Protein Expression and Purification 130 (2017) 90–99

Contents lists available at ScienceDirect

Protein Expression and Purification

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

Recombinant expression, purification and antimicrobial activity of a novel antimicrobial peptide PaDef in *Pichia pastoris*

De-Mei Meng [a], Jing-Fang Zhao [a], Xiao Ling [a], Hong-Xia Dai [a], Ya-Jun Guo [a], Xiao-Fang Gao [a], Bin Dong [a], Zi-Qi Zhang [a], Xin Meng [d], Zhen-Chuan Fan [a, b, c, *]

[a] China International Science and Technology Cooperation Base of Food Nutrition/Safety and Medicinal Chemistry, Key Laboratory of Food Nutrition and Safety, Ministry of Education of China, Tianjin University of Science & Technology, Tianjin, 300457, People's Republic of China
[b] Tianjin Food Safety & Low Carbon Manufacturing Collaborative Innovation Center, Tianjin, 300457, People's Republic of China
[c] Obesita & Algaegen LLC, College Station, TX, 77845, USA
[d] College of Biotechnology, Tianjin University of Science & Technology, Tianjin, 300457, People's Republic of China

ARTICLE INFO

Article history:
Received 22 June 2016
Received in revised form
29 September 2016
Accepted 8 October 2016
Available online 11 October 2016

Keywords:
PaDef
Antimicrobial peptide
Pichia pastoris
Secreted expression
Antibacterial activity

ABSTRACT

The antimicrobial peptide PaDef was isolated from Mexican avocado fruit and was reported to inhibit the growth of *Escherichia coli* and *Staphylococcus aureus* in 2013. In this study, an N-terminal 6 × His tagged recombinant PaDef (rPaDef) with a molecular weight of 7.5 KDa, for the first time, was expressed as a secreted peptide in *Pichia pastoris*. The optimal culture condition for rPaDef expression was determined to be incubation with 1.5% methanol for 72 h at 28 °C under pH 6.0. Under this condition, the amount of the rPaDef accumulation reached as high as 79.6 μg per 1 ml of culture medium. Once the rPaDef peptide was purified to reach a 95.7% purity using one-step nickel affinity chromatography, its strong and concentration-dependent antimicrobial activity was detected to be against a broad-spectrum of bacteria of both Gram-negative and Gram-positive. The growth of these bacterial pathogens was almost completely inhibited when the rPaDef peptide was at a concentration of as low as 90 μg/ml. In summary, our data showed that rPaDef derived from Mexican avocado fruit can be expressed and secreted efficiently when *P. pastoris* was used as a cell factory. This is the first report on heterologous expression of PaDef in *P. pastoris* and the approach described holds great promise for antibacterial drug development.

1. Introduction

Antimicrobial peptides (AMPs) generally are short peptides generated by the innate immune system of many different species including animal, plant and even fungus and confer specific immunity against microbial invaders [1,2]. Thus far, AMPs are known to be able to effectively prevent pathogen invasion, possess broad-spectrum antimicrobial activity against Gram-positive bacteria, Gram-negative bacteria, fungi, parasites and viruses and were also shown to inhibit the growth of even tumor cells [3–6]. One of the most notable features of AMPs is that these functional short peptides rarely induce bacterial resistance which is a serious problem with conventional antibiotics [7,8]. Therefore, AMPs have emerged as one of the most promising candidates for a new class of antibiotics to be clinically used in the future [9–11].

Preparative isolation of antimicrobial peptides from natural sources is obviously not an economically efficient way since a large quantity of antimicrobial peptides is potentially required for pharmaceutical applications [12,13]. Over the past several decades, several cell factory systems have been applied for the economical production of antimicrobial peptides [14,15]. As one of the major systems, *Escherichia coli* have been taken advantage to produce recombinant antimicrobial peptides of more than 80% cases globally [15]. However, the problem for this prokaryotic system is that *E. coli* is not a suitable host for small peptides to be expressed at high concentrations and be recovered from the

* Corresponding author. College of Food Engineering and Biotechnology, Tianjin University of Science & Technology, No. 29 13rd Rd., Tianjin Economy-and-Technology Development Area, Tianjin, 300457, People's Republic of China.
E-mail address: fanzhen@tust.edu.cn (Z.-C. Fan).

http://dx.doi.org/10.1016/j.pep.2016.10.003
1046-5928/

expression system, especially for the toxic peptides like AMPs [16]. In the past two decades, the methylotrophic yeast, *Pichia pastoris*, has been developed as an excellent cell factory for large-scale expression of proteins including short peptides of different sources [17]. Different from *E. coli*, *P. pastoris* serves as an eukaryotic expression system and has been used successfully to produce a large variety of functional recombinant proteins of human, animal, plant, fungal, bacterial and viral origins [18]. There were also numerous reports showed that *P. pastoris* is a suitable cell factory to produce AMPs including shrimp paenedin, human cathelicidin CAP18LL-37, Enterocin P, Cecropin A and so on [19–22]. Of most importance, the expression of a secreted form of recombinant AMPs in *P. pastoris* further offers several advantages over bacterial expression systems. These include appropriate folding of AMP molecules, disulfide bond formation and correct execution of post-translational modifications which conserve protein functions [23]. In addition, secretion of recombinant AMPs circumvents intracellular accumulation in *P. pastoris* cells and simplifies the purification by avoiding contamination with intracellular proteins [24–27]. These advantages together make secreted recombinant AMP production in *P. pastoris* a popular strategy for scientific research.

Persea americana var. *drymifolia* defensin (PaDef) is a 45 amino acid peptide originally isolated from Mexican avocado fruit in 2013 [28]. The amino acid sequence of PaDef has a sequence homology even as high as 80% to plant defensins, suggesting that PaDef is a type 1 defensin [28]. In the same study, the recombinant PaDef expressed in the bovine endothelial cell line BVE-E6E7 was shown to be able to inhibit the growth of *Escherichia coli* and *Staphylococcus aureus*, suggesting that PaDef is a functional AMP against not only Gram-positive bacteria but also Gram-negative bacteria. Regarding the effects on *S. aureus* viability, they observed a 52–65% inhibition of viability when 100 μg/ml total proteins from clones were used to treat bacteria [28]. In this study, an expression construct with the insertion of an N-terminal 6 × His tagged PaDef coding sequence was created in a *P. pastoris* expression vector pPICZαA backbone and transformed into an engineered yeast strain *P. pastoris* GS115. By taking advantage of this eukaryotic protein expression system, we seek to investigate, for the first time, whether *P. pastoris* is a suitable cell factory system of high efficiency to produce functional recombinant PaDef and if so, how broad the antibacterial spectrum of the yeast-expressed recombinant PaDef briefly is by testing it on several representative bacteria of both Gram-positive and Gram-negative.

2. Materials and methods

2.1. Plasmids, strains and growth medium

The yeast expression vector pPICZαA was bought from Invitrogen (Carlsbad, CA, USA). *Pichia pastoris* strain GS115 (ATCC 20864), *Listeria monocytogenes* (ATCC 21633), *Salmonella* (ATCC 10467), *Escherichia coli* O157 (ATCC 35150), *Escherichia coli* (ATCC 10305) and *Staphylococcus aureus* (ATCC 25923) were purchased from America Type Culture Collection (http://www.atcc.org/). *Escherichia coli* strain XL1-blue (Stratagene, La Jolla, CA, USA) were purchased commercially. *Bacillus subtilis* strain 151-1, *Bacillus subtilis* LZZ-133, *Bacillus subtilis* L300-1, *Enterobacter aerogenes*, *Enterococcus faecalis* and *Enterobacter sakazakii* were gifts from China Agricultural University (Beijing, China). *P. pastoris* transformants were selected on YPDS (2% peptone, 1% extract yeast, 2% dextrose, 1 M sorbitol and 2% agar) plus Zeocin (100 or 200 μg/ml) agar plates. Shake-flask expression of recombinant PaDef was achieved by growing *P. pastoris* clones in BMGY (2% tryptone, 1% yeast extract, 1.34% YNB, 4×10^{-5}% biotin, 1% glycerol and 100 mM potassium phosphate, pH 6.0) and BMMY (2% peptone, 1% yeast extract, 100 mM potassium phosphate, 1.34% YNB, 4×10^{-5}% biotin and 1% methanol, pH 6.0) media. All tested strains in the antimicrobial effect assay were grown at Luria-Bertani (5 g/l yeast extract, 10 g/l tryptone, and 10 g/l NaCl) media. The *E. coli* cells transformed with plasmids were cultured in low salt Luria-Bertani medium (5 g/l yeast extract, 10 g/l tryptone, and 5 g/l NaCl) containing 25 μg/mL of Zeocin.

2.2. Reagents and materials

DNA marker, T4 DNA ligase and the restriction enzymes *Eco*RI, *Kpn*I and *Sac*I were purchased from Fermentas (Carlsbad, CA, USA). Zeocin™ was obtained from Invitrogen (Carlsbad, CA, USA). The Gel Extraction kit, Plasmid Miniprep kit and Cycle-Pure kit were purchased from Solarbio (Beijing, China). Ni Sepharose™ 6 Fast Flow were obtained from GE Healthcare (Fairfield, MA, USA). Ampicillin was purchased from Solarbio (Beijing, China). Anti-6 × His antibody monoclonal antibody was purchased from Beyotime Institute of Biotechnology (Shanghai, China). Peroxidase AffiniPure Goat Anti-Rat IgG (H + L) secondary antibody was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). Soluble TMB (3′,3′,5′,5'-tetramethylbenzidine) kit was purchased from Beijing ComWin Biotech Co., Ltd. (Beijing, China).

2.3. Recombinant plasmid construction

The amino acid (APD ID: AP 02332) and gene (Accession KC007441) sequence of PaDef was obtained from the Antimicrobial Peptide Database (http://aps.unmc.edu/AP/) and GenBank (https://www.ncbi.nlm.nih.gov/genbank/), respectively. The optimized codon sequence of PaDef for *P. pastoris* was synthesized by GENEWIZ (Suzhou, China) and inserted into pUC19 vector. To facilitate the upcoming purification of the recombinant PaDef, a 6 × His tag-encoding sequence was in-frame fused to the 5′-end of the PaDef coding sequence. Other than this, the nucleotide sequence for the restriction site *Eco*RI were sequentially incorporated at the 5′-end of the synthesized oligonucleotides and the *Kpn*I restriction site sequence were in order added to the 3′-end of the PaDef coding sequence, thus resulting in a 186 bp DNA fragment shown as in Fig. 1A. This DNA fragment was then inserted into pUC19 backbone, resulting in pUC19-PaDef. Afterwards, the inserted fragment was digested with restriction enzymes *Eco*RI and *Kpn*I and ligated into the linearized pPICZαA (Invitrogen, CA, USA), leading to the generation of the *P. pastoris* expression vector pPICZαA-PaDef, which was verified by both restriction endonuclease analysis and direct nucleotide sequencing. The strategy for pPICZαA- PaDef construction was shown in Fig. 1B.

2.4. P. pastoris transformation and PCR analysis of P. pastoris transformants

P. pastoris was transformed by electroporation as described previously [29]. In brief, 2 μg of *Sac*I-linearized pPICZαA-PaDef was mixed with 80 μl of competent *P. pastoris* cells. The cell mixture was then transferred to an ice-cold 0.2 cm electroporation cuvette (Bio-Rad Laboratories Inc, Philadelphia, PA, USA) and kept on ice for 5 min. The cell mixture was then pulsed at 1500 V, 25 μF of capacitance and 200 Ω of resistance for 5 msec by using a Gene Pulser Xcell apparatus (Bio-Rad Laboratories Inc, Philadelphia, PA, USA). One milliliter of ice-cold sorbitol (1 M) was immediately added to the cuvette following electroporation. At last, every 200 μl of aliquots were spread on separate yeast YPDS plates containing 100 μg/ml of Zeocin. Plates were incubated for 2–3 days at 28 °C until colonies formed.

The rPaDef-positive *P. pastoris* transformants were screened by