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A eukaryotic expression strategy for producing the novel antimicrobial peptide PRW4

Na Zhan [1] · Tianyu Wang [1] · Licong Zhang [1] · Anshan Shan [1]

Abstract

The antimicrobial peptide PMAP-36 is a cationic peptide derived from porcine myeloid. The N-terminally paired lysine of PMAP-36 was substituted with tryptophan, and the C-terminal hydrophobic tail was deleted, thereby obtaining the antimicrobial peptide PRW4. PRW4 is a α-helical antimicrobial peptide with broad-spectrum antimicrobial activity. In this study, PRW4 was fused to the 6× His-Trx, and the fusion protein was successfully expressed in *Pichia pastoris* GS115 from the vector pPICZαA. The maximal induction of recombinant protein occurred in the presence of 1% methanol after 96 h at pH 6.0. After purification by a Ni-NTA resin column and digestion by enterokinase protease, 15 mg of recombinant PRW4 with a purity of 90% was obtained from 1 L of fermentation culture. The results indicated that recombinant PRW4 had similar antimicrobial activity as synthetic PRW4 against bacteria such as *Escherichia coli* ATCC 25922, *Escherichia coli* UB 1005, *Salmonella typhimurium* C7731, *Salmonella typhimurium* 7913, *Salmonella typhimurium* ATCC 14028, *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 12228, and *Streptococcus faecalis* ATCC 29212. We have successfully expressed PRW4 in *P. pastoris*, and this work provides a reference for the production of modified antimicrobial peptides in *P. pastoris*.

Keywords Antimicrobial peptides · PRW4 · *Pichia pastoris* · Antimicrobial activity · Gene expression

Introduction

For decades, edible antibiotics have been used in the livestock industry. However, the long-term use of antibiotics, as well as the abuse of these compounds, has contributed to the increase in bacterial resistance, threatening public health [1, 2]. Therefore, it is necessary to find new types of antibiotic substitutes that can be applied to livestock production [3].

In recent decades, antimicrobial peptides (AMPs) have drawn increasing attention due to their rapid and broad-spectrum activities against gram-negative and gram-positive

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bacteria, fungi, viruses, etc. In addition, the antimicrobial mechanism of AMPs is different from that of antibiotics. AMPs physically destroy the cell membrane structure of bacteria and leak the contents of cells to kill cells, so it is difficult to acquire drug resistance [4–6]. Therefore, AMPs are an effective alternative to antibiotics [6].

However, the expensive synthesis cost of AMPs greatly limits their application in livestock production. Recombinant DNA technology as a low-cost, high-efficiency method for large-scale protein production provides a possible opportunity for AMPs to be used in livestock production [7]. For decades, many protein expression systems, such as *E. coli*, *Bacillus subtilis*, and *P. pastoris*, have been widely used to generate recombinant proteins [8, 9]. As a eukaryotic expression system, *P. pastoris* can provide suitable environment and conditions for the folding, glycosylation, and other post-translational modifications of foreign proteins to ensure the activity of the proteins, which are not available in prokaryotic expression systems such as *E. coli* and *B. subtilis* [10]. In addition, the *P. pastoris* expression system can secrete the expressed foreign protein extracellularly, so it is beneficial for later separation and purification. Therefore, the *P. pastoris* expression system is more convenient, practical,

and efficient for the expression of AMPs and has more industrial development potential than other expression systems. Many natural AMPs have been successfully expressed in the *P. pastoris* expression system, such as plectasin, PMAP-36, fowlicidin-2, snakin-1, and abaecin [11–17].

The antimicrobial peptide PRW4, designed and engineered by our laboratory, is a short peptide derived from amino acids 2–17 of the porcine antimicrobial peptide PMAP-36, and lysines at positions 7 and 11 are substituted with tryptophan to obtain better stability. PRW4 (RFRRLRWKTRWRLKKI-NH$_2$), with a molecular weight of 2299.8 Da, shows broad-spectrum antimicrobial activity; therefore, PRW4 has the potential to be a new class of antimicrobial drugs [18]. However, there are no means to produce PRW4 in large quantities at a low cost, which is the most important factor limiting its application in production.

In this study, we established an efficient method to express the antimicrobial peptide PRW4 in the *P. pastoris* expression system.

Materials and methods

Strains, vectors, culture medium, and reagents

E. coli DH5α used to construct the recombinant vector was purchased from Invitrogen (Carlsbad, USA). *P. pastoris* GS115 used as the expression host was kept by our laboratory. *E. coli* ATCC 25922, *E. coli* UB 1005, *S. typhimurium* C7731, *S. pullorum* 7913, *S. typhimurium* ATCC 14028, *S. aureus* ATCC 29213, *S. epidermidis* ATCC 12228, and *S. faecalis* ATCC 29212 were kept by our laboratory and were used for the antimicrobial activity assay. The expression vector pPICZαA was purchased from Invitrogen (Carlsbad, USA). All of the above strains were stored in 30% glycerol at − 80 °C.

Luria-Bertani (LB) medium, yeast extract peptone dextrose medium (YPD), and Mueller-Hinton broth medium (MHB) were used to cultivate *E. coli* DH5α, *P. pastoris* GS115, *E. coli* ATCC 25922, *E.coli* UB 1005, *S. typhimurium* C7731, *S. pullorum* 7913, *S. typhimurium* ATCC 14028, *S. aureus* ATCC 29213, *S. epidermidis* ATCC 12228, and *S. faecalis* ATCC 29212.

The *P. pastoris* containing the plasmid pPICZαA was cultured in buffered glycerol complex (BMGY) medium, and for the inducible expression of 6× His-Trx-PRW4, cells were grown in buffered methanol complex (BMMY) medium. The restriction enzymes *Xho*I, *Xba*I and *Sac*I, T4 DNA ligase, *Taq* DNA polymerase, and low range protein ladder were purchased from Fermentas (Carlsbad, USA). DNA markers (5000 bp) were purchased from Takara Biomedical Technology (Dalian, China). Ni sepharose™ 6 fast flow resin and recombinant enterokinase were purchased from Sangon

Biotech Co., Ltd. (Shanghai, China). Zeocin™ and ampicillin were purchased from Invitrogen (Carlsbad, USA). The gel extraction kit and plasmid mini kit were purchased from GenStar Co., Ltd. (Beijing, China). Synthetic PRW4 was synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). All other chemicals and reagents used in this study were of analytical grade or higher.

Construction of the recombinant plasmid

A gene sequence encoding the N-terminal 6× His-Trx-PRW4 fusion with a stop codon containing *Xho*I and *Xba*I restriction enzyme sites at its 5′- and 3′-ends, respectively, was synthesized by Shanghai Sangon Biotechnology Co., Ltd. and was cloned into the *Pichia* expression vector pPICZαA, which resulted in the expression vector pPICZαA-PRW4. Based on the codon preference of *P. pastoris*, the online software Condon Usage Database (http://www.kazusa.or.jp/codon/) was used to codon optimize the genes. pPICZαA-PRW4 was then transformed into *E. coli* DH5α and selected on LB plates with Zeocin (25 μg/mL). Restriction enzyme analysis and DNA sequencing analysis were used to ensure that the plasmid harbored the expected nucleotide sequence and the sequence was in the correct in-frame orientation.

P. pastoris transformation and positive transformant selection

pPICZαA-PRW4 and the empty pPICZαA plasmid were linearized by *Sac*I. Then, the linearized pPICZαA-PRW4 plasmid and the linearized empty pPICZαA plasmid were transformed into GS115 competent cells at 1500 V, 25 μF capacitance, and 200 Ω resistance. Colonies containing the resistance gene were screened on YPDS plates (YPD medium + 1.5% agar powder) containing 100 μg/mL Zeocin. Positive transformants were confirmed by PCR using the following primers.

Primer 5′AOX1:5′-GACTGGTTCCAATTGACAAGC-3′
Primer 3′AOX1: 5′-GCAAATGGCATTCTGACATCC-3′

Expression of recombinant proteins in *P. pastoris* GS115 and shake-flask cultivation optimization

The 6× His-Trx-PRW4 positive transformants were selected and cultured in 10 mL of YPD for 24 h at 30 °C with a rotation speed of 250 rpm. The cells were then grown in 50 mL of BMGY medium until the OD$_{600}$ of the culture was 2–6. The cells were collected by centrifugation at 10,000×g for 5 min to remove the supernatant. Cell pellets obtained in the previous step were then re-suspended in BMMY medium, and the OD$_{600}$ of the culture was adjusted to 1.0 to induce the expression of the recombinant protein. The positive transformants were expressed at 30 °C with a rotation speed of 250 rpm and

the methanol was added every 24 h during the 120 h induction period. The culture supernatants were harvested by centrifugation at different time intervals. The molecular weight of the target protein was determined by Tricine-SDS-PAGE and the supernatants were collected and assessed by Bradford assay to determine the quantity of total extracellular protein.

To optimize the induction time for total protein yield at initial pH 6.0 under the induction by 1.0% methanol, the culture was continued for up to 120 h, and fermentation supernatants were collected at 24, 48, 72, 96, and 120 h post-induction. The changes in the amount of total proteins secreted into the culture supernatants were determined by Bradford assay as described above. When the initial pH of the fermentation medium was 6.0, fermentation supernatants were collected for 96 h after the addition of 0.5%, 1.0%, 1.5%, 2.0%, and 2.5% (v/v) methanol to optimize the methanol concentration. In order to explore the effect of the initial pH of the medium on the expression of protein, fermentation cultures were performed at different initial pH values of the medium (5.5, 6.0, 6.5, 7.0, and 7.5) under the 1.0% methanol induction and we collected the fermentation supernatants at 96 h post-induction. The concentrations of protein secreted into the supernatant were evaluated by Bradford assay as described above.

Purification of 6× His-Trx-PRW4 and enterokinase cleavage

The 6× His-Trx-PRW4 was purified with a Ni-NTA resin column and the yield of 6× His-Trx-PRW4 was assessed by Bradford assay. The column was pre-equilibrated with 4 column volumes of binding buffer (20 mM Tris-HCl, 500 mM NaCl, 20 mM imidazol; pH 8.0). The culture supernatant was mixed with 5× binding buffer (0.1 M Tris-HCl, 2.5 M NaCl, 0.1 M imidazole; pH 8.0). The mixture was passed through a 0.22-μm filter and then applied to the column. The 6× His-Trx-PRW4 was eluted with 5 column volumes of elution buffer containing 20, 50, 80, 200, 300, or 500 mM imidazole at a flow rate of 1 mL/min.

The 6× His-Trx-PRW4 obtained by affinity chromatography with Ni-NTA was subjected to overnight dialysis in enterokinase buffer (150 mM Tris-HCl, 15 mM NaCl, 2.5 mM CaCl$_2$; pH 7.4). The 6× His-Trx-PRW4 was digested with enterokinase and incubated at 25 °C for 16 h. The cleaved recombinant PRW4 (rPRW4) was then re-purified by affinity chromatography on a Ni-NTA column.

The purified and cleaved rPRW4 was detected using Tricine-SDS-PAGE followed by Coomassie Blue staining. The concentration of cleaved rPRW4 was determined by Bradford assay as described above. The purity of the target protein bands was evaluated with Quantity One Software (BioRad, USA).

Antimicrobial activity assay of rPRW4 in vitro

The antimicrobial activity of rPRW4 in vitro was determined against several bacteria. The minimum inhibitory concentrations (MICs) were determined using a modified standard microtiter dilution method as described previously [19]. The bacteria were incubated overnight at 37 °C until the logarithmic growth phase was reached. The bacteria were then diluted to 10^5 CFU/mL. A 50-μL volume of bacteria in Mueller-Hilton broth (MHB) was mixed with 50 μL of a 2-fold serial dilution of the peptides that were dissolved in 0.01% (v/v) acetic acid and 0.2% (w/v) bovine serum albumin (BSA). The mixtures were incubated at 37 °C for 16–24 h. All of the experiments were performed in triplicate.

Statistical analysis

The statistical means and standard deviation (SD) were computed with the SAS 9.3 software (SAS Institute, Inc., Cary, NC, USA). The minimum inhibitory concentrations (MICs) were presented as the means ± SD.

Results

Construction of the *P. pastoris* expression plasmid pPICZαA-PRW4

The yeast expression vector pPICZαA-PRW4, containing the N-terminal codon-optimized 6× His-Trx tag and the codon-optimized PRW4 gene, was successfully constructed (Fig. 1; Supplementary Fig. S1). Restriction enzyme analysis showed that the DNA fragments digested by *Xho*I and *Xba*I were in accordance with the expected size (Table 1). The constructed vector was sequenced, and the sequenced result was used for alignment by BLAST, which indicated that the synthesized gene was in accordance with the design (data not shown). The pPICZαA-PRW4 plasmid was transformed into *P. pastoris* GS115 competent cells, and the transformants were selected by colony PCR. The results showed that the 6× His-Trx-PRW4 coding sequence was successfully inserted into the Zeocin-resistant *P. pastoris* transformants (Fig. 2).

Effects of induction conditions on the expression of the recombinant protein

A band at approximately 17 kDa was observed from Tricine-SDS-PAGE and Coomassie Blue staining from samples collected between 24 and 120 h after induction (Fig. 3a). The Bradford assay determined that the concentration of total protein secreted in the culture supernatant after 24 h of induction was 30.2 μg/mL. After 96 h of induction, the maximum yield of total protein was 215.5 μg/mL. The results showed that the

Fig. 1 Construction of the *P. pastoris* recombinant plasmid pPICZαA-PRW4
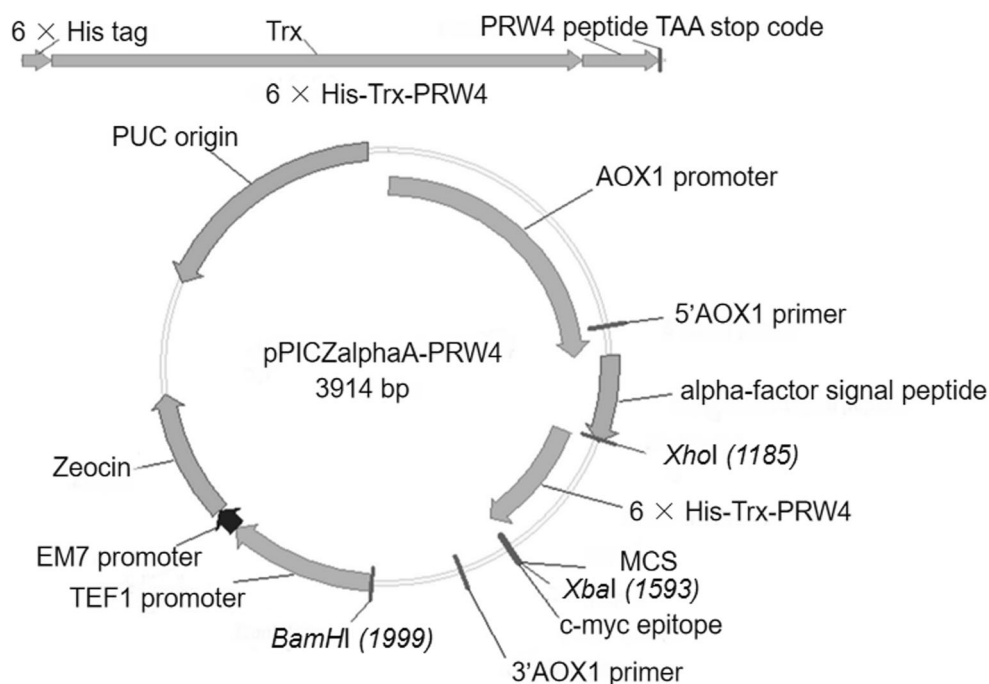
6× His-Trx-PRW4 was expressed in the *P. pastoris* GS115 cells, and the expression yield of the total protein in yeast was correlated with the induction time (Fig. 4a).

To optimize the methanol concentration and increase the expression level of the total protein, we chose five different concentrations of methanol to induce total protein expression, and the other induction conditions and medium remained unchanged. After collecting the culture supernatant, the amounts of secreted total protein were measured. The results showed that the concentration of total protein in the culture supernatant induced by 1.0% methanol was the highest compared to the induction by 0.5%, 1.5%, 2.0%, or 2.5% methanol, which indicated that the 1.0% methanol-induced secretion of the total protein reached the highest yield (Fig. 4b).

To optimize the medium pH for total protein yield, fermentation supernatants were collected after 96 h of induction at different pH values (5.5, 6.0, 6.5, 7.0, and 7.5). Then, we measured the amounts of secreted total protein after the culture supernatants were obtained. It was found that the expression level of total protein in the culture supernatant was the highest when the pH was 6.0, which indicated that different medium pH values might have different effects on the expression yield of secreted total proteins (Fig. 4c).

Expression and antimicrobial activity of purified and cleaved 6× His-Trx-PRW4

The 6× His-Trx-PRW4 was purified with a Ni-NTA resin column and the yield of 6× His-Trx-PRW4 was 160.8 μg/

Table 1 Amino acid sequence and DNA sequences of 6× His-Trx-PRW4

	Sequences	Number of amino acids/ gene length (bp)
Amino acid sequences	<u>KR</u>*HHHHHHMAIVKATDQSFSAETSEGVVLADFWAPWC GPCKMIAPVLEELDQEMGDKLKIVKIDVDENQETAGKYGVMSI PTLLVLKDGEVVETS0VGFKPKEALQELVNKHL*<u>DDDDK</u>RFRRLRWKTRWRLKKI	131
DNA sequences	**<u>CTCGAG</u>**AAAAGA*CATCACCACCACCACCACATGGCTATCGTTAAAGCTACAGATCAATCTTT CTCTGCTGAAACATCTGAAGGCGTTGTTCTTGCTGATTTCTGGGCTCCTTGGTGCGGCCC TTGCAAAATGATCGCTCCTGTTCTTGAAGAACTTGATCAAGAAATGGGCGATAAACTTAA AATCGTTAAAATCGATGTTGATGAAAACCAAGAAACAGCTGGCAAATACGGCGTTATGTC TATCCCTACACTTCTTGTTCTTAAAGATGGCGAAGTTGTTGAAACATCTGTTGGCTTCAA ACCTAAAGAAGCTCTTCAAGAACTTGTTAACAAACATCTT*<u>GATGATGATGATAAAC</u>GTT TTCGCCGATTACGGTGAAAACTTAGATGGAGGTTGAAGAAAATT**<u>TAATCTAGA</u>**	414

The underlined and bold regions are the *Xho*I and *Xba*I cleavage site; the underlined regions are the Kex2 cleavage site, enterokinase digestion sequences; the italic region is the 6× His-Trx tag sequence; the shaded region is the target peptide PRW4 sequence; the bold region is the stop codon

Fig. 2 Identification of recombinant expression vector pPICZαA-PRW4 by PCR amplification. M: low range prestained protein marker; lanes 1–5: the recombinant expression vector pPICZαA-PRW4 electroporation transformation of yeast PCR products
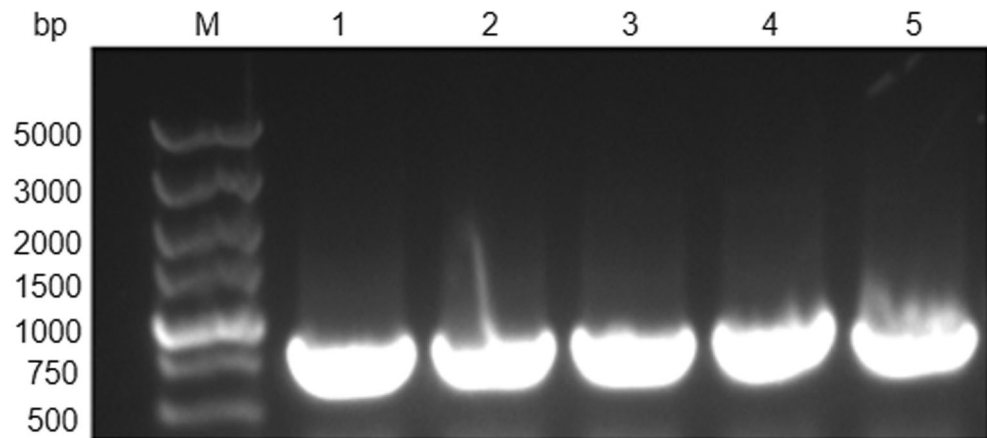

mL. We used enterokinase to cleave the 6× His-Trx-PRW4 to obtain rPRW4 with the molecular weight of 4.0 kDa (Fig. 3b). A yield of 15 mg of rPRW4 with a purity of 90% was obtained from 1 L of fermentation culture. We then tested the antimicrobial activity of PRW4 against *E. coli* ATCC 25522, *E. coli* UB 1005, *S. typhimurium* C7731, *S. aureus* ATCC 29213, *S. epidermidis* ATCC 12228, and *S. faecalis* ATCC 29212. As presented in Table 2, the MICs assay demonstrated that rPRW4 exhibited antimicrobial activity against those strains, which were consistent with previous conclusions.

Discussion

With the rapid development of genetic engineering technology, different heterologous expression systems, such as *P. pastoris* and *E. coli*, are chosen for large-scale preparation of AMPs [20, 21]. However, foreign proteins expressed by the *E. coli* expression system easily form inclusion bodies. And endotoxins produced by *E. coli* can contaminate target proteins [22]. In addition, as a eukaryotic expression system,

P. pastoris can provide suitable environment and conditions for the folding, glycosylation, and other post-processing of foreign proteins to ensure the activity of the protein. Therefore, compared with the *E. coli* expression system, the *P. pastoris* expression system has a higher expression yield and the expression product can show better biological activity [23]. Fan et al. used the *E. coli* expression system and *P. pastoris* expression system to express Beauveria bassiana chitinase (Bbchit1), respectively. The results showed that *P. pastoris* could secrete recombinant Bbchit1 directly into the fermentation broth. However, recombinant Bbchit1 expressed by *E. coli* existed in the form of inclusion bodies, which was undoubtedly not conducive to later isolation and purification. In addition, the yield and specific activity of recombinant Bbchit1 produced by *P. pastoris* were higher than those of recombinant Bbchit1 produced by *E. coli*, respectively, 153 mg/L versus 50 mg/L and 3.9 U/mg versus 2.8 U/mg [23]. Therefore, in recent years, *P. pastoris* has become a very successful expression system for the production of active heterogeneous proteins [24]. The methanol metabolism promoter alcohol oxidase 1 (AOX1) is one of the most potent
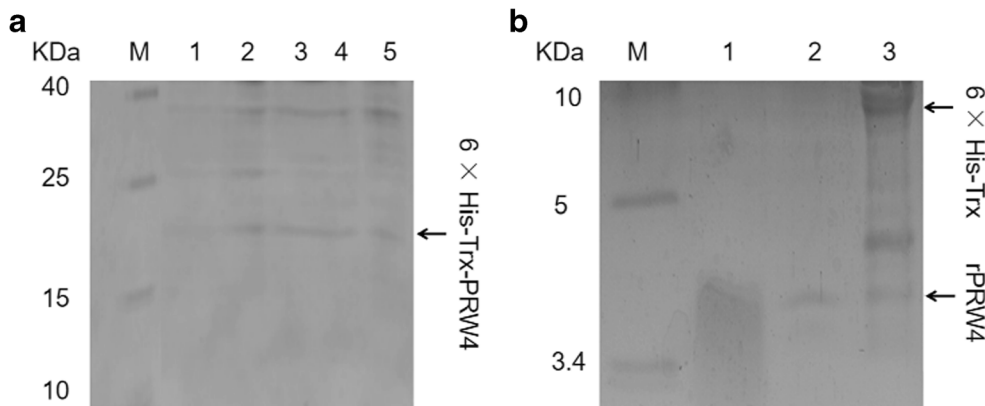
Fig. 3 **a** Expression of the 6× His-Trx-PRW4 fusion protein in *Pichia pastoris* GS115. M: low range prestained protein marker (5 μL); lanes 1–5: supernatant samples taken at 24 h (40 μL, 1.208 μg), 48 h (40 μL, 2.820 μg), 72 h (40 μL, 4.620 μg), 96 h (40 μL, 8.620 μg), and 120 h (40 μL, 7.380 μg) of induction at pH 6.0 by 1.0% (*v/v*) pure methanol,

respectively. **b** Tricine-SDS-PAGE of purified and cleaved 6× His-Trx-PRW4. M: low range prestained protein marker (5 μL); lane 1: chemically synthesized PRW4 sample (40 μL, 4 μg); lane 2: purified rPRW4 sample (40 μL, 0.6 μg); lane 3: 6× His-Trx-PRW4 digestion system (40 μL)

Fig. 4 a Effect of post-induction
time on the expression of the total
protein in supernatant at pH 6.0
under 1.0% methanol induction. **b**
Effect of methanol concentration
on the expression of the total
protein in supernatant at pH 6.0
for 96 h. **c** Effect of culture me-
dium pH on the expression of the
total protein in supernatant for
96 h under 1.0% methanol
induction
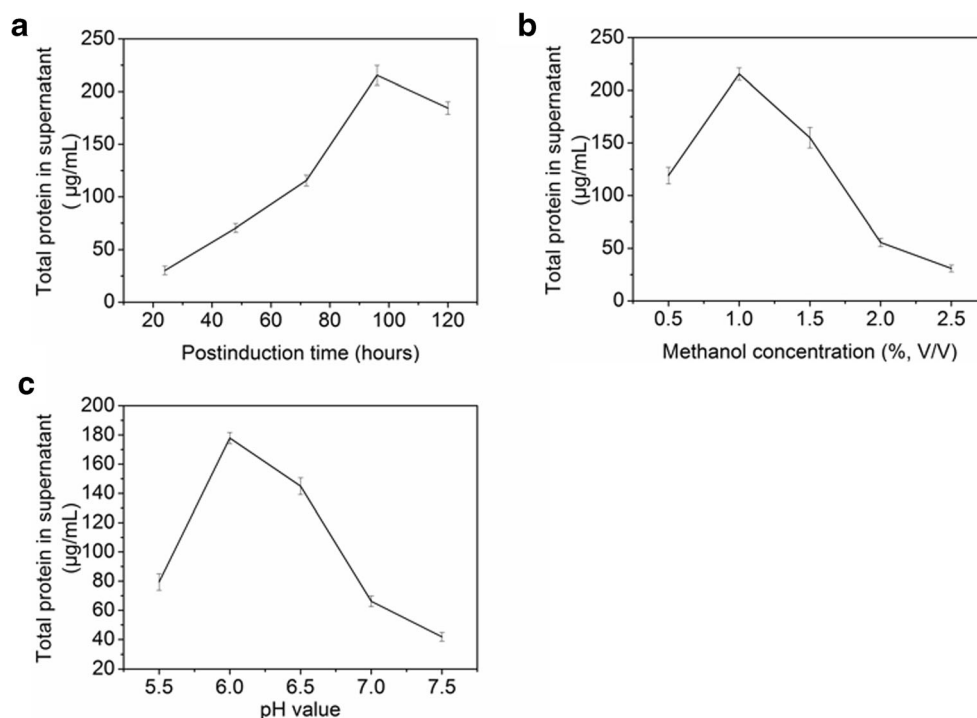
promoters. Under the induction of methanol, AOX1 tran-
scripts account for more than 30% of the whole cell transcript
[25]. In addition, codon bias of *P. pastoris* varies from species
to species, and the efficiency of foreign genes in the host can
affect expression [17, 26]. It has been found that replacing
rarely occurring codons according to the preferred codon
usage of *P. pastoris* can increase the expression level of
foreign genes [27]. Therefore, we optimized codons for the
fusion protein gene 6 × His-Trx-PRW4. The 6× His-Trx-
PRW4 could be secreted into the fermentation supernatant
with an α-mating factor (α-MF) signal peptide, and
endoprotease Kex2 targeted the peptide and was responsible

for the cleavage of α-MF [28]. Therefore, we used the
methanol-inducible yeast expression vector pPICZαA with
the α-MF and AOX1 promoters to produce the fusion protein
in *P. pastoris.* After purification by a Ni-NTA resin column
and digestion by enterokinase protease, rPRW4 was obtained
and had a similar antimicrobial activity as synthetic PRW4
against bacteria, such as *E. coli* ATCC 25922, *E. coli* UB
1005, *S. typhimurium* C7731, *S. pullorum* 7913, *S.
typhimurium* ATCC 14028, *S. aureus* ATCC 29213, *S.
epidermidis* ATCC 12228, and *S. faecalis* ATCC 29212.

Fusion expression is commonly used in the production of
genetically engineered peptides in *P. pastoris* [29]. Fusion
expression is an effective strategy to protect small molecule
AMPs from proteolysis and reduce the toxicity of AMPs to
host cells. To date, many fusion protein tags, such as His [30],
GST [31], thioredoxin (Trx) [32], and SUMO [33], are often
used in the expression of AMPs in *P. pastoris*, *E. coli*, and
B. subtilis. These tags, to a large extent, make AMPs conve-
nient for analysis and separation.

Among these fusion protein tags, Trx has been used to
express AMPs existing in nature, such as plectasin, cecropin
A, and LL-37 [34–36]. Trx is a non-toxic, small, and highly
soluble protein [32]. Trx is employed as a fusion tag with the
ability of increasing the solubility of recombinant proteins
[37]. Hlavac et al. used the *E. coli* expression system to ex-
press the tyrosine kinase Lck and found that the solubility of
Trx-Lck fusion protein was significantly better than that of
GST-Lck fusion protein. Moreover, the solubilization technol-
ogy would lead to high degradation of GST-Lck, but for Trx-
Lck fusion protein, even if there was no protease inhibitor,

Table 2   Antimicrobial activity of recombinant and synthetic PRW4

Strains	MICs (μM) (means ± SD)	
	Recombinant PRW4	Synthetic PRW4
Gram-negative bacteria		
E. coli 25922	2.44 ± 0.83	2.00 ± 0.00
E. coli UB1005	4.00 ± 0.00	4.00 ± 0.00
S. typhimurium C7731	4.83 ± 1.90	2.83 ± 0.98
S. typhimurium ATCC14028	4.67 ± 2.49	3.25 ± 0.96
S. pullorum 7913	2.22 ± 1.31	3.66 ± 3.09
Gram-positive bacteria		
S. aureus 29213	4.00 ± 0.00	4.00 ± 0.00
S. epidermidis 12228	7.33 ± 1.49	8.00 ± 0.00
S. faecalis 29212	10.67 ± 3.77	10.67 ± 3.77

Data are shown as the means ± SD of three independent tests

there was no sign of degradation of Trx-Lck fusion protein [38]. In addition, its small size makes it particularly suitable for peptide production because it allows the target peptide to account for a relatively large percentage of the fusion. An earlier study showed that compared to other tags tested, the target peptide had the highest relative yield, although the overall expression level of the fusion protein was not the highest [39]. Moreover, the positive charge of AMPs may interfere with the interaction between peptide and the host cell DNA and RNA, which may result in unstable transcription and translation [40]. Trx (PI 4.67) can be used as an acidic fusion tag to neutralize the highly purified positive charge of AMPs to ensure smooth transcription and translation processes [36]. Therefore, Trx is recognized as a suitable tag for fusion expression of AMPs [32, 37]. In this study, we used the 6× His-Trx fusion tag to express a novel antimicrobial peptide PRW4, and this work provided a reference for the large-scale and safe production of AMPs in *P. pastoris*. Moreover, in this experiment, we added an enterokinase cleavage site (DDDDK) so that the recombinant protein obtained after cleavage had no additional amino acid residues, and the structure and antimicrobial activity were protected to the greatest extent.

There are many factors that affect the production of recombinant proteins in *P. pastoris*, and the main factors are the concentration of methanol inducer, the induction time, and the pH of the medium [41, 42]. Methanol concentration is a critical factor to determine because AOX1 promoter activity responds to methanol induction, thus regulating the expression levels of foreign genes contained within the pPICZαA plasmid. However, excessively high methanol concentration increases the accumulation of methanol metabolites such as formaldehyde and hydrogen peroxide. The methanol metabolites will have a toxic effect on *Pichia pastoris*, thereby increasing cell death and lysis and affecting heterologous protein expression [26, 43]. Indeed, our results showed that when the concentration of methanol was too high, it could not increase the protein expression of recombinant proteins but reduced the expression yield.

In addition to the concentration of methanol inducer, the induction time is also a factor affecting the expression level. Too long fermentation time will undoubtedly increase costs, and too short fermentation time may affect fermentation efficiency. Therefore, it is important to choose a suitable fermentation time. With the extension of the induction time, the expression products in the culture medium accumulate continuously. The nutrients contained in the medium continue to decrease until they are completely consumed, which prevents the microorganisms from continuing to survive, eventually leading to the death of the microbial cells [44–46]. Our results showed that the protein expression level continued to increase after induction, reaching a maximum at 96 h, and then the protein expression level began to decrease. The reason for this condition might be that the medium at the initial stage of induction could provide a large amount of nutrients, when *P. pastoris* grew vigorously and had a relatively strong ability to secrete proteins. However, as time went by, the nutrients in the medium were gradually consumed, and *P. pastoris* could not obtain enough nutrients, which resulted in a decline in its ability to secrete proteins. Meanwhile, the metabolites of *P. pastoris* were relatively complex, and the secreted proteases might degrade the expressed heterologous proteins [47].

In addition to the above two factors, the pH of the medium also affects the expression of the protein, and too high or too low are not conducive to protein expression [41, 48]. *P. pastoris* can express foreign proteins in a wide range of pH (3.0–7.0). This range will have minimal effect on the growth rate of *P. pastoris* but will significantly affect protease activity and the stability of expressed proteins [49, 50]. In our experiments, the expression level of the recombinant protein was highest at the optimum pH of 6.0. This result was consistent with the result of Lee and co-workers using the *P. pastoris* expression system to express ice-binding proteins [41]. This might be because the protease activity was low and the stability of the recombinant protein was high at pH 6.0, thereby ensuring the yield of the recombinant protein to the greatest extent. High pH had been reported to reduce cell viability and might reduce the stability or activity of recombinant products [51]. In our experiment, when the pH of the medium was increased to 7.5, the protein expression level continued to decrease. This might be because the cell viability and the stability of the recombinant protein were decreased affected by pH. Therefore, the optimal conditions for the expression of recombinant proteins in *Pichia pastoris* were 96 h with 1.0% (*v/v*) methanol and pH 6.0.

The use of Ni-NTA affinity chromatography to separate the 6× His-tagged proteins is a simple and efficient method for protein purification. Moreover, the 6× His tag does not affect the secretion, folding, or function of the recombinant protein [52]. In this study, rPRW4 with a purity of 90% was obtained. This purity value was similar to the results of a large number of studies using this purification method [53, 54]. Approximately 56.1% rPRW4 was successfully released from the 6× His-Trx-PRW4 after enterokinase cleavage. This result was ideal compared to other studies using similar purification strategies. For example, Xu and co-workers used *E. coli* expression system to express the fusion protein Trx-6× His-Mdmcec. The soluble fusion protein (48.0 mg/L) was obtained. The fusion protein was treated with enterokinase and recombinant Mdmcec (11.2 mg/L) was recovered and purified. In their study, approximately 55% recombinant Mdmcec was successfully released after enterokinase cleavage [55].

Antimicrobial peptide activity is generally evaluated with the MICs. The rPRW4 in this experiment had antimicrobial activity against *E. coli* ATCC 25922, *E. coli* UB 1005, *S. typhimurium* C7731, *S. pullorum* 7913, *S. typhimurium* ATCC 14028, *S. aureus* ATCC 29213, *S. epidermidis* ATCC

12228, and *S. faecalis* ATCC 29212, which was consistent with the results of chemically synthetic PRW4 in vitro [18]. This was likely due to the ability of the *P. pastoris* expression system to express the foreign protein and carry out protein folding and processing [56], thus ensuring the integrity of the original structure of the peptides and maintaining the original biological activity of the peptides. Although the antimicrobial activity of rPRW4 has been verified, other biological activities such as the antimicrobial mechanism of rPRW4 still require further study.

Conclusions

In this study, the PRW4 gene was designed and synthesized, and the fusion gene was inserted into the expression vector pPICZαA after restriction enzyme digestion. The expression vector was successfully constructed and transformed into *P. pastoris* by electroporation. Recombinant expression plasmids were successfully achieved, and the expression conditions were optimized. The amount of accumulated recombinant protein reached the highest level with the 1% methanol fermentation for 96 h at pH 6.0. The maximum expression was approximately 215.5 mg/L. After purification and digestion with enterokinase protease, 15 mg of rPRW4 with a purity of 90% was obtained from a 1 L fermentation culture. In this study, rPRW4 had antimicrobial activity against *E. coli* ATCC 25922, *E. coli* UB 1005, *S. typhimurium* C7731, *S. pullorum* 7913, *S. typhimurium* ATCC 14028, *S. aureus* ATCC 29213, *S. epidermidis* ATCC 12228, and *S. faecalis* ATCC 29212. The results showed that the *P. pastoris* expression system was a suitable expression system for extracellular expression of biologically active recombinant proteins compared with the *E. coli* expression system.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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