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RESEARCH PAPER

Coexpression of Kex2 Endoproteinase and *Hac1* Transcription Factor to Improve the Secretory Expression of Bovine Lactoferrin in *Pichia pastoris*

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Abstract The large-scale production of functional recombinant lactoferrin has become a major goal because of its medicinal value and global demand. Secreting recombinant proteins into a culture medium offers a way to simplify protein purification and avoid toxicity from intracellularly accumulated materials. In this study, after 84 h of induction with methanol in a shaking flask, the recombinant bovine lactoferrin (rbLf) titer in the culture supernatant of the strain that integrated two copies of the rbLf gene was only 121.6 μg/L. A bottleneck might have existed in the folding and secretion pathways of rbLf. We then attempted to further improve the rbLf titer by overexpressing the transcription factor Hac1p and α-signal peptide-cutting protease Kex2p with different promoters. Results showed that the inducible coexpression of Hac1p and Kex2p linked with the 2A sequence improved the rbLf titer 5.0-fold (735.8 μg/L) after 84 h of induction with methanol. The maximal titer in a shaking flask was 1,150.5 μg/L after 120 h of induction. The rbLf titer achieved 35.6 mg/L in a 5 L fed-batch fermenter. Thus, *Kex2* and *Hac1* overexpression driven by methanol-induced promoter alleviated the bottleneck in the folding and secretion pathways and greatly improved the secretory expression of rbLf in *Pichia pastoris.*

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1. Introduction

Lactoferrin, an 80 kDa iron-binding glycoprotein belonging to the transferrin protein superfamily, is found in colostrum and milk at high concentrations [1]. Its functions include the immunomodulation and regulation of the gastrointestinal flora [2]. Lactoferrin can inhibit the proliferation of pathogens, such as bacteria, fungi, parasites, and viruses [3]. Lactoferricin, which is located in the N-terminal region of lactoferrin, is released when lactoferrin is digested with pepsin and exerts a high antimicrobial activity [4,5]. Given lactoferrin's medicinal value and global demand, the large-scale production of functional recombinant lactoferrin has become a major goal.

Lactoferrin has been successfully expressed in several microscopic cells. Recombinant bovine lactoferrin (rbLf) has been expressed in *Escherichia coli*, and the functional antibacterial activity of rbLf fractions has been obtained [6]. However, the use of prokaryotic systems cannot prevent protein toxicity that interferes with growth and causes cell death [7]. Lactoferrins from swines, horses, yaks, and goats have been expressed in *Pichia pastoris* [8-12]. The N-terminal region of bovine lactoferrin fused with other antioxidant peptides is expressed in *P. pastoris* and exhibits antibacterial activity [13].

In yeast, secreting recombinant proteins into the culture medium provides a way to simplify protein purification and avoid toxicity from intracellularly accumulated materials. However, protein folding and secretion are the two rate-

limiting steps in heterologous protein expression [14,15]. Various strategies have been investigated to improve the folding and secretion of proteins. Such approaches involve the overexpression of the active transcription factor *Hac1* for the regulation of unfolded protein response (UPR) [16] and the enhancement of the expression of endoplasmic reticulum (ER) chaperone [17] and disulfide isomerase [18].

In this study, we observed the effect of coexpressing transcription factor Hac1p and α-signal peptide cutting protease Kex2p on rbLf extracellular expression in *P. pastoris*.

2. Materials and Methods

2.1. Reagents and materials

Restriction enzymes and reverse transcriptase were purchased from Takara (Dalian, China). ClonExpress MultiS One-Step Cloning Kit was obtained from Vazyme Biotech (Nanjing, China). TRIzol and DNA/protein markers were purchased from Tiangen (Beijing, China). RealSuper Mixture for real-time polymerase chain reaction (PCR) was procured from Toyobo (Tokyo, Japan). A gel extraction kit and a plasmid miniprep kit were obtained from Tsingke (Hangzhou, China). Anti-6×His monoclonal antibody and mouse IgG antibodies coupled with horseradish peroxidase were purchased from Santa Cruz Biotechnology (California, USA). Soluble 3′,3′,5′,5′-tetramethylbenzidine (TMB) reagent and pepsin were purchased from Solarbio (Beijing, China). A His tag enzyme-linked immunosorbent assay (ELISA) detection kit was obtained from GenScript (Nanjing, China). Yeast nitrogen base (YNB), Zeocin, and Geneticin were purchased from Sangon (Shanghai, China). All the primers were synthesized by Tsingke (Hangzhou, China).

2.2. Strains and culture medium

E. coli DH5α was used in the process of vector construction. *E. coli* K88 and *Staphylococcus aureus* ATCC25923 were utilized to determine the antimicrobial activity of the expression products. These prokaryotic microbes were cultured in a Luria–Bertani (LB) medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl).

P. pastoris host strain GS115 was cultivated in a yeast extract peptone dextrose (YPD) medium (1% yeast extract, 2% peptone, and 2% dextrose). *P. pastoris* transformants were selected on a minimal medium (MD) (0.34% YNB, 1% $(NH_4)_2SO_4$, 2% dextrose, 4×10^{-5}% biotin, and 2% agar) or a yeast extract peptone dextrose medium with sorbitol (YPDS) medium (2% peptone, 1% extract yeast, 2% dextrose, 1 M of sorbitol, and 2% agar) containing Zeocin (100 μg/mL). The rbLf-expressing strains were incubated in a minimal dextrose and buffered complex glycerol (BMGY) medium (2% tryptone, 1% yeast extract, 1.34% YNB, 4×10^{-5}% biotin, 1% glycerol, and 100 mM potassium phosphate; pH 6.0) and induced in a buffered complex methanol (BMMY) medium (2% peptone, 1% yeast extract, 100 mM potassium phosphate, 1.34% YNB, 4×10^{-5}% biotin, and 1.5% methanol; pH 6.0).

Precultures for bioreactor cultivations were performed in 250 mL shake flasks and 30 mL BMGY medium. The trace metal solution PTM1 ($CuSO_4 \cdot 5H_2O$ 0.6%, $CoCl_2$ 0.05%, KI 0.009%, $FeSO_4 \cdot H_2O$ 6.5%, $MnSO_4 \cdot H_2O$ 0.3%, H_3BO_3 0.002%, H_2SO_4 0.5%, $ZnCl_2$ 2%, $MoNa_2O_4 \cdot 2H_2O$ 0.024%, and biotin 0.02%) and basal salt medium (BSM) (glycerol 4%, K_2SO_4 1.82%, CaSO4 0.093%, $MgSO_4 \cdot 7H_2O$ 1.49%, KOH 0.413%, H_3PO_4 2.67%, and PTM1 0.435%) were used in fed-batch cultivation.

2.3. Vector construction and *P. pastoris* transformation

The gene sequence encoding lactotransferrin from *Bos taurus* (*rbLf*, GenBank accession no. NP_851341.1) without the signal peptide (first 19 aa) was synthesized in accordance with the codon usage bias of *P. pastoris* with a His tag at the C terminus. The encoding sequences of the α-signal peptide cutting protease Kex2p and the novel methanol-induced promoter P_{0547} [19] were cloned from the genome of *P. pastoris* GS115. HAC1p became the active form after alternative splicing [16]. Two DNA fragments of *Hac1* were cloned from *P. pastoris* GS115 genome and fused via overlap PCR to obtain the active

Table 1. Strains used in this study

Strain	Description	Source
GS115	his4$^-$ Mut$^+$	Invitrogen
PJF01	Integrated the plasmid pPIC9K:P_{AOX}-rbLf	This study
PJF02	Integrated the plasmids pPIC9K:P_{AOX1}-rbLf and pPICZαA:P_{AOX1} -rbLf	This study
PJF03	Integrated the plasmids pPIC9K:P_{AOX1}-rbLf and pPICZαA:P_{AOX1} -rbLf_P_{GAP}-kex2	This study
PJF04	Integrated the plasmids pPIC9K:P_{AOX1}-rbLf and pPICZαA:P_{AOX1}-rbLf_P_{0547}-kex2	This study
PJF05	Integrated the plasmids pPIC9K:P_{AOX1}-rbLf and pPICZαA:rbLf_P_{GAP}- kex2-2A-Hac1	This study
PJF06	Integrated the plasmids pPIC9K:P_{AOX1}-rbLf and pPICZαA:P_{AOX1} -rbLf_P_{0547}- kex2-2A-Hac1	This study
PJF07	Integrated the plasmids pPIC9K:P_{AOX1}-rbLf and pPICZαA:P_{0547}- kex2-2A-Hac1	This study

spliced Hac1. Specific DNA fragments were inserted into the plasmid on the basis of homologous recombination *in vitro* by using ClonExpress MultiS One-Step Cloning Kit, and the plasmid backbones were obtained via PCR.

The plasmid pPIC9K backbone was linearized by *Sal* I, and the transformant resistant to 0.25 mg/mL geneticin was selected from the His^+ transformants. When the plasmid pPICZαA backbone was used, the linearized plasmid was produced by *pme* I, and the transformants were grown on YPDSZ plates. The positive clones were confirmed through PCR and DNA sequencing. The constructed strains are listed in Table 1. The detailed construction processes are summarized in supplementary file, and the primers used are listed in Table S1.

2.4. Methanol-induced expression in flask and fermenter

The selected colonies were initially cultivated overnight at 30°C and 250 rpm in a BMGY medium until OD_{600} reached approximately 2.0. The cultures were then inoculated into 20 mL of BMMY to induce expression in a 250 mL flask, and 1% methanol was added every 24 h. Yeast culture media were sampled and assayed every 24 h.

The fermentation of the recombinant *P. pastoris* was performed in a 5 L standard fermenter (Sartorius Stedim Biotech, Göttingen, German) containing 3 L BSM. The cultivation parameters were as follows: growth temperature 30°C, pH 5.0. The initial dissolved oxygen (DO) concentration was maintained at 20% of air saturation by regulating the stirrer speed and airflow. The cultivation started in BSM for initial cell growth and lasted for approximately 18–24 h at 30°C and pH 5.0. After the glycerol in the medium was exhausted, limited glycerol was feed until OD_{600} reached approximately 160. Then a carbon source starvation period of 15 min was observed. At the same time, the broth pH was adjusted to 6.0 by adding ammonia solution (100%, v/v). Then, 10.8 mL/h methanol (100% methanol containing 1.2% PTM1, v/v) as carbon source was added, whereas dissolved oxygen was kept constant at approximately 20–30%. OD_{600} and expression level were monitored throughout a 4-day induction.

2.5. Western blot analysis

Yeast culture supernatants were collected and concentrated through methanol/chloroform precipitation [20]. Then, the samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). For Western blot, antibodies against 6× His-tag were used as primary antibodies. Mouse IgG antibodies coupled with horseradish peroxidase were used as secondary antibodies. TMB chromogenic reagent was used for detection.

2.6. ELISA for rbLf quantification

The concentration of 6× His-tagged rbLf protein in the supernatants was quantified in accordance with the instruction of a His Tag ELISA detection kit. A standard curve with 6× His-tagged 12.7 kD protein (>97% purity) against an ELISA signal was graphed. OD_{450} was read on an ELISA reader (SpectraMax M5, Molecular Devices, USA). All assays were performed in triplicate.

2.7. Real-time quantitative PCR analysis

The total RNA of PJF01 and PJF07 strains was extracted with TRIzol, and cDNA was synthesized with oligo$(dT)_{18}$ and MMLV reverse transcriptase. Real-time PCR primers (Table S2) were designed to detect the transcripts of *rbLf*, *Kex2*, and *Hac1*. Relative quantification was performed on a Mastercycler Realplex2 cycler (Eppendorf, Hamburg, Germany). Measurements were performed in triplicate. All results were normalized to the *GADP* gene and presented relative to the transcription level of the genes in the PJF01 strain (value = 1) by using the method of Livak and Schmittgen [21].

2.8. rbLf Purification

Sequentially, the fermentation supernatants were equilibrated with 0.5 M NaCl and 10 mMimidazole, filtrated through a 0.45 μm filter, loaded onto a 25 mL metal chelate Ni^{2+} XK16 column (GE Healthcare, Piscataway, NJ, USA), and washed with an extract buffer. The protein was eluted with a gradient of 40–250 mM imidazole. The purified rbLf protein was freeze dried and stored at −20°C for further analysis.

2.9. Antimicrobial assay

The liquid test tube method [22] was used to determine the antimicrobial activity and to evaluate the antimicrobial activity of purified rbLf and pepsin-digested rbLf. *E. coli* K88 and *S. aureus* ATCC25923 were cultured in a LB medium to the stationary phase and diluted with the LB medium to a concentration of 10^5 cells per mL. The pepsin-digested product of rbLf was evaluated the antimicrobial activity of the released bioactive peptides in accordance with the protocol of García-Montoya *et al.* [6]. Culture suspensions (950 μL) and sample solution (50 μL purified rbLf or pepsin-digested rbLf, containing 25 μg of protein) were mixed and incubated at 37°C for 6 h. Ampicillin was used at 100 μg/mL as a positive control of growth inhibition. The sterile liquid culture was used as the control. Experiments were made in triplicate. Antimicrobial activity was calculated using the following formula:

$$\text{Antibacterial ratio} = \frac{\text{OD600 Control} - \text{OD600 Sample}}{\text{OD600 Control}} \times 100\%.$$

3. Results

3.1. rbLf expression in *P. pastoris*

PJF01 was obtained by linearizing the plasmid pPIC9K:P_{AOX}-rbLf with *Sal* I and transforming it into GS115 through electroporation. The results were confirmed by polymerase chain reaction (PCR) and DNA sequencing, then the positive colonies of PJF01 were selected for fermentation in the shake flask. The culture supernatant was obtained after methanol/chloroform precipitation for the production of a 10-fold concentrate for Western blot. The molecular weight of rbLf expressed by PJF01 after 84 h of induction was approximately 80 kDa, as assayed by Western blot (Fig. 1A), and the amount of rbLf was 51.9 μg/L, as determined by ELISA. These results indicated that rbLf was successfully expressed extracellularly, but the titer was limited.

3.2. Adding another copy of rbLf expression cassette

The plasmid pPICZαA:P_{AOX1}–rbLf was constructed and integrated into PJF01 to form PJF02 and increase the expression of the rbLf protein (Table 1). After 84 h of methanol induction, ELISA showed a 134.3% increase, which was 121.6 μg/L in the rbLf titer (Fig. 1B).

3.3. Enhancement of rbLf secretion

The secretory expression of the rbLf protein was further increased by constructing the plasmids of pPICZαA-rbLf_P_{GAP}-Kex2 and pPICZαA-rbLf_P_{0547}-Kex2 and individually integrating them into PJF02 (Table 1). The two plasmids modified the strain by *Kex2* overexpression. Consequently, *Kex2* overexpression with constitutive promoter (PJF03) and methanol-induced promoter (PJF04) enhanced the rbLf titer by 59.7% and 188.9%, respectively (Fig. 1B).

The effect of *Hac1* overexpression on the rbLf titer was observed by constructing plasmids pPICZαA-rbLf_P_{GAP}-Kex2-2A-Hac1 and pPICZαA-rbLf_P_{0547}-Kex2-2A-Hac1. The 2A sequence from *Thosea asigna* virus (T2A) [23] was used as a linker between Kex2 and Hac1. The rbLf *Hac1* titer in PJF05 with pPICZαA-rbLf_P_{GAP}-Kex2-2A-Hac1 unexpectedly showed a 20.9% decrease compared with that in PJF03. *Hac1* overexpression in PJF06 with pPICZαA-rbLf_P_{0547}-Kex2-2A-Hac1 enhanced the rbLf level by 109.5% compared with that the rbLf level in PJF04 and reached 735.8 μg/L (Fig. 1B). In PJF06, *Kex2* and *Hac1* overexpression driven by the methanol-induced promoter produced considerably more rbLf than that that driven by the Gap promoter in PJF05. After 120 h of induction with methanol, rbLf was 1150.5 μg/L in PJF06,

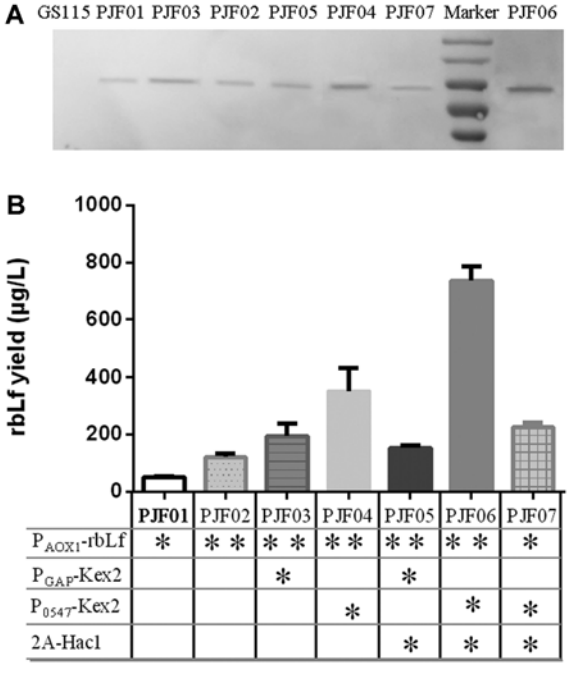

Fig. 1. Expression of rbLf in different *Pichia pastoris* strains after 84 h methanol induction in a flask. (A) Western blot analysis of culture supernatant. Protein markers obtain 180, 130, 100, 70, and 55 kDa individually. (B) rbLf titer in different strains. * denotes single copy of genes or expression cassettes listed in the left column of table, and ** denotes double copies.

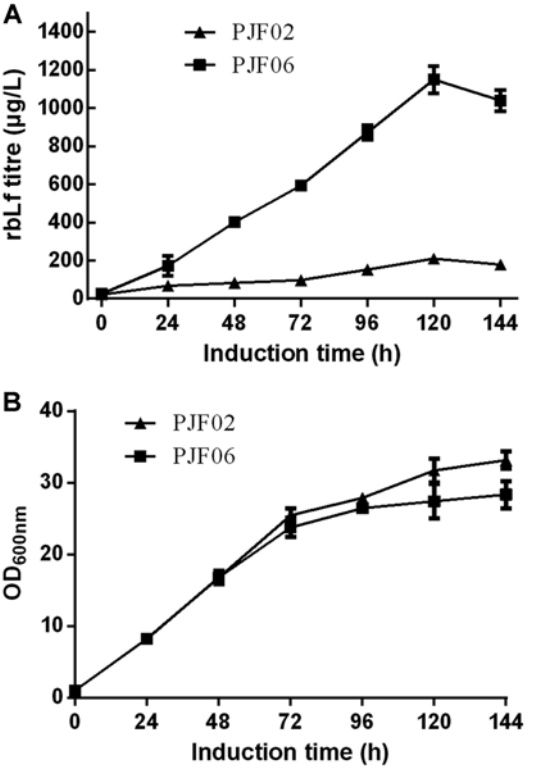

Fig. 2. Time course of the rbLf titer (A) and OD_{600} (B) of PJF02 and PJF06.

and this value was 5.5-fold of that of rbLf produced by PJF02 (Fig. 2A). The higher expression of rbLf inhibited the growth of PJF06 and decreased the biomass compared with that of PJF02 after 72 h induction (Fig. 2B).

Kex2 and *Hac1* were overexpressed by the methanol-induced promoter in PJF07 strain with a single copy of the *rbLf* gene to determine the combined effects of the two genes on the rbLf titer, thereby producing 225.3 μg/L rbLf after 84 h methanol induction (Fig. 1B). The rbLf titer was 4.3-fold higher than that produced by PJF01.

3.4. Gene transcription analysis

To verify whether the overexpression of *Hac1* and *Kex2* increased the rbLf expression at the transcription level, we determined the transcription levels of *rbLf*, *Hac1*, and *Kex2* in PJF02 and PJF06 after 72 h of induction by real-time PCR (Fig. 3). No difference in the transcription levels of rbLf was observed between the two strains. The transcription levels of *Kex2* and *Hac1* increased 76.6- and 124.2-fold in PJF06 relative to the transcription level in PJF02, confirming the successful integration of the expression cassettes of *Kex2* and *Hac1* into the genome, and *Kex2* and *Hac1* functioned without the attribution to transcriptional regulation on *rbLf*.

3.5. rbLf production in fermenter

To obtain a high yield, we conducted high-density fermentation of PJF07 strain in a 5 L fermenter starting with 3 L of BSM supplemented with trace salts and glycerol (Fig. 4). The total induction time with methanol was 96 h. OD_{600} of cells reached > 600, and wet cell weight reached 460 g/L after 96 h induction. We observed the maximum level of rbLf (36.5 mg/L) after 84 h methanol induction.

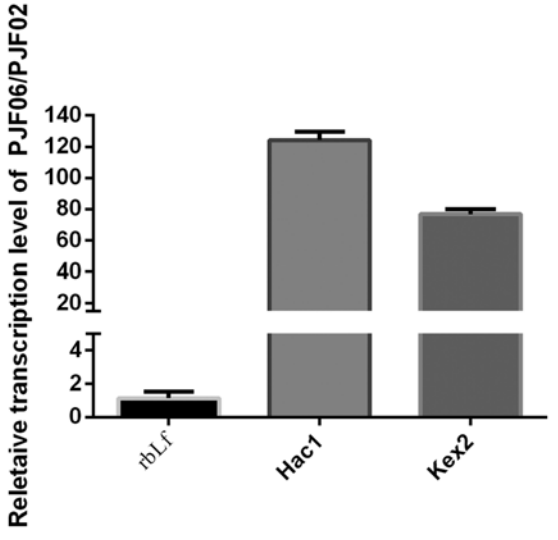

Fig. 3. Transcription of the genes manipulated in this study. The results are normalized to *GADP*, and the transcription levels of PJF06 are related to PJF02. Values are expressed as the mean ± standard error of three independent experiments.

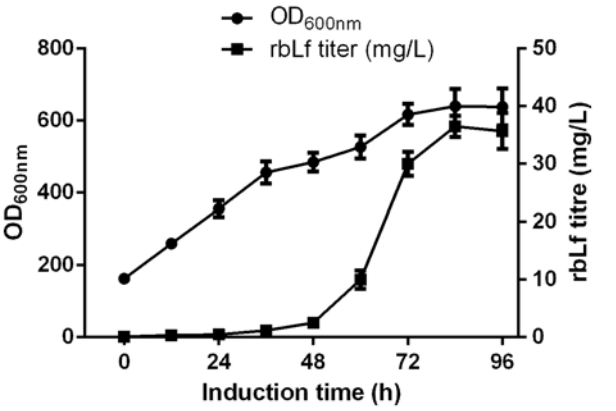

Fig. 4. Growth and rbLf production time course of *Pichia pastoris* PJF07 in a 5 L fermenter. The rbLf titer is detected by using collected culture supernatant through ELISA.

3.6. Purification and antibacterial activity analysis of rbLf

P. pastoris culture supernatant was harvested through centrifugation after fermentation. The filtrate supernatant containing rbLf was applied to Ni^{2+} column purification, and the bound proteins were eluted. The rbLf protein was 125-fold purified, and 16.9% of the total protein was recovered after Ni^{2+} column affinity purification. The culture supernatant and purified rbLf protein with 87.7% purity were subjected to SDS-PAGE and Western blot assay (Fig. 5A and B).

Most of the antimicrobial activities of lactoferrin result from its highly cationic N-terminal region containing two antimicrobial domains, namely, lactoferricin (17–30 aa)

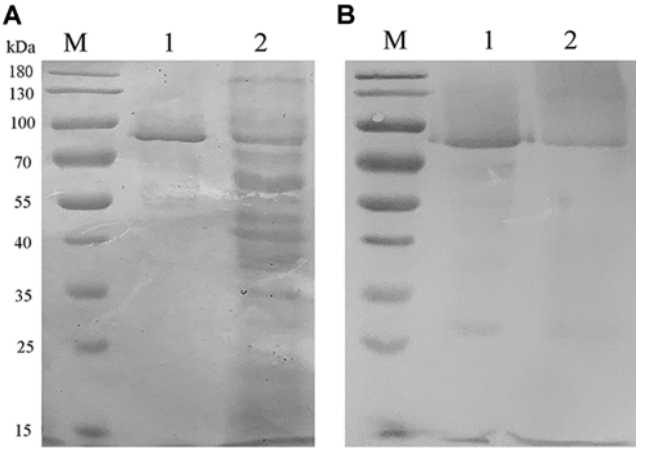

Fig. 5. SDS-PAGE analysis (A) and Western blot (B) of the rbLf expressed by *Pichia pastoris*. Lane M: protein marker; lane 1: purified rbLf; lane 2: culture supernata nt.

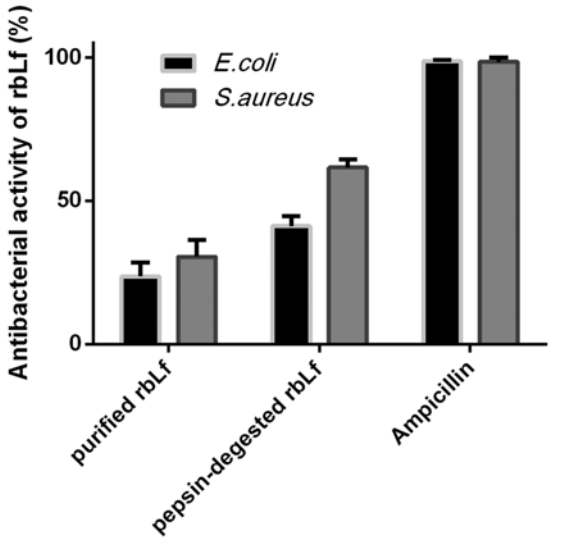

Fig. 6. Antibacterial activity of purified rbLf and pepsin-digested rbLf. Bacteria are incubated in the LB broth and treated with the sample solution containing 25 μg/mL protein.

and lactoferrampin (265–284 aa), which can be released from bovine lactoferrin by proteolytic digestion [24]. The antimicrobial activities of purified rbLf and pepsin-digested rbLf against *E. coli* K88 and *S. aureus* ATCC25923 were observed (Fig. 6). After 6 h of cultivation, 23.7% of *E. coli* and 30.5% of *S. aureus* were inhibited by the purified rbLf. For pepsin-digested rbLf, *E. coli* and *S. aureus* were inhibited by 41.2% and 61.6%, respectively. Therefore, the functional rbLf was successfully expressed, and the digested form of rbLf was more active against microorganisms than the undigested form.

4. Discussion

The indiscriminate use of antibiotics results in infections caused by resistant strains. Therefore, some new treatment strategies have been widely explored [25]. Lactoferrin positively affects the development and health of neonates, and adding bovine lactoferrin to infant formulas decreases the incidence of infectious diseases [26]. Oral administration of lactoferrin provides a protective effect against necrotizing enterocolitis in neonates [27].

Alleviating the bottlenecks in the folding and secretion pathways is beneficial to the overexpression of heterologous proteins in *P. pastoris* [28,29]. Thus, some strategies have been used to improve the folding and secretion of rbLf. The yeast *Kex2* gene encodes an endoprotease, cleaving the yeast endogenous preproteins for maturation in the specific site [30]. Cleavage removes the signal peptides of preproteins and facilitates the secretion of mature proteins and thus considered a key step in yeast secretion pathways [31,32]. The secretory yield of heterologous proteins can be improved through genomic integration with additional *Kex2* copies [33]. Our study is consistent with these findings.

Secreted proteins are sent into ER to undergo post-translational modifications. Only correctly folded proteins leave the ER and proceed through the secretory pathway. When unfolded and misfolded proteins accumulate in the ER, the endoribonuclease Ire1p is activated to splice *Hac1* mRNA [34]. The spliced *Hac1* mRNA then encodes an active transcription factor to regulate UPR [35]. UPR regulation is an essential strategy of yeast to defend against protein stresses. The target genes of the transcriptional factor *Hac1* are involved in some important cellular processes, such as carbon metabolism, stress response, and protein folding and secretion [36]. We found that the effect of Hac1p depends on the expression model. The constitutive Gap promoter driving *Hac1* failed to increase rbLf productivity (PJF05 *vs.* PJF03), and the methanol-induced promoter intensely increased rbLf productivity (PJF06 *vs.* PJF04). The coexpression of *Kex2* and *Hac1* increased rbLf productivity 4.3-fold in the strain with a single rbLf copy (PJF07 *vs.* PJF01) and 6.0-fold in the strain with double copies (PJF06 *vs.* PJF02), indicating that the high abundance of target gene copies required the further enhancement of the protein folding and secretion ability of host cells.

Lactoferrin has been expressed in several microscopic cells. The yields of lactoferrin from various animals reported are 12 mg/L from swine [8], 40 mg/L from yaks [10], and 2 mg/L from goats [11]. rbLf has been intracellularly expressed in *P. pastoris*, and the titer is reported to reach 3.5 g/L in a fermenter, achieving the highest expression level in *P. pastoris* [12]. For simplify purification process, rbLf in *P. pastoris* was extracellularly expressed in the present study. However, the maximum secretory expression level of rbLf was only 35.6 mg/L in a 5 L fed-batch fermenter. Boettner *et al.* [37] conducted a comparative analysis on 79 different human cDNAs expressed in *P. pastoris* and observed that the DNA sequence was closely related to the protein expression level. And it is reported that the expression level of some proteins is related with phenotypes of methanol utilization. The titer of recombinant human interferon gamma is nearly 10-fold higher in *P. pastoris* KM71 (Mut^S phenotypes) than that in strain GS115 (Mut^+ phenotypes) [38]. The reference of lactoferrin intracellular expression (3.5 g/L) mentioned above also use *P. pastoris* KM71 as host. We will redesigning the DNA sequence of lactoferrin according to codon degeneracy and try other host cell with different

phenotypes to improve the lactoferrin secretory titer. Moreover, optimizing fermentation conditions will further increase yield.

5. Conclusions

Numerous genetic modification strategies were used to enhance the secretory production of rbLf in *P. pastoris*. Increasing the copy of the *rbLf* gene and the overexpression of Kex2p and Hac1p immensely enhanced the rbLf titer. The inducible coexpression of Hac1p and Kex2p linked with the 2A sequence improved the rbLf titer 5.0-fold after 84 h of methanol induction. The maximal titer reached 1,150.5 μg/L in a shaking flask after 120 h of methanol induction. The rbLf titer achieved 35.6 mg/L in 5 L fed-batch fermenter. The results indicated that the production of rbLf in *P. pastoris* was improved by alleviating the bottlenecks in folding and secretion pathways.

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References

1. Groves, M. L. (1960) The isolation of a red protein from milk[2]. *J. Am. Chem. Soc.* 82: 3345-3350.
2. Roberts, A. K., R. Chierici, G. Sawatzki, M. J. Hill, S. Volpato, and V. Vigi (1992) Supplementation of an adapted formula with bovine lactoferrin: 1. Effect on the infant faecal flora. *Acta Paediatr.* 81: 119-124.
3. Jenssen, H. and R. E. Hancock (2009) Antimicrobial properties of lactoferrin. *Biochimie*. 91: 19-29.
4. Bellamy, W., M. Takase, K. Yamauchi, H. Wakabayashi, K. Kawase, and M. Tomita (1992) Identification of the bactericidal domain of lactoferrin. *Biochim. Biophys. Acta.* 1121: 130-136.
5. Arias, M., L. J. McDonald, E. F. Haney, K. Nazmi, J. G. Bolscher, and H. J. Vogel (2014) Bovine and human lactoferricin peptides: chimeras and new cyclic analogs. *Biometals.* 27: 935-948.
6. García-Montoya, I., S. A. González-Chávez, J. Salazar-Martínez, S. Arévalo-Gallegos, S. Sinagawa-García, and Q. Rascón-Cruz (2013) Expression and characterization of recombinant bovine lactoferrin in *E. coli*. *Biometals.* 26: 113-122.
7. Rosano, G. L. and E. A. Ceccarelli (2014) Recombinant protein expression in *Escherichia coli*: advances and challenges. *Front Microbiol.* 5: 172.
8. Wang, S. H., T. S. Yang, S. M. Lin, M. S. Tsai, S. C. Wu, and S. J. Mao (2002) Expression, characterization, and purification of recombinant porcine lactoferrin in *Pichia pastoris*. *Protein Expr. Purif.* 25: 41-49.
9. Paramasivam, M., K. Saravanan, K. Uma, S. Sharma, T. P. Singh, and A. Srinivasan (2002) Expression, purification, and characterization of equine lactoferrin in *Pichia pastoris*. *Protein Expr. Purif.* 26: 28-34.
10. Dong, Z. Y. and Y. Z. Zhang (2006) Molecular cloning and expression of yak (*Bos grunniens*) lactoferrin cDNA in *Pichia pastoris*. *Biotechnol. Lett.* 28: 1285-1292.
11. Chen, G. H., L. J. Yin, I. H. Chiang, and S. T. Jiang (2007) Expression and purification of goat lactoferrin from *Pichia pastoris* expression system. *J. Food. Sci.* 72: M67-M71.
12. Iglesias-Figueroa, B., N. Valdiviezo-Godina, T. Siqueiros-Cendón, S. Sinagawa-García, S. Arévalo-Gallegos, and Q. Rascón-Cruz (2016) High-level expression of recombinant bovine lactoferrin in *Pichia pastoris* with antimicrobial activity. *Int. J. Mol. Sci.* 17: 902.
13. Sun, J., J. Jiang, L. Liu, Z. Wang, and C. Wei (2019) Expression of the hybrid antimicrobial peptide lactoferrin-lysozyme in *Pichia pastoris*. *Biotechnol. Appl. Biochem.* 66: 202-208.
14. Cudna, R. E. and A. J. Dickson (2003) Endoplasmic reticulum signaling as a determinant of recombinant protein expression. *Biotechnol. Bioeng.* 81: 56-65.
15. Damasceno, L. M., C. J. Huang, and C. A. Batt (2012) Protein secretion in *Pichia pastoris* and advances in protein production. *Appl. Microbiol. Biot.* 93: 31-39.
16. Guerfal, M., S. Ryckaert, P. P. Jacobs, P. Ameloot, K. Van Craenenbroeck, R. Derycke, and N. Callewaert (2010) The HAC1 gene from *Pichia pastoris*: characterization and effect of its overexpression on the production of secreted, surface displayed and membrane proteins. *Microb. Cell Fact.* 9: 49.
17. Kim, M. D., K. C. Han, H. A. Kang, S. K. Rhee, and J. H. Seo (2003) Coexpression of BiP increased antithrombotic hirudin production in recombinant *Saccharomyces cerevisiae*. *J. Biotechnol.* 101: 81-87.
18. Shusta, E. V., R. T. Raines, A. Plückthun, and K. D. Wittrup (1998) Increasing the secretory capacity of *Saccharomyces cerevisiae* for production of single-chain antibody fragments. *Nat. Biotechnol.* 16: 773-777.
19. Xu, N., J. Zhu, Q. Zhu, Y. Xing, M. Cai, T. Jiang, M. Zhou, and Y. Zhang (2018) Identification and characterization of novel promoters for recombinant protein production in yeast *Pichia pastoris*. *Yeast.* 35: 379-385.
20. Wessel, D. and U. I. Flügge (1984) A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal. Biochem.* 138: 141-143.
21. Livak, K. J. and T. D. Schmittgen (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods.* 25: 402-408.
22. Tian, J., S. Shen, C. Zhou, X. Dang, Y. Jiao, L. Li, S. Ding, and H. Li (2015) Investigation of the antimicrobial activity and biocompatibility of magnesium alloy coated with HA and antimicrobial peptide. *J. Mater. Sci. Mater. Med.* 26: 66.
23. Szymczak, A. L., C. J. Workman, Y. Wang, K. M. Vignali, S. Dilioglou, E. F. Vanin, and D. A. Vignali (2004) Correction of multi-gene deficiency in vivo using a single'self-cleaving'2A peptide–based retroviral vector. *Nat. Biotechnol.* 22: 589-594.
24. Tang, X. S., Z. R. Tang, S. P. Wang, Z. M. Feng, D. Zhou, T. J. Li, and Y. L. Yin (2012) Expression, purification, and antibacterial activity of bovine lactoferrampin-lactoferricin in *Pichia pastoris*. *Appl. Biochem. Biotechnol.* 166: 640-651.
25. Chatterjee, M., C. P. Anju, L. Biswas, V. A. Kumar, C. G. Mohan, and R. Biswas (2016) Antibiotic resistance in Pseudomonas

aeruginosa and alternative therapeutic options. *Int. J. Med. Microbiol.* 306: 48-58.
26. Chen, K., L. Chai, H. Li, Y. Zhang, H. M. Xie, J. Shang, W. Tian, P. Yang, and A. C. Jiang (2016) Effect of bovine lactoferrin from iron-fortified formulas on diarrhea and respiratory tract infections of weaned infants in a randomized controlled trial. *Nutrition.* 32: 222-227.
27. Manzoni, P., M. Meyer, I. Stolfi, M. Rinaldi, S. Cattani, L. Pugni, M. G. Romeo, H. Messner, L. Decembrino, N. Laforgia, F. Vagnarelli, L. Memo, L. Bordignon, M. Maule, E. Gallo, M. Mostert, M. Quercia, L. Bollanii, R. Pedicino, L. Renzullo, P. Betta, F. Ferrari, T. Alexander, R. Magaldi, D. Farina, F. Mosca, and M. Stronati (2014) Bovine lactoferrin supplementation for prevention of necrotizing enterocolitis in very-low-birth-weight neonates: a randomized clinical trial. *Early Hum. Dev.* 90: S60-S65.
28. Weis, R., R. Luiten, W. Skranc, H. Schwab, M. Wubbolts, and A. Glieder (2004) Reliable high-throughput screening with *Pichia pastoris* by limiting yeast cell death phenomena. *FEMS Yeast Res.* 5: 179-189.
29. Vogl, T., G. G. Thallinger, G. Zellnig, D. Drew, J. M. Cregg, A. Glieder, and M. Freigassner (2014) Towards improved membrane protein production in *Pichia pastoris*: general and specific transcriptional response to membrane protein overexpression. *N. Biotechnol.* 31: 538-552.
30. Fuller, R. S., R. E. Sterne, and J. Thorner (1988) Enzymes required for yeast prohormone processing. *Annu. Rev. Physiol.* 50: 345-362.
31. Rockwell, N. C., D. J. Krysan, T. Komiyama, and R. S. Fuller (2002) Precursor processing by kex2/furin proteases. *Chem. Rev.* 102: 4525-4548.
32. Rockwell, N. C. and J. W. Thorner (2004) The kindest cuts of all: crystal structures of Kex2 and furin reveal secrets of precursor processing. *Trends Biochem. Sci.* 29: 80-87.
33. Yang, S., Y. Kuang, H. Li, Y. Liu, X. Hui, P. Li, Z. Jiang, Y. Zhou, Y. Wang, A. Xu, S. Li, P. Liu, and D. Wu (2013) Enhanced production of recombinant secretory proteins in *Pichia pastoris* by optimizing Kex2 P1'site. *PLoS One.* 8: e75347.
34. Schröder, M., R. Clark, and R. J. Kaufman (2003) IRE1- and HAC1-independent transcriptional regulation in the unfolded protein response of yeast. *Mol. Microbiol.* 49: 591-606.
35. Foti, D. M., A. Welihinda, R. J. Kaufman, and A. S. Lee (1999) Conservation and divergence of the yeast and mammalian unfolded protein response. Activation of specific mammalian endoplasmic reticulum stress element of the grp78/BiP promoter by yeast Hac1. *J. Biol. Chem.* 274: 30402-30409.
36. Lin, X. Q., S. L. Liang, S. Y. Han, S. P. Zheng, Y. R. Ye, and Y. Lin (2013) Quantitative iTRAQ LC–MS/MS proteomics reveals the cellular response to heterologous protein overexpression and the regulation of HAC1 in *Pichia pastoris*. *J. Proteomics.* 91: 58-72.
37. Boettner, M., C. Steffens, C. von Mering, P. Bork, U. Stahl, and C. Lang (2007) Sequence-based factors influencing the expression of heterologous genes in the yeast *Pichia pastoris*—A comparative view on 79 human genes. *J Biotechnol*. 130: 1-10.
38. Razaghi, A., E. Tan, L. H. L. Lua, L. Owens, O. P. Karthikeyan, and K. Heimann (2017) Is *Pichia pastoris* a realistic platform for industrial production of recombinant human interferon gamma? *Biologicals*. 45: 52-60.