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# Purification of a mannose-binding lectin *Pinellia ternata* agglutinin and its induction of apoptosis in Bel-7404 cells

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ABSTRACT

A novel high-throughput purification method for a monocot mannose-binding lectin, *Pinellia ternata* agglutinin (PTA), from tubers of *P. ternata* was established by mannose-Sephrose 4B affinity chromatography. The total protein was extracted from tubers of *P. ternata* using phosphate buffered saline (PBS) buffer. The extracted total protein was precipitated completely at 65% ammonium sulfate saturation and dissolved in different concentrations of NaCl solution to activate its binding affinity toward the column. PTA was bound to the affinity column by loading of the total protein into the column and elution using PBS buffer. The maximum purification yield (35.5 mg/g) was obtained when PTA was treated with 25% (w/v) NaCl solution, and the purity of PTA analyzed by SDS–PAGE was ~97%. The agglutination property of purified PTA was confirmed by mouse erythrocytes, which indicates its biological function. Nuclear staining assay and DNA fragmentation demonstrated that PTA could induce apoptosis of Bel-7404 cells, which further demonstrates its biological and pharmacological activities. Induction of apoptosis in the human tumor Bel-7404 cell line by PTA indicates its possible use in cancer therapy. The present investigation reports a significantly improved isolation method to obtain highly purified mannose-binding plant lectin proteins. The proposed method has great potential for industrial application because of its advantages, which include rapid isolation, high purity, high yield, low cost, and minimal requirement of chemical materials.



## Introduction

Plant lectins are proteins or glycoproteins possessing at least one non-catalytic domain that can bind reversibly to specific mono- or oligosaccharides [1,2]. Lectins can be found in all kingdoms of life ranging from viruses through bacteria and plants to animals. In particular, they occur widely in plants. Among monocotyledonous plants, lectins have been reported from families such as Araceae, Gramineae, Amaryllidaceae, Alliaceae, Liliaceae and Orchidaceae. Over the last few years, Araceae has emerged as a lectin-rich family, with lectins constituting 70–80% of the stored proteins in tubers [3,4]. Previous studies have shown that some plant lectins exhibit various pharmacological and biological activities such as termination of pregnancy, anti-pest, anti-tumor, anti-virus and anti-fungal [5–9]. These bioactivities are believed to be related to the carbohydrate-binding property of lectins, which endows them with binding activity towards certain carbohydrate components in the cell surface. Although the exact biological function of these proteins has yet to be elucidated, their high concentrations and source tissues suggest a role in storage. Lectins can also serve as defense molecules against insect herbivores and pathogens in plants and animals [10–12]. Three major lectin families, namely, the legume lectins, the type II ribosome-inactivating proteins (RIP-sII), and the *Galanthus nivalis* agglutinin (GNA)[1]-related lectins, have been studied intensively because of their significant biological activities [13,14]. The anti-cancer properties of lectins have been widely demonstrated *in vitro*, *in vivo*, and in human case studies, and results suggest their role as therapeutic agents [5,13,15]. Lectin molecules are known to bind to cancer cell membranes or receptors, thereby causing inhibition of tumor growth, cytotoxicity, and apoptosis. To date, numberous plant lectin proteins have been crystallized, and the crystal structures of members of the different plant lectin families have revealed a wide variety of lectin folds and carbohydrate binding site architectures.

*Pinellia ternata* (Thunb.) Breit, a traditional Chinese medicine plant belonging to Araceae family, is widely used in China [16]. *P. ternata* is an edible herb native to China. Its raw or semi-cooked tubers and leaves have been consumed for over 2000 years in ancient China as a medicinal plant for relieving cough and

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[1] *Abbreviations used:* PTA, *Pinellia ternata* agglutinin; PBS, phosphate buffered saline; GNA, *Galanthus nivalis* agglutinin; RIPsII, the type II ribosome-inactivating proteins; DMEM, Dulbecco's modified eagle's medium; SDS, sodium dodecyl sulfate.

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inflammation [8]. *P. ternata* agglutinin (PTA), a monocot mannose-binding lectin isolated from the tubers of *P. ternata*, is regarded as a mannose-specific glycoprotein lectin because of its sugar-binding specificity. The structure of PTA was recently determined in our laboratory (data not shown). PTA is a homodimer composed of two N-terminus domains and two C-terminus domains, and each domain contains three carbohydrate-binding sites with a molecular weight of ~12 kDa. In an earlier study, the full-length gene of PTA was cloned and expressed in *Bombyx mori* cells [17]. Other reports have shown that recombinant PTA expressed in *Escherichia coli* exerts hemagglutination and antifungal activities against *Alternaria alternata*, *Bipolaris sorokiniana*, *Gibberella saubinetii* and *Curvularia lunata* [18,19]. Recent insect bioassay and transgenic studies show that PTA has significant insecticidal activity against aphid and armyworm [8,16,20,21]. However, native PTA isolated from the tubers of *P. ternata* has yet to be examined in terms of its biological function, such as antiproliferation activity and induction of apoptosis in human tumor cell lines. Although a number of plant lectins have been purified using purification methods such as gel filtration chromatography, ion-exchange chromatography, and affinity chromatography, few reports focus on the purification study of the plant lectin. In addition, several plant lectins are available commercially, but they are expensive because of their complicated purification procedures and low yields. These disadvantages limit the research and application of plant lectins. The aim of the present study is to establish a novel single-step purification method for the plant mannose-binding lectins PTA by mannose-Sepharose 4B affinity chromatography procedure and investigate its biological activity in anti-proliferation activity and capability of induction of apoptosis in the human cancer cell lines Bel-7404. The proposed method features low purification costs and high yields of the desired lectin. This work improves the purification method of plant lectins and provides a rational basis for its industrial purification and clinical application.

## Materials and methods

### Materials

Tubers of *P. ternata* (Thumb.) Breit were purchased from Tianmu Mountain (Zhejiang Province, China). Mannose-Sepharose 4B affinity chromatography column and human liver tumor Bel-7404 cells were saved in our lab (Institute of Bioengineering, College of Life Sciences, Zhejiang Sci-Tech University). Mouse erythrocytes were obtained from the local animal house. All chemicals used were analytical reagent grade.

### Precipitation of total protein by ammonium sulfate

Fresh tubers of *P. ternata* (10 g) were peeled, homogenated with 40 ml of PBS buffer (0.01 M, pH 7.4), and then soaked overnight at 4 °C with shaking (150 rpm). The extract was filtered by passing through muslin cloth, and then the filtrate was centrifugated at 13,800*g* and 4 °C for 30 min. The clear supernatant was collected, and ammonium sulfate ranging from 35% to 75% saturation was added to the supernatant with magnetic stirring in an ice-box. The precipitated protein was collected by centrifugation at 13,800*g* and 4 °C for 10 min and subsequently dissolved in PBS buffer (0.01 M, pH 7.4). The protein concentration was determined using spectrophotometer system (NANODROP 2000, Thermo). After determination of the optimized ammonium sulfate saturation at which the total protein of *P. ternata* tubers was completely precipitated, the total protein was precipitated. The total protein was collected by centrifugation at 13,800*g*, and 4 °C for 10 min.

### Mannose-Sepharose 4B affinity purification

All purification steps were carried out at 0–4 °C. A mannose-Sepharose 4B affinity column was used for the purification of PTA. To determine optimal NaCl concentrations, the precipitated protein was dissolved in NaCl solutions of different concentrations of 10%, 15%, 20%, 25% and 30% (w/v). The dissolved total protein solution (10 ml, 25 mg/ml) was loaded into a mannose-Sepharose 4B affinity column (1 × 10 cm) that had been previously equilibrated with the same concentration of NaCl solution using an AKTA purifier at a flow rate of 0.5 ml/min. Unbound proteins were eluted by washing the column with the same concentration of NaCl solution at a flow rate of 1 ml/min until the absorbance of the eluted fractions read zero at 280 nm. The affinity-bound lectin protein was then eluted with PBS buffer (0.01 M, pH 7.4). Elution collection was begun when the absorbance of the eluted fraction increased and ceased when the absorbance of the eluting fraction reached zero at 280 nm. The collected fractions were concentrated and desalted by dialysis against PBS buffer (0.01 M, pH 7.4) at 4 °C for 16 h. The concentration of the protein solution was determined, and the obtained lectin protein was calculated. SDS–PAGE gel was used to analyze the molecular weight of purified PTA as well as its purity.

### Hemagglutination assay

The hemagglutination activity of purified PTA was determined by mouse erythrocytes in a 96-well plate. A total volume of 50 μl of solution was used in each well, including 25 μl of purified PTA prepared in 0.9% (w/v) NaCl solution and 25 μl of a mouse erythrocyte suspension in 0.9% (w/v) NaCl solution. The PTA solution and mouse erythrocyte suspension were mixed gently, and agglutination was visually assessed using an inverted microscope after allowing the mixture to settle at room temperature for 30 min.

### Determination of apoptosis of Bel-7404 cells

To examine the biological and pharmacological activities of PTA against the human liver tumor cell line Bel-7404, nuclear staining assay and DNA fragmentation were carried out. Briefly, Bel-7404 cells suspended in 80 μl of DMEM medium containing 10% fetal bovine serum were seeded at a density of $1 \times 10^4$ cells/well into 96-well plates and incubated at 5% $CO_2$ and 37 °C for 24 h. After the cultivation, PTA solutions prepared in PBS buffer (0.01 M, pH 7.4) of different concentrations were added to the wells. Further incubation was performed at 5% $CO_2$ and 37 °C for 48 h. Cells treated without PTA solution (replaced by 0.01 M, pH 7.4 PBS buffer) were designated as the negative control. After cultivation, the medium was removed from both groups. Untreated and treated cells were then washed twice in PBS buffer (0.01 M, pH 7.4) and stained with 50 μl of Hoechst 33342 (10 μg/ml) for 2 h at 37 °C in darkness. Morphological aspects of the nucleus were observed and photographed by a fluorescence microscope.

Bel-7404 cells were cultured in 6-well plates at a density of $1 \times 10^5$ cells/well for the DNA fragmentation assay. After treatment with PTA for 48 h, the cells were collected and treated with 500 μl of lysis buffer (1% SDS, 10 mM EDTA, 10 mM Tris–HCl, pH 7.4) for 10 min at room temperature. After centrifugation at 13,800*g* for 10 min, the supernatant was collected and then treated with RNase A (final concentration 0.1 mg/ml) for 20 min at 37 °C. The DNA of Bel-7404 cells was isolated using a phenol/chloroform/isopentanol method by vortex-mixing and centrifugation and then precipitated at −20 °C for 2 h with ethanol/sodium acetate. DNA samples were separated by 1.5% agarose gel electrophoresis.