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Prokaryotic expression and bioactivity analysis of N-terminus domain of *Pinellia ternata* agglutinin using alkaline phosphatase signal peptide

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ABSTRACT

Pinellia ternata agglutinin (PTA) from the tubers of *P. ternata* is a two-domain monocot mannose-binding lectin. *Pta-n* encoding N-terminus domain of PTA (PTA-N) was fused with *Escherichia coli* alkaline phosphatase signal peptide (APSP) gene by polymerase chain reaction (PCR) for secretion expression. The fused nucleotide sequence *apsp-pta-n* was inserted into pET-28a prokaryotic expression vector by restriction enzyme digest sites (*Nco* I and *Xho* I), and then overexpressed in *E. coli* BL21(DE3) cells by isopropyl β-D-1-thiogalactopyranoside (IPTG) induction. Expressed APSP targeted the recombinant protein APSP-PTA-N into the periplasmic space, and then APSP was recognized and automatically cleaved by the membrane-bound signal peptidase. Ni–NTA chromatography was used for the purification and about 20 mg/L purified PTA-N was obtained. The minimum agglutination concentration of PTA-N determined by mice erythrocytes was 6.33 ± 0.47 μg/ml. The carbohydrate inhibition assay was carried out to determine the carbohydrate-binding property indicating PTA-N bound to specific sugars. The *in vitro* anti-proliferative activity towards human tumor cell lines and anti-fungal activity against *Gibberella saubinetii* were also demonstrated. Nuclear staining assay was performed to demonstrate PTA-N induced cell apoptosis. The results showed that PTA-N had significant biological functions, similar to native PTA. This strategy was the first time used to express plant mannose-binding lectin proteins and the product induced human tumor cell apoptosis, suggesting its potential application in biomedicine research.

Introduction

Plant lectins are proteins possessing at least one non-catalytic domain that bind reversibly to specific mono- or oligosaccharides and their derivatives [1,2]. The history of plant lectins can be dated back to 1888 when Stillmark discovered a highly toxic protein in aqueous extracts of castor bean, which is a plant lectin, named ricin [1]. Plant lectins as defense proteins are widely distributed in tuber, seed and vegetative tissue of higher plants [1,3,4]. Although the precise physiological role of lectins in nature is still unknown, they have proved to be very valuable in a wide variety of applications *in vitro* including clinical diagnostics, identifying blood serotypes, preparation of bone marrow for transplantation, mitogenic stimulation of lymphocytes, and identification of stem cells *in vitro* [5,6]. The agglutination activity of these highly specific carbohydrate-binding molecules is usually inhibited by monosaccharides, but for some lectins, di, tri, and even polysaccharides are required [7]. The different agglutination properties of lectins are based on a specific carbohydrate-binding activity, and almost all of the carbohydrate-binding sites are conserved [8,9]. Plant lectins belong to a very heterogeneous group of glycoproteins which recognize and bind to carbohydrates or glycoproteins even in presence of various detergents. They belonging to different lectin families have very similar sequences and structural features [10,11]. In recent years, several plant lectins, especially monocot mannose-binding lectins have been studied to possess cytotoxic activity and to induce tumor cell apoptosis because they can interact with the complex carbohydrates on the cell membrane of tumor cell lines [7,12,13].

The mannose-binding lectin is the most commonly studied in the field of plant lectins. The snowdrop bulb lectin, which named *Galanthus nivalis* agglutinin (GNA) is the first monocot mannose-binding lectin isolated and characterized in details [14]. Nowadays the monocot mannose-binding lectins have been generally classified as GNA (*G. nivalis* agglutinin)-related lectins, which are distributed ubiquitously in higher plants and play a significant role in recognition of the high mannose type glycans [1].

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Pinellia ternata is a traditional Chinese medicinal plant belonging to *Pinellia*, *Araceae* species. *P. ternata agglutinin (PTA)*[1], a storage protein, accumulates in the tissues of *P. ternata*, especially in the tubers [15]. Yao [16,17] firstly obtained the full-length cDNA of *P. ternata* lectin and expressed PTA in tobacco. The result showed transgenic tobacco had enhanced resistance towards aphids. Jin [18] expressed PTA in tobacco chloroplasts, and also exhibited broad spectrum resistance against aphid, whitefly, lepidopteran insects, bacterial and viral pathogens. Bhat [19] cloned *Remusatia vivipara* lectin (RVL) from the tubers of *Remusatia vivipara* belonging to *Araceae* species, which exhibited the nematicidal activity. Later, the structure of RVL was determined by Shetty [20]. Recently, PTA and another plant mannose-binding lectins belonging to *Araceae* species have been cloned. Most of them are directly cloned into prokaryotic expression vector and expressed in *Escherichia coli*. However, the recombinant lectin proteins are insoluble inclusion bodies [16,21–25]. To date, no report shows plant mannose-binding lectins are solubly expressed in *E. coli*.

E. coli expression system is one of the most extensively used prokaryotic expression system. It has several advantages, including growth on inexpensive medium, high cell density and rapid biomass accumulation [26]. Secretion of expressed proteins into periplasm of *E. coli* has several advantages over intracellular production including enhanced biological activity, higher product stability and solubility, and N-terminus authenticity of the recombinant protein [27]. Biological activity is dependent on protein folding and, particularly if disulfide bonds must be formed, proper folding is unlikely in the reducing environment of cytoplasm. A series of enzymes such as disulfide-binding proteins and petidyl-prolyl isomerases exist in the *E. coli* periplasm [27,28]. In most cases, but not all the times, targeting the protein into the periplasmic space promotes downstream processing and folding [27,29]. Alkaline phosphatase signal peptide (APSP) has been proposed to play a significant role in the initial step of protein secretion in bacteria. Most secreted proteins of bacteria are known to be synthesized as cytoplasmic precursors containing an additional N-terminus extension called the signal peptide that is essential for protein membrane translocation. After translocation, the signal peptide is specifically recognized and automatically cleaved by a membrane-bound signal peptidase [26,30–32].

In a previous study, our group cloned the *pta* gene by RT-PCR and sequence analysis shows *pta* encodes a 257-amino acid peptide [33]. Native PTA purified from the tubers of *P. ternata* by mannose-Sepharose affinity column has been crystallized in our lab. The protein structure has been determined by X-ray diffraction (unpublished). The structure data of PTA shows that PTA is tetramer composed of two N-terminus domains (PTA-N) and two C-terminus domains (PTA-C) with a total molecular weight of 48 kDa. The natural signal peptide (M_1–A_{23}) at N-terminus, 8 amino acid residues (L_{134}–G_{141}) between two domains and 7 amino acid residues (S_{251}–A_{257}) at C-terminus of the lectin protein are not observed in the structure, indicating *pta* encodes a precursor, and then the precursor may be post-translationally cleaved. Finally, a tetramer is obtained containing two N-terminus domains and two C-terminus domains. It has already been reported in several plant lectins that they are tetramers composed of 4 domains (two N-terminus domains and two C-terminus domains) without the natural signal peptides [10,20]. To date, no report shows these mannose-binding plant lectins are solubly expressed in *E. coli*. In this paper, based on the structure information of PTA, we designed special PCR primers cloning *apsp* gene to the 5′ end of *pta-n*. Depending on the secretion function of the signal peptide, PTA-N is solubly expressed and targeted into the periplasm. The product exhibits biological functions, similar to native PTA purified from tubers of *P. ternata*.

Materials and methods

Materials

The tubers of *P. ternata*, human tumor cell lines, human normal cell line AAV-293, *Gibberella saubinetii*, sugars and pEASY-*pta* vector harboring *pta* gene were saved in the Institute of Bioengineering, College of Life Sciences, Zhejiang Sci-Tech University, China. The restriction enzymes and T4 DNA ligase were purchased from Fermentas. Primers and PCR reagents were synthesized or purchased from Shanghai Sangon. Ni–NTA column, *E. coli* strain DH5α, *E. coli* strain BL21 (DE3) and pET-28a expression vector were purchased from Novagen.

Construction of E. coli expression vector

PCR was performed to clone *apsp* (VKQSTIALALLPLLFTPVTKA, GenBank accession No. M13763.1) to the 5′ end of *pta-n* by special primers (Table. 1). Special forward primers (FP1, FP2 and FP3) containing *apsp* gene were used in 3-step PCR. Step I: pEASY-*pta* vector harboring *pta* gene (Genbank accession No. JF293072.1) as template, FP1 as a forward primer, RP as a reverse primer, PCR was performed under following condition: The template was denatured at 94 °C for 3 min, followed by 30 cycles of amplification (94 °C for 1 min, 55 °C for 30 s, 72 °C for 45 s), and finally, 72 °C for a 10-min extension. Step II: Amplified DNA fragment (F1) as template, FP2 as a forward primer, RP as a reverse primer, PCR condition was similar to Step I. Step III: Amplified fragment (F2) as template, FP3 as a forward primer, and RP as a reverse primer, PCR condition was also similar to Step I. Amplified DNA fragment (F3) was purified and doubly digested by restriction enzymes *Nco* I and *Xho* I, and then inserted into pET-28a expression vector which had been predigested by the same restriction enzymes. The recombinant expression vector pET-28a-*apsp-pta-n* was transformed into *E. coli* DH5α cells. The clone picked up from a solid Luria–Bertani (LB) medium plate containing kanamycin (100 μg/ml) was confirmed by DNA sequencing. The positive clone was finally transformed into *E. coli* BL21(DE3) cells for the expression of recombinant protein.

Overexpression and purification of PTA-N

E. coli BL21(DE3) cells harboring pET-28a-*apsp-pta-n* expression vector were grown in LB medium containing kanamycin (100 μg/

Table 1
Primers used for fusing apsp with pta-n in 3-step PCR. Forward primers (FP1, FP2, FP3) harboring apsp sequence and reverse primer (RP) were used for cloning apsp to 5′ end of pta-n by 3-step PCR. The full-length nucleotide sequence (66 bp) of apsp was underlined. The overlap regions of FP1 and pta-n, RP and pta-n were indicated by wavy line. The overlap regions of primers were indicated by shadow. The restriction enzyme digest sites (*Nco* I and *Xho* I) were bolded and italic.

Primer	Primer sequence (5′–3′)
FP1	5′-AATTTACCCCTGTGACAAAAGCCGCAGTGGGCACC-3′
FP2	5′- TAGCACTGGCACTCTTACCGTTACTGTTTACCCCTG-3′
FP3	5′-AG***CCATGG***TGAAACAAAGCACTATTGCACTGGCAC-3′ ***Nco* I**
RP	5′- AATAATA***CTCGAG***GCCGGGGAC -3′ ***Xho* I**

[1] *Abbreviations used:* PTA, Pinellia ternata agglutinin; PTA-N, N-terminus of *Pinellia ternata* agglutinin; PTA-C, C-terminus of *Pinellia ternata* agglutinin; APSP, Alkaline phosphatase signal peptide; PBS, Phosphate buffered saline; ORF, Open reading frame; LB, Luria-Bertani medium; RT-PCR, Reverse transcription-polymerase chain reaction; pI, Isoelectric point; IPTG, Isopropyl β-D-1-thiogalactopyranoside; MIC, Minimum inhibitory concentration; PDA, Potato dextrose agar; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO, Dimethyl sulfoxide; DMEM, Dulbecco's modified eagle's medium; FBS, Fetal bovine serum.