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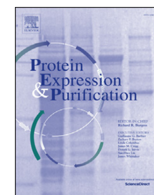

New findings in potential applications of tobacco osmotin

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The osmotin protein is involved in both monocot and dicot plant responses to biotic and abiotic stress. To determine the biological activity of osmotin, the gene was amplified from tobacco genomic DNA, fused with the hexahistidine tag motif and successfully expressed in *Escherichia coli*, after which the recombinant osmotin was purified and renatured. Various activities were then tested, including hemolytic activity, toxicity against human embryonic kidney cells, and the antifungal activity of the recombinant osmotin. We found that osmotin had no adverse effects on human kidney cells up to a concentration of 500 $\mu g.ml^{-1}$. However, the purified osmotin also had significant antimicrobial activity, specifically against fungal pathogens causing candidiasis and otitis, and against the common food pathogens.

Using the osmotin-*Agrobacterium* construct, the osmotin gene was inserted into tobacco plants in order to facilitate the isolation of recombinant protein. Using qPCR, the presence and copy number of the transgene was detected in the tobacco plant DNA. The transgene was also quantified using mRNA, and results indicated a strong expression profile, however the native protein has been never isolated. Once the transgene presence was confirmed, the transgenic tobacco plants were grown in high saline concentrations and monitored for seed germination and chlorophyll content as indicators of overall plant health. Results indicated that the transgenic tobacco plants had a higher tolerance for osmotic stress. These results indicate that the osmotin gene has the potential to increase crop tolerance to stresses such as fungal attack and unfavorable osmotic conditions.

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1. Introduction

Biologically active compounds have been extensively investigated for use in crops protection and against emerging antibiotic resistance. Currently there are two main approaches for studying such biologically active compounds, through either artificial synthesis or isolating the active compounds from natural sources. In general, plants serve as an ideal source of such biologically active compounds, including both antibiotics and compounds with other bioactivities [1]. The plant immune system consists of many different small compounds, peptides and proteins with specific or systematic roles which can be obtained by isolation or recombinant production.

Osmotin is an antifungal protein belonging to the 5th class of pathogenesis-related proteins and its structure is similar to thaumatin. Osmotin was first isolated from tobacco cells and described by Singh et al. (1987). Later, osmotin was also found in other plant species both in monocots and dicots, and many different types of osmotin functions have since been elucidated. Examples of the biological activity of osmotin include protection against osmotic stress response caused by higher salt concentration [2], cold [3,4] and drought [5,6]. Osmotin also plays a dual role in plant immune systems and it has a broad range of antifungal activity as well [7–9]. Since osmotin has such immune functions, it has potential applications for use as an anti-fungal preservative in the food industry, if it can be determined to be non-toxic. Additionally, due to the homology of the osmotin receptor to the adiponectin receptor [10,11], osmotin may be potentially used as a drug or drug template for pharmaceuticals associated with obesity, atherosclerosis and insulin resistance [12,13]. Many other potential applications for osmotin may exist, as most of its interactions remain unexplored (e.g. interaction with cytokinins [14], ecdysteroids or brassinosteroids [15]).

Despite the multitude of potential uses associated with osmotin, many important questions remain unanswered. Its antifungal activity has not been studied in relation to its activity against yeasts,

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an important food contaminants [9]. If the usage of osmotin is to become widespread, recombinant expression could provide a solution for its large-scale production. However, in recombinant bacterial production, osmotin's secondary structure contains eight disulfide bonds and it is almost impossible to prepare a native protein under the reductive conditions of *E. coli* cells. Some publications describe the usage of renaturation buffers after denaturation of osmotin [9,16] but we were not able to reproduce their results. Despite repeated efforts, we have never obtained renaturated protein. In another report, the authors did not consider the denaturated osmotin structure to be a problem and they did not take care about its structure [17].

The preparation of transgenic plants with improved resistance to adverse environmental conditions can play a significant role in resolving some global problems. The potential applications of the tobacco osmotin gene has been summarized in the literature [18]. However, the isolation of recombinant His-tagged osmotin from transgenic plant sources has never been published. Another unanswered question that remains is what happens when transgenic tobaccos overproduce osmotin genes. Liu et al. [19] reported that the constitutive accumulation of osmotin in transgenic tobacco did not lead to enhanced fungal resistance. On the other hand, research by Barthakur et al. [20] indicated an improved resistance of transgenic tobacco to high salt concentrations, and the same result was observed by Goel et al. and Sokhansanj et al. [2,21]. These publications and others have focused on the fact that osmotin expression is post-transcriptionally regulated through miRNAs [22–24].

The objective of this study is to find a renaturation buffer to produce the recombinant protein in its native structure using both bacterial and plant expression system, assess its activity against important yeasts, determine its toxicity, prepare transgenic tobacco and quantify its osmotin mRNA in correlation with its impact on salt stress resistance.

2. Material and methods

2.1. Cloning of osmotin gene into the bacterial expression system

The osmotin gene (accession number M29279 in NCBI database) was amplified from isolated template DNA (DNeasy® Plant Mini Kit, Qiagen, USA) of *Nicotiana tabacum* cv. Wisconsin 38 using the primers *OSM*/*Nde*I/F (5′-CGG AAT TCC ATA TGG CAA CTA TCG AGG TCC GAA AC-3′), *OSM*/*Bam*HI/R (5′-CTG CGG GAT CCC GGC TAA CCA TTA GGA CAA AAG ATA ACC-3′), and *Pfu* DNA polymerase (Promega, USA). The 643-bp-long amplicon was digested with the appropriate restriction enzymes (New England Biolabs, UK), *Nde*I and *Bam*HI respectively, and ligated with the vector pET-19b (Merck, Germany) using T4 DNA ligase (Promega, USA). The obtained vector was multiplied in *E. coli* DH5α cells (New England BioLabs, UK), isolated (QIAprep® Spin Miniprep Kit, Qiagen, USA) and used for the transformation of *E. coli* BL21 (DE3) cells (New England BioLabs, UK).

2.2. Expression of osmotin, its isolation and purification from bacterial cells

Recombinant osmotin was expressed in cells of the prepared bacterial strain *E. coli* BL21 (DE3)/pET-19b/*OSM*. The cells were cultivated in LB medium (Oxoid, USA) until the early exponential phase, when expression was induced by the addition of 0.4 mM IPTG and lasted for 3 h. After expression, cells were harvested by centrifugation and lysed in the following buffer: pH 8, 50 mM Tris, 1 mM EDTA (4 ml of buffer per 1 g of cells). The soluble and insoluble parts of the lysed cells were fractionated by centrifugation. The insoluble part was denatured for 1 h in the following buffer: pH 8, 0.1 M Tris, 6 M urea. Denatured osmotin was bound to immobilized nickel and purified using affinity chromatography (IMAC, Qiagen, USA). The binding to the column was caused by the presence of the hexahistidine tag from the pET-19b vector. The isolated protein was desalted using a PD 10 column (Sephadex G-25, GE Healthcare, USA) by an elution buffer: pH8, 0.1 M tris, 6 M Urea.

2.3. Renaturation of osmotin

In the first step, denatured osmotin was reduced with sodium sulfite, which was added to the final concentration of 0.3 M. The reduction was performed for 5 days at 4 °C. Immediately after reduction, osmotin was renatured via the method below. For the renaturation, the buffer composition was optimized by testing the addition of a range of different molecules. The choice of buffer additives was based on iFOLD® Protein Refolding Systems (Merck, Germany): Tris (50 mM, pH 7.5), citrate-phosphate (100 mM, pH 5), acetate (20 mM, pH 5.2), NaCl (100 mM, 250 mM), glutathione (ox./red. 3.8/1.2), Triton X-100 (1% v/v), glycerol (20%, v/v), PEG 6000 (0.1%, v/v), L-arginine (400 mM), glucose (500 mM), a cofactors cocktail (1 mM of NADH, thiamine HCl, biotin, $CaCl_2$, $MgCl_2$, $CuSO_4$, $ZnCl_2$, $CoSO_4$, ADP, $NiCl_2$), β-cyclodextrin (12.5 mM), DMSO (30%, v/v), guanidine HCl (500 mM), β-mercaptoethanol (10 mM), and EDTA (1 mM). Before use, each buffer was deaerated by bubbling with nitrogen for 10 min. The optimization of renaturation buffer composition was done in a microplate with a total volume of 200 μl. After the preparation of buffers and the addition of denatured osmotin in the ratio of 10:1, the microplate was incubated at room temperature for 24 h, after which the difference in absorbance at 340 nm between the buffer alone and the buffer with osmotin was calculated. The difference in absorbance values was less than 0.02%, meaning that osmotin did not precipitate. The large-scale renaturation was done via the dialysis of denatured osmotin (Spectra/por, Spectrum Laboratories, Canada) against a large excess of renaturation buffer for 24 h at 4 °C with continual stirring.

2.4. Confirmation of osmotin native structure

The obtained renatured recombinant protein was digested with trypsin, desalted and then analyzed using a MALDI TOF mass spectrometer (Bruker Daltonic, Germany). Peptide masses were assigned using the MASCOT software search engine (Matrix Science, London, UK) and compared with theoretical digestions of the sequence of recombinant osmotin and with the Swiss-Prot database (Swiss Bioinformatics Institute, Geneva, Switzerland) with taxonomy restricted to green plants. Protein score was calculated using MASCOT as $-10*\log(P)$, where P is the probability that the observed match is a random event. Protein scores greater than 58 were evaluated as significant ($P < 0.05$). The conformation of renatured protein structure was confirmed by reflectance measurement using an FTIR spectrometer (Nicolet 6 700, Thermo-Nicolet, USA) in tandem with an ATR cell (GladiATR, PIKE, USA) and DTGS detector with a distinction of 4 cm^{-1}. Infrared spectra were collected between 1700 and 1600 cm^{-1} (the area of secondary structures) using 64 scans per sample. The evaluation of secondary structure was based on the book „Infrared and Raman Characteristic Group Frequencies: Tables and Charts“ [25]. The number of disulfide bridges was determined by Raman spectroscopy. Spotted samples were analyzed with a disperse Raman microscope (DXR Nicolet, Thermo-Nicolet, USA) with excitation laser 532 nm and performance 10 mW, 5 × 10 s. The spectral range was 200–3500 cm^{-1} with the separation of peaks in the range 480–560 cm^{-1}. The number of disulfide bonds was determined

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