcccggccatccttggcctcgtcattcctcggacagcc
       S  K  H  L  L  L  L  L  P  A  I  L  G  L  V  I  P  R  T  A
 121 gcgacagtgggcaccaactacctgctgaccggtgaaaccctaaacacgaacgagcatctc
       A  T ↑V  G  T  N  Y  L  L  T  G  E  T  L  N  T  N  E  H  L
 181 cggaacggcgacttcgacttggtcatgcaggaggactgcaacctcgtcctttacaacggc
       R  N  G  D  F  D  L  V  M  Q  E  D  C  N  L  V  L  Y  N  G
 241 aattggcagtccaacaccgccaacagaggaaaggagtgcaagctcaccctgaccgaccgc
       N  W  Q  S  N  T  A  N  R  G  K  E  C  K  L  T  L  T  D  R
 301 ggcgagctcctcatccaagaccgcgatggatccaacgtctggagcagcggctcccagtca
       G  E  L  L  I  Q  D  R  D  G  S  N  V  W  S  S  G  S  Q  S
 361 gagaggggcaactacgccgccgtcctccatccggaggggagactggtcatctacggccca
       E  R  G  N  Y  A  A  V  L  H  P  E  G  R  L  V  I  Y  G  P
 421 tccgtcttcaagatcaacccttgggcccccccgcctcaacagcctgctgctcggcgacgtc
       S  V  F  K  I  N  P  W  A  P  R  L  N  S  L  L  L  G  D  V
 481 cctttcacgaacaacatgctcttctccggccagatcctgcacaacgacggcatgctcact
       P  F  T  N  N  M  L  F  S  G  Q  I  L  H  N  D  G  M  L  T
 541 gcgaggaaccacagactggtcatgcaggaggactgcaacctcgtcctgtacggcggcaaa
       A  R  N  H  R  L  V  M  Q  E  D  C  N  L  V  L  Y  G  G  K
 601 ttggggtggcagtccaatacccacggcaacggcgagcactgcttcctaaggcttacccac
       L  G  W  Q  S  N  T  H  G  N  G  E  H  C  F  L  R  L  T  H
 661 aagggcgaactcatcatcaaagacgacgacttcaagaccatctggagcagcaggtccaag
       K  G  E  L  I  I  K  D  D  D  F  K  T  I  W  S  S  R  S  K
 721 tccaaacaaggcgactacgccttcatcctgcaggacgacggcttcgccgtcatctacggc
       S  K  Q  G  D  Y  A  F  I  L  Q  D  D  G  F  A  V  I  Y  G
 781 cctgccatcttttcgaccggctcgaagagctcagtcgctgcagaggagatgatgatcggc
       P  A  I  F  S  T  G  S  K  S  S ↑V  A  A  E  E  M  M  I  G
 841 atggtgacggagaaggtgaatgcgaagtaaagtgggaaaactgcatgctccccagcaagc
       M  V  T  E  K  V  N  A  K  *
 901 cggttcatggtctgttcgataatcgcgcgtgcatgagcagtgggtaccgcgattattccg
 961 tgttgtacgtacgtagctactgtgtctttcgtcccagtaccgtctttgttaaagaggctt
1021 tgatccgccttttgctttcgtaagccatcggtctggcttctgctttctctcgtgtaactg
1081 catgtggtgcttcgaataaatatggctacgtaccaaaaaaaaaaaaaaaaaaaaaaaaa
1141 aaaaa
```

Fig. 4. cDNA sequence and deduced amino acid sequence of TDL. The start codon and the stop codon were underlined italically, the arrows indicate cleaving sites. The putative polyadenylation signal is boxed, and poly A was underlined.

those of other *Araceae* species such as *Colocasia esculenta* (Hirai *et al*., 1993) and *Arum maculatum* (Van Damme *et al*., 1995a). The cleavage site of the C-terminal was between S_{253} and V_{254}. Translated with DNA Tools 6.0, the mature protein of TDL has two putative domians with a theoretic isoelectric point of 4.99 and 7.13, and a calculated molecular weight of 11.89 kD and 12.09 kD respectively. The richest amino acid residues were Leu (12.9% by frequency), Gly (9.6%), and Asn (7.7%).

Homology analysis shows TDL has high homology with many other mannose-binding lectins. Database retrieval with PSI-Blast (National Center for Biotechnology Information databases) showed that TDL is a member of the monocot mannose-binding lectin superfamily. Homologous analysis showed that the identity between TDL and *A. heterophyllum* agglutinin, *A. lobatum* agglutinin, *P. ternate* agglutinin, *P. pedatisecta* agglutinin and *A. maculatum* agglutinin was 72.5, 73.3, 79.3, 74.1 and 66.1%, respectively (Fig. 5). Recently, the molecular cloning of another lectin from *Typhonium divaricatum* (L.) Decne has been reported (Kong *et al*., 2006). The multi-alignment of *Typhonium divaricatum* agglutinin (TDA) and TDL sequences revealed only 21% homology between them (data not shown), indicating that they might be different lectins co-exist in the same plant.

Analysis of the secondary and three-dimensional structures of TDL. The analysis of secondary structure of TDL (Fig. 6) show that β-sheets, connected with turns and coils, occurred predominantly in the structure of TDL. The structural organization of β-sheets found in SCA (Wrigh *et al*., 1999) was generally conserved in TDL (data not shown) as revealed by hydrophobic cluster analysis (HCA) (Gaboriaud *et al*., 1987). Analysis ExPASy Molecular Biology Server (http://swiss-model.expasy.org) indicated that the overall structure of both domains (Fig. 7) are composed of three subdomains connected by pseudo-three-fold symmetry with each subdomain composed of a 4-stranded antiparallel β-sheet. The mannose-binding sites were located in the clefts formed by the three bundles of β-sheets. This was very similar

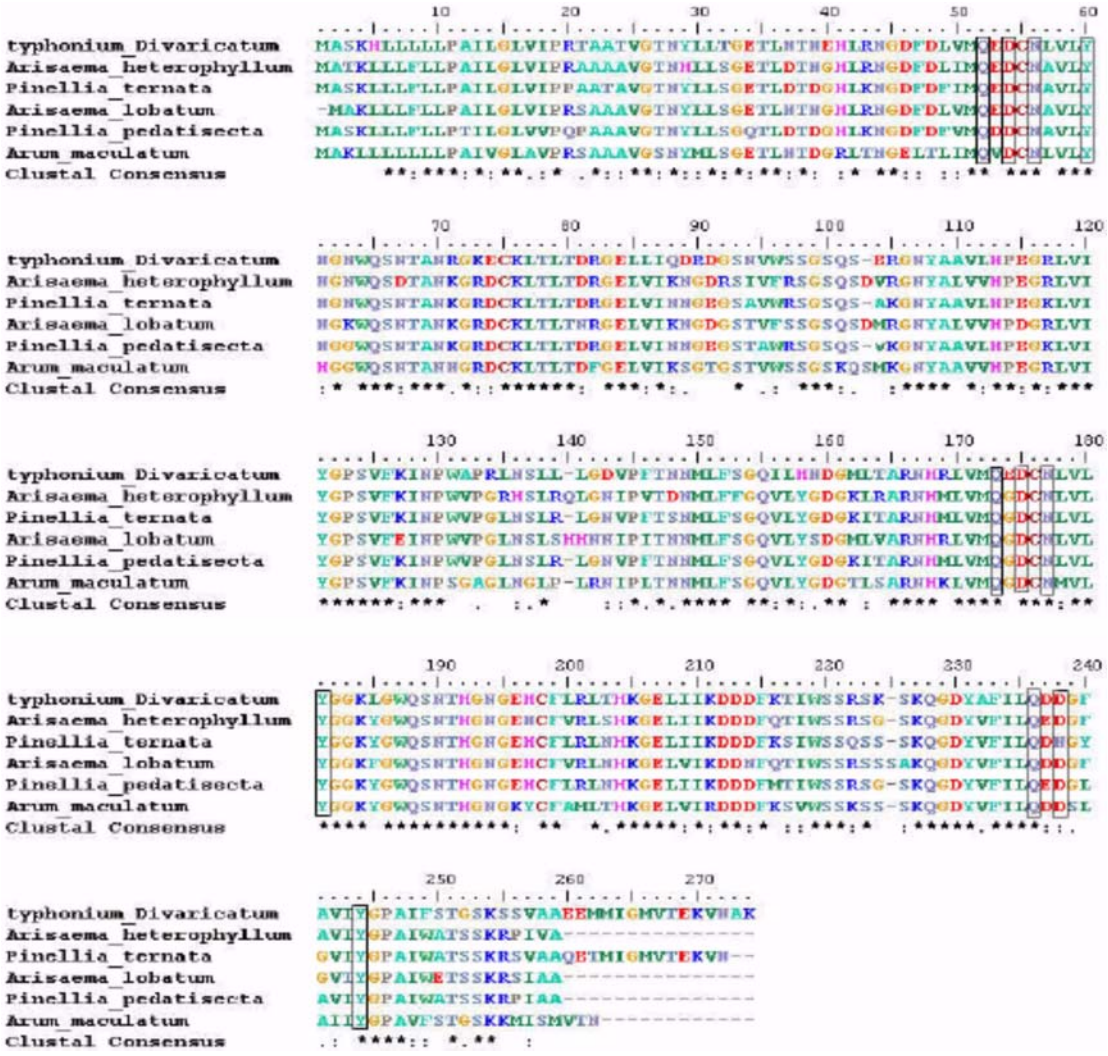

Fig. 5. Multi-alignment of the predicted *T. divaricatum* lectin (TDL) amino acid sequence with other monocot mannose-binding lectins from family Araceae. *Arisaema lobatum* (AY557617), *Arisaema heterophyllum* (AY338965), *Pinellia ternata* (AY451854), *Pinellia pedatisecta* (AY451853), *Arum maculatum* (AMU12198). The amino acid residues identical among any two of the six lectins were indicated with asterisk (*). One amino acid residue different from those of the other five lectins were indicated with colon (:). Two amino acid residues different from those of the other four lectins were indicated with dot (.). Mannose-binding sites (QDNY) were boxed.

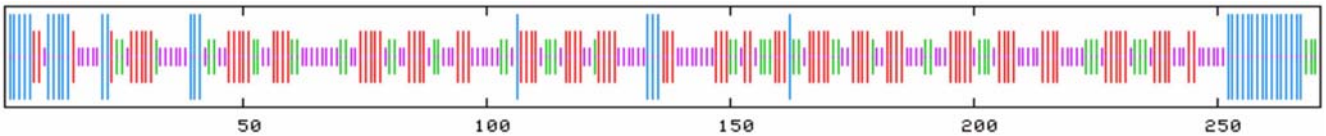

Fig. 6. The secondary structure of TDL. Helix, sheet, turn and coil were indicated, respectively, with cyan, red, green and purple vertical lines.

to the 3-D structure of many other mannose-binding lectins (Barre *et al*., 1996).

The database search indicated that the two putative conserved domains share 46.2% identity. Domain A was between T_{27} and P_{132} amino acid and domain B was between N_{146} and S_{253}. Combined with the biochemical analysis showing TDL was a tetramer, it was deduced that TDL was first synthesized as a large precursor of 29-kDa peptide that undergoes post-translational removal of the 24-residue leader sequence the second cleavage near the center into two fragments. Thereafter, the proprotein undergoes further proteolytic modifications with the linker sequence and the 18-residue C-terminal peptide cleaved, resulting in two domains with nearly identical size, which finally assemble into the $(AB)_2$-heterotetramer

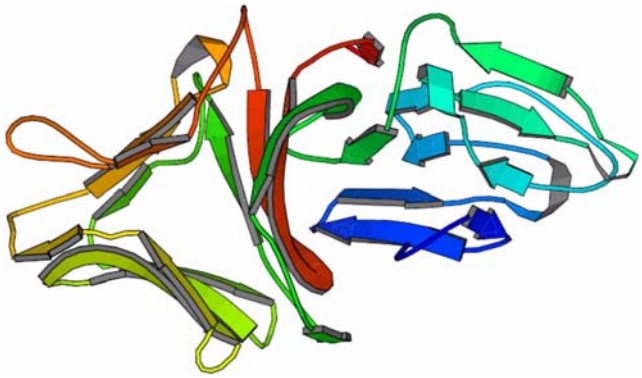

Fig. 7. The predicted tertiary structure of TDL. β--sheets were indicated by patches. Turns and loops were indicated by lines.

after the cleavage. This kind of post-translational process also occurs in other mannose-binding lectins, especially those from family Araceae (Van Damme *et al*., 1998), resulting in heterotetramers composed of four polypeptide chains, and each chain had similar size of 11-14 kDa (Van Damme *et al*., 1995b).

Only three of six subdomains of the two domains are made up of evolutionally conserved amino acid residues, mainly constituted by Gln (Q), Asp (D), Asn (N), Tyr (Y). Asn (N) was substituted by hydrophobic amino acid Phe (F) in the third mannose-binding pocket in TDL (Fig. 5). It should also be noted that there is a very good correlation between the number of active binding sites and the sugar-binding activity of mannose-binding lectins (Van Damme *et al*., 1998). The lack of interaction of TDL with simple sugars (i.e. mannose) may be due to the result of the replacement (deletion and insertion) of key amino acid residues within the monosaccharide-binding pockets during evolution. This kind of mutation results in the steric hindrances that prevent the binding of mannose into the active site through the network of hydrogen bonds. This mutation also occurs in many Araceous lectins such as AMA (Van Damme *et al*., 1995a) and CEA (Hirai *et al*., 1993). By the above comparison, it was found that TDL had many common characters possessed by mannose-binding lectins, especially those from Araceae.

This novel Araceous lectin, showing anti-proliferative activity towards various cancer cell lines and anti HSV-II effect of Vero cell line, may provide us with a rational basis for its efficiency in cancer and virus research and applications in modern clinical diagnosis of these diseases. Recently, TDL was found to have potent anti-insect activity against aphid by the experiment undertaking in our laboratory (data not shown). The purification, characterization and molecular cloning of TDL will enable us to study its potential insect resistance function in the future as well.

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