Protein Expression and Purification 125 (2016) 43–52

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

Production of bioactive wheat puroindoline proteins in *Nicotiana benthamiana* using a virus-based transient expression system

Azadeh Niknejad [a], Diane Webster [b], Mrinal Bhave [a,*]

[a] Faculty of Science, Engineering and Technology, Swinburne University of Technology, Melbourne, Victoria, Australia
[b] School of Biological Sciences, Monash University, Melbourne, Victoria, Australia

ARTICLE INFO

Article history:
Received 22 July 2015
and in revised form 20 August 2015
Accepted 8 September 2015
Available online 9 September 2015

Keywords:
Puroindolines
Grain texture
Antimicrobial proteins
magnICON® system

ABSTRACT

The emergence of antibiotic resistant pathogenic strains of bacteria has necessitated the development of novel antimicrobial agents. The puroindoline A and B (PINA and PINB) proteins of wheat, well-known for their roles in determining the important phenotype of grain texture, are also antimicrobial, making them attractive as natural bio-control agents. However, the biochemical basis of PIN functionality remains unclear due to limitations in expressing them at the required yield and purity and lack of accurate tertiary structure. This study focussed on rapid transient expression of PINs targeted to different subcellular compartments (chloroplast, apoplast, endoplasmic reticulum and cytosol) of *Nicotiana benthamiana* leaf cells using the deconstructed tobacco mosaic virus-based 'magnICON®' system. The expressed recombinant PINs were characterised by Western blot using the Durotest anti-friabilin antibody, enzyme-linked immunosorbent assays (ELISA) and antimicrobial activity tests. Maximum yield of the His-tagged PINs occurred when targeted to the chloroplast. Both PINs exhibited oligomeric and monomeric forms on gels, but Western blots with the widely used Durotest anti-friabilin antibody identified only oligomeric forms. Only the PINs purified by a hydrophobic interaction method exhibited monomeric forms with the anti-His tag antibody, indicating correct folding. Interestingly, the Durotest antibody did not bind to monomers, suggesting their epitope may be obscured. PINs purified by His-tag affinity purification under native conditions or by the hydrophobic method exhibited antimicrobial activities. The successful *in planta* expression and optimisation of purification will enable future studies to examine the detailed structure of the PINs and explore novel bio-control applications in health, food and/or agriculture.

1. Introduction

The emergence of antibiotic resistant pathogenic bacteria ('superbugs') worldwide and their implications to healthcare have flagged a need for the development of novel antimicrobial agents. Antimicrobial proteins and peptides (AMPs) are a key component in the innate immune responses of many organisms, evolved over millions of years, and often show a broad-spectrum activity [36]. Due to the minimal likelihood of emergence of resistance by a single mutation, these are amongst the leading candidates to substitute or complement antibiotic treatments. AMPs are typically short (⩽100 amino acid residues), amphipathic, and often rich in hydrophobic (e.g., tryptophan, phenylalanine) and/or basic residues [16]. Tryptophan in particular has unique biochemical properties that allow it to interact with and insert into biological membranes [39].

Two small (148 residue) proteins called puroindoline A (PINA; 16.387 kDa) and puroindoline B (PINB; 16.79 kDa), expressed in the grains of common wheat (*Triticum aestivum* L.) fall amongst AMPs. These are encoded by 447 bp intronless genes, *puroindoline a* and *b* (*Pina* and *Pinb*) [12]. Alleles of *Pin* genes are being investigated extensively due to the causative roles of PIN proteins in determining soft (wild-type) or hard (mutant) endosperm texture, a parameter critical for food functionality and world wheat trade. The molecular genetics of this trait has been reviewed [5]. However, the PINs are also unique in being basic, cysteine-rich, helicoid and antimicrobial. Their putative antimicrobial domain is considered to be the tryptophan-rich domain (TRD), and our previous work shows that the synthetic peptides designed based on it are antibacterial and antifungal, including against wheat stripe rust (*Puccinia striiformis*) and leaf rust (*Puccinia triticina*) spores and/or leaf infections [34,1,2]. The antimicrobial properties have also been

Abbreviations: Pin, *puroindoline* gene; PIN, puroindoline protein; TRD, tryptophan rich domain.

* Corresponding author at: Faculty of Science, Engineering and Technology, Swinburne University of Technology, PO Box 218, John Street, Hawthorn, Victoria 3122, Australia.
E-mail address: mbhave@swin.edu.au (M. Bhave).

http://dx.doi.org/10.1016/j.pep.2015.09.009
1046-5928/

confirmed *in vitro* for wheat flour-purified PINs and recombinant PINs expressed in *Escherichia coli* host cells [6,27] and also transgenically in wheat [19] and cereal crops which do not innately encode PINs, e.g., corn [49]. PINB expressed in *Nicotiana tabacum* chloroplasts also exhibits activity against *E. coli* [41].

Many investigations confirming specificity to bacterial and/or fungal cells makes PINs attractive as natural biocidal agents for applications in health, hygiene, food and/or agriculture industries. However, despite this potential, PINs have not been purified in high enough yields and purity as yet from wheat flour or the bacterial or eukaryotic expression systems. Hence in-depth analysis of the biochemical basis of their effects on grain texture, and antimicrobial properties and explorations of such applications, remain elusive. The biochemical basis behind their intriguing functionality, i.e., interdependence of PINA and PINB in determining the wheat grain texture [43], is largely unresolved. Expression in a yeast two-hybrid system suggests involvement of a smaller hydrophobic domain and/or residues outside the TRD [4]. However, the two proteins occur together in the grain and cannot be purified individually, and each needs to be expressed in appropriate quality and quantity, making this analysis difficult. Protein expression in adequate quantities and purity is essential for a number of biochemical investigations of proteins such as sequencing, epitope mapping, testing of functionality including enzymatic and antimicrobial activities, and tertiary structure determinations. Several industrially and therapeutically important proteins have been expressed successfully in plant systems in recent years; however, low yield and inefficient purification methods are the major challenges for their economical production [8]. The present work aimed to clone and transiently over-express wheat PINA and PINB in leaves of *Nicotiana benthamiana*, using the magnICON® expression system [25]. The proteins were targeted to different intracellular compartments, and various strategies were applied for optimising their expression and purification. The work has led to successful expression of PINs that exhibited some unique biochemical properties and antimicrobial activity.

2. Materials and methods

2.1. The magnICON® vector modules

The magnICON® system, based on deconstructed tobacco mosaic virus (TMV) and designed for *in planta* assembly of separate modules (ICON Genetics, Germany; http://www.icongenetics.com/html/home.htm; last accessed April 2015) was used for transient expression of PINA and PINB in the leaves of *N. benthamiana*, a close relative of tobacco that is indigenous to Australia. The following modules were used: (i) two 3′ modules, i.e., pICH11599 for cloning of *Pin* genes, and pICH7410 that encodes the Green Fluorescent Protein (GFP); (ii) three 5′ modules, i.e., pICH15879, pICH12190 and pICH8420, containing the viral replicase encoding gene and the targeting pre-sequences for localisation of the expressed protein in the cytosol, chloroplast or apoplast, respectively; (iii) the module pICH14011 encoding integrase, for *in planta* recombination of a 3′ and a 5′ module, followed by transcription of the resulting DNA and splicing of an intron and the recombination sites, to create an RNA replicon expressing the desired protein (Fig. S1). The vectors and their past applications have been described elsewhere [13,25,45].

2.2. Amplification of Pin genes and cloning into the pICH11599 module

The genes *Pina-D1a* (Genbank DQ363911) and *Pinb-D1b* (DQ363913), encoding the putative wild-type full-length PINA and PINB, were amplified from the genomic DNA of the soft wheat cultivar *T. aestivum* cv. Rosella. These PCR products were used as templates to amplify the sections encoding the putative mature PINA (363 bp, 120 residues, 13.44 kDa) and PINB (360 bp; 119 residues; 13.6 kDa) proteins (i.e., without their 28 and 29 residue signal peptides, respectively), and with C-terminal His or His-SEKDEL tags (Table 1). For cloning of *Pina*, the sequence for the EcoRI restriction site was incorporated at the 5′ end of the forward primer and that for BamHI at the 5′ end of the reverse primer. For *Pinb*, an NcoI site was incorporated in the forward primer and SacI in reverse primer. Both reverse primers contained a sequence for a C-terminal six-histidine tag (His tag). Another reverse primer was also designed for each gene such that it also encoded the conserved eukaryotic C-terminal endoplasmic reticulum (ER) retention signal [29], SEKDEL (Ser-Glu-Lys-Asp-Glu-Leu) (a His-SEKDEL tag). A few spacer nucleotides were added flanking the restriction sites to allow proper digestions (Table 1). The PCR products were purified using HiYield™ gel/PCR fragments extraction kit (Real Biotech Corporation, Taiwan). The PINA-His, PINA-His-SEKDEL and PINB-His-SEKDEL PCR products were double-digested with EcoRI + BamH1, the PINB-His products double-digested with NcoI + SacI, and all digests ligated with respectively double-digested pICH11599. The

Table 1
Primers used for amplification and cloning of *Pin* genes.

Amplicon	Primer name and sequence
Pina-D1a full length	Pina-D1 Forward: 5′-**ATG**AAGGCCCTCTTCCTCA-3′ Pina-D1 Reverse: 5′-**TCA**CCAGTAATAGCCAATAGTG-3′
Pinb-D1a full length	Pinb-D1 Forward: 5′-**ATG**AAGACCTTATTCCTCCTA-3′ Pinb-D1 Reverse: 5′-**TCA**CCAGTAATAGCCACTAGGGAA-3′
Pina-His	Pina-D1H Forward: 5′-GGTA*GAATTC*G*ATG*GATGTTGCTGGCGGGGGTG-3′ Pina-D1H Reverse: 5′-CCG*GGATCCTCA***ATGGTGATGGTGATGGTG**CCAGTAATAGCCAATAGTGCC-3′
Pinb-His	Pinb-D1H Forward: 5′-GAA*CCATG*GAAGTTGGCGGAGGAGGTGG-3′ Pinb-D1H Reverse: 5′-CCG*GAGCTCTCA***ATGGTGATGGTGATGGTG**CCAGTAATAGCCACTAGG-3′
Pina-His-SEKDEL	Pina-D1-HS Forward: 5′-GGTA*GAATTC*G*ATG*GATGTTGCTGGCGGGGGTG-3′ Pina-D1-HS Reverse: 5′CCG*GGATCCTCA***TAGCTCATCTTTCTCAGAATGGTGATGGTGATGGTG**CCAGTA3′
Pinb-His-SEKDEL	Pinb-D1-HS Forward: 5′-GAA*GAATTC*G*ATG*GAAGTTGGCGGAGGAGGTGG-3′ Pinb-D1-HS Reverse: 5′CCG*GGATCCTCA***TAGCTCATCTTTCTCAGAATGGTGATGGTGATGGTG**CCAGTA3′

Restriction sites incorporated for directional cloning are shown in italics and shade. Nucleotides in bold and shade encode His-tag in the sense strand. Nucleotides in bold and underline encode SEKDEL in the sense strand. Nucleotides in italics and wave underline encode start and stop codons. The first residue of mature PINs is boxed.