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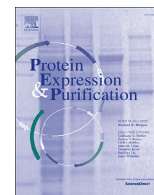

Comparison of inducible versus constitutive expression of plectasin on yields and antimicrobial activities in *Pichia pastoris*

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ABSTRACT

Background: Plectasin might serve as a substitute for traditional antibiotics, but its yields and antimicrobial activities warrant further investigation.
Objective: To identify the influence of inducible versus constitutive expression of plectasin on yields and antimicrobial activities.
Methods: Through SOE-PCR, a recombinant plectasin gene was generated and inserted into inducible (pPICZαA) and constitutive (pGAPZαA) vectors in order to create *Pichia pastoris* GS115 strains. After 120 h of fermentation, supernatants were purified by an AKTA purifier using nickel columns. Minimal inhibitory concentration (MIC) and inhibition zone assays were performed after Tricine-SDS-PAGE.
Results: After 120 h of fermentation, the yield of constitutive plectasin (370 μg/ml) was much lower than that from inducible vector (880 μg/ml) ($P < 0.05$). However, constitutive strain reached its plateau phase faster and keep more consistent yield ($P < 0.05$). The MICs of inducible plectasin against Methicillin-resistant *Staphylococcus aureus* (MRSA) 15471118, vancomycin-resistant *Enterococcus feces* (VREF), and penicillin-resistant *Streptococcus pneumonia* (PRSP) 31355 were 64, 32, and 64 μg/ml, respectively, while those of constitutive plectasin were 4, 4, and 16 μg/ml. No significant differences were observed in antimicrobial activities between inducible and constitutive plectasin for MRSA 15471118, VREF and PRSP 31355 (all $P > 0.05$). However, constitutive plectasin had a larger inhibition zone than inducible plectasin with the same mass.
Conclusions: Although *P. pastoris* GS115 (pGAPZαA-Plectasin-GS115) had lower expression than *P. pastoris* GS115 (pPICZαA-plectasin-GS115), it reached the plateau phase faster, had steadier yields and showed superiority in antimicrobial activities. Therefore, pGAPZαA might be more suitable for expression of plectasin in GS115 compared with pPICZαA.

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1. Introduction

Antimicrobial resistance (AMR) has become a global problem in medicine, especially in China. A report from Chinese Ministry of Health National Antimicrobial Resistance Investigation Net (Mohnarin) 2004–2005 showed that 40.7% of the *Streptococcus pneumoniae* strains were resistant (10.5%) or intermediate (30.2%) to Penicillin. Furthermore, five strains of *Enterococci* were reported to be Vancomycin-intermediate and Methicillin-resistant *Staphylococci aureus* also turned out to be one of most sharp problems [1].

The crisis of AMR has triggered researches on the efficacy of treatment with antibiotic substitutes such as antimicrobial peptides (AMPs). In 2014, over 100 new peptides were added to the AMP database, expanding the total number of species representation to 2493 and some have been validated to serve as new therapeutic drugs [2,3]. Among them, plectasin is the first AMP extracted from fungus in 2005. It has verified advantages, such as strong pH stability, high-thermal stability, and potent antimicrobial activity against gram-positive bacteria, including *Staphylococcus*

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aureus, *S. pneumoniae* and *Streptococcus suis*, even some antibiotic-resistant strains [4,5].

Up to now, most researches on plectasin have focused on molecular modifications to enhance its yields and antimicrobial activity. The popular expression platforms were commonly known as prokaryotic expression system such as *Escherichia coli* and eukaryotic expression system such as *Pichia pastoris*. Unfortunately, the fact that expression platforms might also exert an influence on yields and antimicrobial activities of plectasin has been under-evaluated [5,6].

Our previous studies built on *E. coli* have shown high yields but less effective antimicrobial activities [7,8], which is mainly attributed to the limitation that *E. coli* lacks the ability to modify protein's advanced structures. In contrast, *P. pastoris* possesses the ability to modify protein, utilize signal peptides and yield endogenous protein [9].

In *P. pastoris* expression system, the most commonly used expression vectors include inducible vector pPICZαA and constitutive vector pGAPZαA. pPICZαA contains the pAOX-1 promoter, while pGAPZαA contains pGAP promoter. Many researchers have utilized pAOX-1 to adjust plectasin expression, and achieved positive results to some degree [6,11,12]. However, choice of the pAOX-1-adjusted *P. pastoris* platform requires rapid growth of recombinant strains under conditions with addition of methanol [13]. Methanol would be the only carbon sources during the expression of plectasin [10]. Furthermore, methanol in the medium might influence the activity of the secreted protein. However, application of the strong pGAP promoter could avoid the limitations associated with pAOX-1, influence the *P. pastoris* platform to produce target protein in a steady and highly efficient manner, and remove the potential negative consequences of methanol addition [14]. These positive features of pGAP suggest its good potential for promoting the efficient expression of plectasin in industrial applications.

Until now, the relative influence of inducible versus constitutive expression systems on yields and antimicrobial activities of plectasin has not been elucidated. Therefore, we make a comparison with respect to the expression of recombinant plectasin in *P. pastoris*, with the aim of identifying a better expression platform for future industrial applications.

2. Materials and methods

2.1. Sources of strains, vectors, reagents, and restriction enzymes

2.1.1. Strains

DH5α (TaKaRa, Dalian, China), *P. pastoris* GS115 (Invitrogen, Carlsbad, CA, USA). Methicillin-resistant *S. aureus* (MRSA 15471118), vancomycin-resistant *Enterococcus feces* (VREF), and penicillin-resistant *Streptococcus pneumonia* (PRSP 31355) were isolated from clinical infections with support by the Southern Hospital (Guangzhou, China).

2.1.2. Vectors

pPICZαA and pGAPZαA (Invitrogen).

2.1.3. Reagents

Premix Taq (TaKaRa, Shiga, Japan), Gel Extraction Kit, Cycle-Pure Kit, Plasmid Mini Extraction KitI (Omega Bio-Tek, Norcross, GA, USA), Tricine-SDS-PAGE kit (CWBIO, Guangzhou, China), Zeocin (Invivogen, Toulouse, France), sorbitol (Solarbio, Beijing, China), dithiothreitol (DTT) (Genview Scientific Inc., El Monte, CA, USA), lithium acetate (Aladdin, Shanghai, China), yeast nitrogen base YNB, biotin, and vancomycin (Xiang Bo Biological, Guangzhou, China), fetal calf serum (FPS) (Gibco, Gaithersburg, MD, USA), agarose (Gene Company, Hong Kong, China), DNA marker (TaKaRa, Shiga, Japan), HisTrap FF crude (GE Healthcare, Life Sciences, Piscataway, NJ, USA), and Mueller-Hinton Broth (Oxoid, Waltham, MA, USA).

2.1.4. Enzymes

*Xho*I, *Xba*I, and *Bgl*II (TaKaRa, Shiga, Japan), T4 DNA Ligation Kit Ver.2.1(TaKaRa, Shiga, Japan), and protein marker (TaKaRa, Shiga, Japan).

2.2. Expression of the synthetic plectasin gene

According to Mandal et al. [12], the amino acid sequence of plectasin is: GFGCN GPWDE DDMQC HNHCK SIKGY KGGYC AKGGF VCKCY. According to the codon preference for *P. pastoris* expression [15], the gene sequence of plectasin was designed as follows:

5′ − GGT TTC GGT TGT AAC GGT CCA TGG GAC GAG GAC GAC ATG CAA TGT CAC AAC CAC TGT AAG TCC ATC AAG GGT TAC AAG GGT GGT TAC TGT GCT AAG GGT GGT TTC GTC TGT AAG TGT TAC − 3′.

To generate this fragment, we used gene splicing by overlap extension PCR (SOE-PCR) [16], and designed the templates and the primers, which were synthesized by Beijing Genomics Institute (Shenzhen, China) (Table 1). The amplification protocol was as follows: pre-denaturation for 4 min at 94 °C, denaturation for 1 min at 94 °C, renaturation for 30 s at 55.4 °C, and extension for 30 s at 72 °C for 28 cycles, followed by continuous extension for 10 min at 72 °C and holding for 10 min at 4 °C. The templates of the second PCR were based on the product of the SOE-PCR, and the protocol was as follows: pre-denaturation for 4 min at 94 °C, denaturation for 1 min at 94 °C, renaturation for 30 s at 50 °C, extension for 30 s at 72 °C for 30 cycles, followed by continuous extension for 10 min at 72 °C and holding for 10 min at 4 °C. After the target gene was synthesized by SOE-PCR, 1% agarose gel electrophoresis was performed to identify the product, which was excised and purified.

2.3. Construction and identification of recombinant plasmids

pPICZαA-DH5α and pGAPZαA-DH5α were each incubated in 5 ml LB medium, 37 °C, 75× g. After shaking overnight, a Plasmid Mini Extraction Kit was used to extract the plasmid (Fig. 1) and obtain purified pPICZαA and pGAPZαA. After 2.5 h digestion with *Xho*I and *Xba*I at 37 °C, the products of digestion were identified by electrophoresis on a 1% agarose gel and purified. The target gene and vectors were each ligated in a mixture with a mass ratio of 10:1 in 10 μl final volume and incubated at 16 °C for 30 min. The ligation products were each chemically transformed into competent DH5α to construct the recombinant plasmids. After growing on Low salt LB plates with 25 μg/ml Zeocin, the constructs in single colonies were identified by colony-PCR and were sent to Beijing Genomics Institute (Shenzhen, China) to determine their sequences.

2.4. Construction of recombinant Pichia

After linearization of the pPICZαA-plectasin and pGAPZαA-plectasin constructs with *Bgl*II, the products purified from mixture by the Cycle-pure kit were transformed into competent GS115 yeast by electroporation. The mixtures were incubated on yeast extract, peptone, and dextrose with 1.2 M sorbitol (YPDS) plates with 0, 10, 30, 70, and 100 μg/ml Zeocin. The genomic DNAs of grown single colonies were exposed via the “Cooking-Frozen-Cooking” method and were identified by PCR to identify positive recombinants [17]. Mixtures of each positive recombinant and glycerol were preserved at −20 °C for future expression analysis.

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