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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Engineering protein translocation and unfolded protein response enhanced human PH-20 secretion in *Pichia pastoris*

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Abstract

Hyaluronidases catalyze the degradation of hyaluronan (HA), which is finding rising applications in medicine, cosmetic, and food industries. Recombinant expression of hyaluronidases in microbial hosts has been given special attention as a sustainable way to substitute animal tissue–derived hyaluronidases. In this study, we focused on optimizing the secretion of hyaluronidase from *Homo sapiens* in *Pichia pastoris* by secretion pathway engineering. The recombinant hyaluronidase was first expressed under the control of a constitutive promoter P_{GCW14}. Then, two endoplasmic reticulum–related secretory pathways were engineered to improve the secretion capability of the recombinant strain. Signal peptide optimization suggested redirecting the protein into co-translational translocation using the ost1-proα signal sequence improved the secretion level by 20%. Enhancing the co-translational translocation by overexpressing signal recognition particle components further enhanced the secretory capability by 48%. Then, activating the unfolded protein response by overexpressing a transcriptional factor ScHac1p led to a secreted hyaluronidase activity of 4.06 U/mL, which was 2.1-fold higher than the original strain. Finally, fed-batch fermentation elevated the production to 19.82 U/mL. The combined engineering strategy described here could be applied to enhance the secretion capability of other proteins in yeast hosts.

Key points

- *Improving protein secretion by enhancing co-translational translocation in P. pastoris was reported for the first time.*
- *Overexpressing Hac1p homologous from different origins improved the rhPH-20 secretion.*
- *A 4.9-fold increase in rhPH-20 secretion was achieved after fermentation optimization and fed-batch fermentation.*

Keywords *Pichia pastoris* · Hyaluronidase · Protein secretion · Cotranslational translocation · Unfolded protein response

Introduction

Hyaluronidase is a group of carbohydrate-active enzymes that possess major degradation activity toward hyaluronan (HA) (Yao et al. 2023; Zhang et al. 2022). Hyaluronidases are widely applied in the food industry, cosmetic industry, and medical field. They have been applied in preparing bioactive HA oligomers with defined molecular mass (Han et al. 2022; He et al. 2020), and in clinical therapies, to degrade extracellular HA for better subcutaneous drug diffusion (Bookbinder et al. 2006; Pavan et al. 2016), as well as in cosmetic surgeries to correct HA overfilling (Jung 2020). Also, hyaluronidases from bovine, ovine, or human have been used in identifying bioactive molecules with hyaluronidase-inhibiting activities, whose application could be found in developing anti-inflammatory, anti-aging food ingredients, and cosmetic ingredients (Koch et al. 2019;

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Pardau et al. 2017), as well as contraceptive agents (Chen et al. 2016).

The recombinantly expressed human hyaluronidase PH-20 (rhPH-20) has been considered a substitution for commercially available bovine testicular hyaluronidase, which is limited in source and might bring clinical safety risks as animal-derived product (Locke et al. 2019). Several studies have reported the recombinant expression of rhPH-20 in different hosts including plant cells, mammalian cells, and yeasts (Table 1). However, most of the expression levels reported in these studies are relatively low, which indicates a large potential to optimize rhPH-20 expression.

The *Pichia pastoris* expression system is a widely used microbial expression system for the high-level expression of recombinant proteins. As a eukaryotic host, it possesses a posttranslational modification system that is vital for the successful functional expression of some eukaryote-origin proteins, for example, glycoproteins (Reitinger et al. 2008). The endoplasmic reticulum (ER) is a vital organelle in which the vast majority of posttranslational modifications occur (Liu et al. 2022). In the process of protein expression, nascent peptides with a signal peptide (SP) first enter the ER lumen via co-translational translocation or posttranslational translocation (Tang et al. 2015). In the ER lumen, proteins fold under the assistance of chaperones, form disulfide bonds under the guidance of PDI, undergo glycosylation, and are transported to the Golgi apparatus for further modification by COPII vesicle (Liu et al. 2022). Extensive studies have shown the potential of engineering these ER-related protein processing pathways in improving recombinant protein production in *P. pastoris*. By overexpressing PDI with P_{AOX1} promoter in recombinant strain, Kerr et al. achieved a tenfold increase in productivity of Murine factor H (Kerr et al. 2021); Huang et al. reported a 6.21-fold improvement in amylase activity after overexpressing six copies of the *HAC1* gene (Huang et al. 2017). Though extensive studies have reported strategies to improve recombinant protein production in *P. pastoris*, there are still efforts to be made to discover new engineering strategies. For example, though engineering the posttranslational translocation has recently been proven to improve the secretion of several proteins (Zahrl et al. 2022), the feasibility of engineering co-translational translocation for enhanced secretion has not been demonstrated.

In this study, we focus on engineering two ER-related secretion pathways, namely translocation and the unfolded protein response (UPR), to facilitate higher secretion levels of rhPH-20. The core region of the protein is first expressed under a strong constitutive promoter P_{GCW14}, and the two pathways are engineered by a three-step modification (Fig. 1). SP engineering and overexpressing components of ER translocation are performed to improve the early secretion pathway; Spliced form of the UPR transcription factor Hac1p from *S. cerevisiae*, *P. pastoris*, and *Homo sapiens* is overexpressed. The activation of UPR and changes in reactive oxygen species (ROS) levels are studied. Fed-batch fermentation is performed to elevate the productivity of recombinant strain. The engineered strain with higher constitutive productivity of rhPH-20 may provide a platform for its large-scale preparation and contribute to its applications in medical and food research. Strategies reported in this study might also be employed to enhance the secretory expression of other enzymes in the *P. pastoris* host.

Materials and methods

Strains, plasmids, and materials

All strains and plasmids used in this study are listed in Table S1. Primers used to construct each vector are listed in Table S2. The encoding sequence of human PH-20 in this study was codon optimized based on PH-20 from *H. sapiens* and is deposited in China National Microbiology Data Center (NMDC) with accession numbers NMDC-N0001DR2 (https://nmdc.cn/resource/genomics/sequence/detail/NMDCN0001DR2). Restriction endonucleases were purchased from Takara, DNA polymerases, DNA purification kit, gel extraction kit plasmid miniprep kit, and reverse transcriptase were obtained from Vazyme. The ROS detection kit was purchased from Solarbio. HA was provided by

Table 1 Overview of the studies reporting rhPH-20 expression in different hosts

Host	Time for cultivation	Method for activity assay	rhPH-20 production	Reference
P. pastoris	120 h	Elson-Morgan method	19.82 U/mL	This study
P. pastoris	72 h	Elson-Morgan method	2 U/mL	(Chen et al. 2016)
Arabidopsis thaliana	-	Turbidimetric method	5.8 IU/mL*	(Li et al. 2014)
HEK293T cells	72 h	Turbidimetric method	3.5 IU/mL*	(Ata-abadi et al. 2022)
Escherichia coli	-	Turbidimetric method	1.7 IU/mL*	(Kaessler et al. 2011)

*10 IU is approximately equal to 1 U under the definition of enzyme activity in this study (El-Safory et al. 2010)

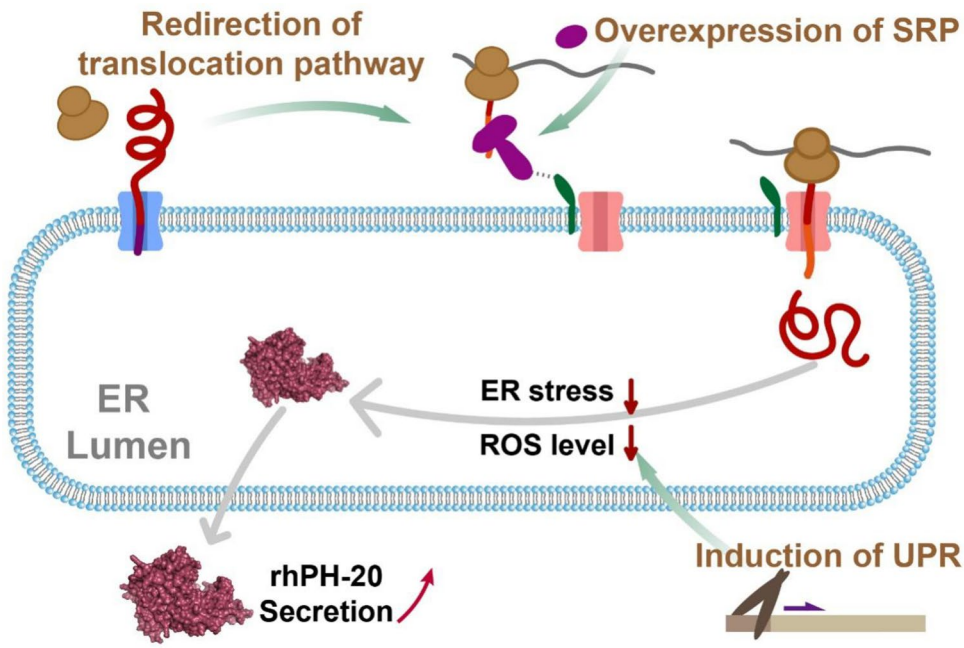

Fig. 1 Overview of the pathways in protein secretion addressed in this study. First, SP engineering redirected rhPH-20 into the co-translational translocation pathway; overexpressing components of signal recognition particle (SRP), improved its entry into ER as a nascent peptide; finally, induction of UPR by overexpressing Hac1p enhanced expression of posttranslational modification proteins, relieved ER stress and reduced ROS level, which resulted in further improved secretory expression of rhPH-20

Shandong Focusfreda Biotech Co., Ltd. All other reagents were purchased from commercial sources.

Construction of recombinant vectors

Primers used in this study are listed in Table S2. The accession number of each introduced gene is listed in Table S3. *Escherichia coli* JM109 was adopted as the clone host for plasmid construction. To construct the vector pGCZα-PH20, pGAPZαA was first linearized by endonuclease *EcoR* I and *Sal* I, and the encoding gene of rhPH-20 was amplified with primers PH20-IF and PH20-IR. Genes were fused with plasmid by ClonExpress II One Step Cloning Kit (Vazyme, Nanjing, China). Then, primers Promoter-VF/R was used to amplify the linearized vector with P_{GAP} deleted, and GCW14-IF/R was used to amplify the P_{GCW14} promoter from the GS115 genome to construct vector pGCZα-PH20. In SP optimization, recombinant vectors with altered SPs were constructed by one-step reverse PCR using primers (F/R), with introduced sequences on homology arms.

For multi-cassette plasmid construction, each single cassette was first constructed based on the pGAP3.5 K backbone: pGAP3.5 K was linearized by *EcoR* I and *Not* I, and the gene of interest was inserted. The plasmid harboring the first cassette was then linearized using primers Cassette insertion-VF/R, and the cassettes for insertion were amplified by primers CF/R. The primers were named employing their positions in the multi-cassette plasmid (for example, 2/3 cassette-CF/R were used to amplify the second cassette in a three-cassette plasmid). These amplified cassettes with homology arms were then fused to vectors with Hieff Clone® Plus Multi One Step Cloning Kit (Yeasen, Nanjing, China).

Construction of recombinant strain

To achieve integrative construction of recombinant strains, plasmids were first extracted and linearized: for pGCZαA derivatives, no proper Endonuclease cleavage sites were found, so reverse PCR was used for linearization; for pGAP3.5 K derivatives, *Nco* I was used to cleave the plasmids at HIS4 site. The purified linearized product was then added to 100 µL of *P. pastoris* GS115 competent cells, and transformed into cells by electroporation at 2000 V for 5 ms. For rhPH-20 expression strain construction, the transformants were screened on YPD agar plates (20-g/L glucose, 10-g/L yeast extract, 20-g/L peptone, and 20-g/L agar) with 0.5 mg/mL, 1.0 mg/mL, 1.5 mg/mL, and 2.0 mg/mL of zeocin, three-copy strains were screened and verified according to methods described by Xiang et al. (2022). For transformants overexpressing pGAP3.5 K derived vectors, single-copy colonies were picked directly from the MD agar plate (20-g/L glucose, 13.4-g/L YNB, and 20-g/L agar).

Shake-flask fermentation of recombinant strains

Recombinant strains rejuvenated in 10 mL of YPD medium (20-g/L glucose, 10-g/L yeast extract, and 20-g/L peptone) were inoculated with a volume of 10% (v/v) to 30-mL BMGY medium (10-g/L glycerol, 20-g/L peptone, 10-g/L yeast extract, 13.4-g/L YNB, 4-g/L $K_2HPO_4{\cdot}3H_2O$, and 11.8-g/L KH_2PO_4) for preculture at 30 °C and 220 rpm. After 24 h, strains were collected, washed with sterile water,

and resuspended in 30 mL of fresh BMGY medium for shake-flask fermentation at 30 °C and 220 rpm. After 72 h, the culture broth was collected for enzymatic activity assay or western blot analysis.

For shake-flask fermentation optimization, the initial conditions were set as follows: BMGY medium, inoculum concentration of 10%, and incubation temperature of 30 °C. Strains rejuvenated in 10 mL of YPD medium were directly inoculated into 30 mL of fermentation broth and cultivated at 220 rpm. The optimal time point for harvest was selected according to the time course of rhPH-20 secretion in the first step of optimization.

Fed-batch fermentation

Fed-batch fermentation was carried out based on the optimization results of shake-flask fermentation. A single colony was picked from an agar plate to 10 mL YPD medium for overnight incubation. Then, it was inoculated into 50 mL BMGY medium with a volume of 10% for 24 h preculture, to reach OD_{600} of 20. 200 mL of cultured seed broth was then inoculated into a 5-L fermenter carrying 1.8 L of modified BMGY medium (40-g/L glycerol, 20-g/L peptone, 10-g/L yeast extract, 13.4-g/L YNB, 4-g/L $K_2HPO_4{\cdot}3H_2O$, and 11.8-g/L KH_2PO_4, pH6.0). The stirring rate and ventilation were adjusted to maintain the dissolved oxygen (DO) level above 20%. The temperature was maintained at 30 °C for the first 24 h and was switched to 20 °C for better target protein production after 24 h. After glycerol depletion indicated by a steep increase in DO, 50% glycerol was supplemented at a constant rate of 3 mL/h. Samples from culture broth were collected every 12 h for further characterization.

Hyaluronidase activity assay

To determine secreted hyaluronidase activity, supernatant from culture broth was first collected and diluted with 0.1 M acetate buffer (pH 5.0) and used for hyaluronidase activity assay described by Chen et al. (2016), and a modified Elson-Morgan method was used for colorimetric determination. One unit (U) of hyaluronidase activity is defined as the amount of reducing end released from the substrate that is equivalent to 1 nmol of N-acetylglucosamine per minute at 37 °C (Wang et al. 2011).

SDS-PAGE and western blot analysis of rhPH-20

SDS-PAGE was performed with a 12% Tris-Gly resolving gel, stained by Coomassie Brilliant Blue G250. For western blot analysis, proteins from the culture supernatant were separated with SDS-PAGE, and then transferred to a polyvinylidene fluoride (PVDF) film and blocked with 5% bovine serum albumin (BSA) in TBST buffer for 2 h. The PVDF film was then washed with TBST buffer, incubated with the anti-6 × His tag antibody (Yeasen, Nanjing, China, 1:5000 diluted with TBST) for 2 h, and finally HRP-conjugated goat anti-mouse lgG (Yeasen, Nanjing, China, 1:5000 diluted with TBST) for 2 h. Fluorography was performed with ECL reagents (ThermoFisher Scientific, MA, USA).

Quantitative analysis of relative gene expression level

Approximately 5×10^6 of yeast cells were first collected from culture broth, and washed with RNAase-free water. The total RNA of each strain was then extracted using Yeast Total RNA Isolation Kit (Sangon Biotech, Shanghai, China). After reverse transcription, total cDNA was collected as the template for real-time PCR (RT-PCR). The reference gene for normalization was the *ACT1* gene, and changes in the transcriptional level of each gene were calculated using the ΔΔCt method. Primers used for RT-PCR are listed in Table S2.

Results

Constitutive expression of rhPH-20 with a constitutive promoter P_{GCW14}

For constitutive rhPH-20 expression, the expression vector was harbored by inserting the rhPH-20 gene into plasmid pGCZαA. pGCZαA was constructed by substituting the P_{GAP} promoter in the pGAPZαA plasmid with another strong constitutive promoter, P_{GCW14}, which was determined to lead to a higher transcription level than the P_{GAP} promoter (Zhang et al. 2013). Reverse PCR was used to linearize the vector to integrate the DNA into the genome of the *P. pastoris* GS115 strain. The successfully constructed strain, *P. pastoris* GS115/PGCZα-PH20, showed a hyaluronidase activity of 2.01 U/mL at 72 h. Based on sequence analysis, the truncated rhPH-20 protein has more than six potential N-glycosylation sites, making it hard to predict its apparent molecular mass. This is supported by SDS-PAGE analysis, as no clear bands at its theoretical molecular mass (53.58 kDa calculated by Expasy Compute pI/Mw tool (https://web.expasy.org/compute_pi/)) were observed (Fig. 2a). Therefore, the western blot analysis was employed, and as shown in Fig. 2b; the signal of His6-tagged rhPH-20 was found to distribute in a range of molecular mass above 100 kDa. This is consistent with the previous studies (Chen et al. 2016; Pang et al. 2022).

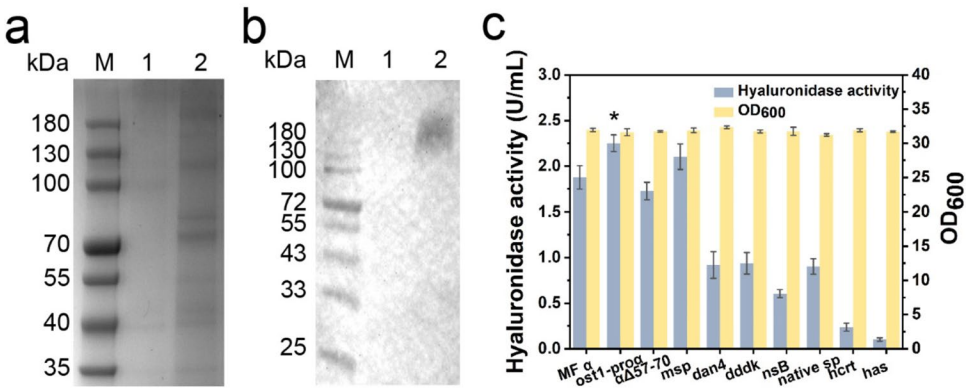

Fig. 2 Expression and SP optimization of rhPH-20 in *P. pastoris* GS115. **a** SDS-PAGE analysis; **b** western blot analysis. Lane M: molecular mass marker; lane 1: culture supernatant of GS115 strain with empty plasmid insertion; lane 2: culture supernatant of recombinant strain expressing rhPH-20. Samples were concentrated for analysis. **c** Secreted rhPH-20 activity and biomass of each strain. Statistical significance to control is indicated with an asterisk (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

Signal peptide screening for optimized secretion expression of rhPH-20

Both substitution and modification of the initial SP MFα were adopted to optimize SP (Table S4). For SP substitution, two endogenous SPs, dan4 and dddk in *P. pastoris*, were picked as they were shown to facilitate efficient secretion of SECA and DDDK protein (Duan et al. 2019; Govindappa et al. 2014); three other SPs from *Candida antarctica* and *H. sapiens* were picked and synthesized according to codon bias in *P. pastoris*. Meanwhile, there are several studies reporting that using the heterologous protein's native SP could also facilitate high-level secretion (Ciplys et al. 2015; Vadhana et al. 2013), thus the native SP from human PH-20 was also tested in this study.

Overexpressing rhPH-20 with different SPs did not exert a significant effect on biomass accumulation, as suggested in Fig. 2c (data used to generate the figure is listed in Table S5). Compared to SP substitution, SP modification based on MFα seems to be more effective for enhancing rhPH-20 expression. Replacing the pre-MFα sequence with ost1 SP (to construct ost1-proα) and mouse salivary α-amylase SP (to construct msp) enhanced the secretory yield of rhPH-20 by 20% and 12.1% compared to MFα (Fig. 2c). The efficient secretion led by ost1-proα could be explained by the fact that ost1 SP guides protein into the ER lumen via co-translational translocation, instead of posttranslational guided by MFα (Fig. 3a) (Besada-Lombana and Da Silva 2019).

It is also noteworthy that, the native SP was found to be able to guide rhPH-20 secretion, although with low efficiency (51.9% compared to MFα). This is partially consistent with several other studies, supporting that some SPs from higher eukaryotes could be recognized by *P. pastoris* and lead the protein to the secretion pathway (Kangwa et al. 2018; Latiffi et al. 2013).

Enhanced rhPH-20 secretion by overexpression of signal recognition particle components

Based on the previously constructed strain expressing rhPH-20 with ost1-proα SP (strain OProα), we set out to test whether strengthening the translocation pathway by overexpressing specific components would help improve the secretory capacity of rhPH-20. Three major components in this pathway, namely signal recognition particle (SRP), SRP receptor (SR), and translocon (Sec61 complex) were overexpressed (Fig. 3a). For overexpression of SRP, three subunits were selected: Srp54p recognizes and interacts with SP (Walter et al. 1982), Srp14p plays a vital role in elongation arrest (Brooks et al. 2009), and Srp68p plays roles in ribosome binding and interacting with SR (Lee et al. 2021). Gene of each protein was amplified from *P. pastoris* GS115 genome according to annotation, cloned into plasmid pGAP3.5 K (Fig. 3b, sequences of plasmids are listed in Table S6), and transformed into the strain constructed in 3.2 harboring ost1-proα-leaded rhPH-20 gene. After 72 h of cultivation, strains overexpressing *SRP14* and *SRP68* were observed to elevate the production of rhPH-20 by 29.7% and 15.3%, and the overexpression of *SRP54* led to the highest secreted hyaluronidase activity of 3.14 U/mL, which amounts to a 1.48-fold increase comparing to the parental strain Oproα (Fig. 3c). In contrast, no positive effect was found in strains overexpressing SR and translocon; this might indicate overexpression of SPR components is a more efficient way to promote co-translational translocation in *P. pastoris*.

Improved rhPH-20 secretion by overexpression of spliced form *HAC1* from *S. cerevisiae*

After translocating into the ER lumen, proteins undergo posttranslational modifications (Liu et al. 2022). When excessive protein enters ER and aggregate, the unfolded

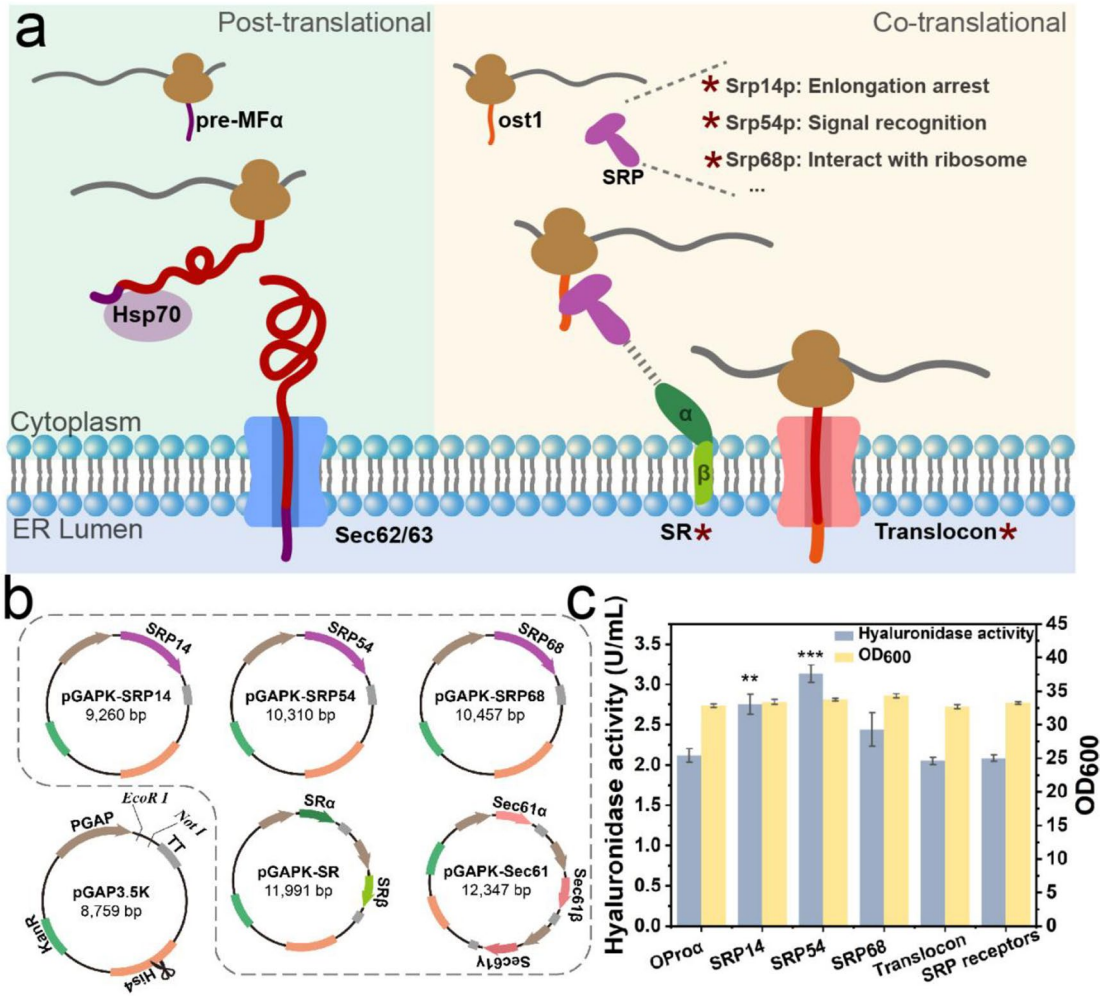

Fig. 3 Optimizing and engineering the translocation pathway for rhPH-20 secretion. **a** Mechanism illustration of two pathways that nascent peptides translocate into the ER lumen, the overexpression targets of the co-translational pathway are marked with asterisks. **b** Construction of vectors harboring SRP subunits, SRP receptor, and Sec61 complex. **c** Secreted rhPH-20 activity and biomass of each strain. Statistical significance was analyzed employing Student's *t* test

protein response (UPR) activates (Fig. 4): The chaperone protein KAR2 senses unfolded proteins, and unbinds the transmembrane Ire1p, activating its endonuclease activity. Ire1p splices the intron in *HAC1* mRNA, which is then translated into Hac1p, a transcriptional factor upregulating the expression of UPR genes related to protein folding, misfolded protein degradation, etc. to relieve ER stress (Guerfal et al. 2010). Direct overexpression of spliced form *HAC1* has been demonstrated as an efficient strategy to induce UPR and improve protein secretion (Liu et al. 2022).

To further improve the posttranslational protein modification process, we selected three *HAC1* genes from *P. pastoris*, *S. cerevisiae*, and *H. sapiens* (*XBP1*, encoding Hac1p homolog, Xbp1p in the human genome), cloned their spliced form gene to construct vectors harboring both *SRP54* and *HAC1* genes. Recombinant vectors were transformed into strain Oproα, resulting in recombinant strains OE54-PpHAC1, OE54-ScHAC1, and OE54-HsHAC1. Results from shake-flask fermentation suggested improvement of rhPH-20 secretion into culture supernatant in all three strains by 23%, 32%%, and 21%. This proves that overexpressing Hac1p homologs from other eukaryotes could elevate protein secretion levels, and might indicate the functional consistency in these three proteins. Among the three strains, OE54-ScHAC1, harboring the *HAC1* gene from *S. cerevisiae*, achieved an activity of 4.06 U/mL (Fig. 5a). The secretion capability of rhPH-20 of this strain indicates a more than twofold increase after three steps of optimization, compared to the original strain GS115/PGCZα-PH20. The effect of overexpressing *HAC1* genes alone was also evaluated. Consistent with results from co-overexpressing *SRP54* and *HAC1*, introducing *ScHAC1* led to the best improvement of rhPH-20 productivity, which is about 1.51-fold compared to control strain Oproα (Fig. 5b). By western blot analysis,

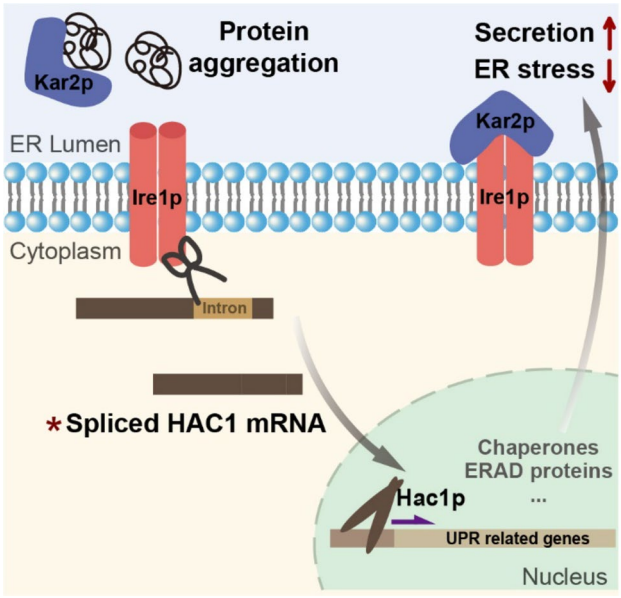

Fig. 4 Illustration of the UPR activation process in yeast, and the effect of overexpressing spliced form *HAC1*. Ire1p splices the intron in *HAC1* mRNA when protein aggregation occurs, the spliced form *HAC1* mRNA is then translated to Hac1p, which activates UPR-related genes that are involved in processes including PTM and endoplasmic reticulum-associated degradation (ERAD). The overexpression target in this study is marked with an asterisk

an increase in the amount of secreted rhPH-20 was observed after three steps of pathway engineering, which is consistent with results from the enzyme activity assay (Fig. 5c).

The enhancement of rhPH-20 secretion facilitated by introducing *HAC1* was greater when overexpressing it alone compared to co-expressing with *SRP54*. This might indicate that the expression level of these two genes can be further optimized.

Activation of UPR and alleviation of ER stress after *HAC1* overexpression

RT-PCR was performed to analyze the activation of UPR before and after *ScHAC1* overexpression. As shown in Fig. 5e, in strain OE54 overexpressing rhPH-20 and Srp54p, a slight increase in UPR level was observed compared to the wild-type GS115 strain, as the expression level of *KAR2*, a UPR-responsive gene (Guerfal et al. 2010), was slightly increased by 1.61-fold. After co-expressing *ScHAC1*, a significant enhancement of transcription level was found in *ERO1*, *PDI1*, and *KAR2*, which encode chaperones that are involved in protein folding and disulfide bond formation (Fig. 5e). The downregulation of endogenous *HAC1* and the stress-responsive gene *MSN2* also indicated alleviated ER stress and cellular stress (Wu et al. 2021). We also examined the transcriptional level of rhPH-20 in the two strains, and no significant change was found, which proved that the enhanced rhPH-20 secretion did not result from the enhanced target gene expression (Fig. 5d). Taken together, these results suggest that activating the UPR by overexpressing *ScHAC1* can strengthen the secretion pathway and help enhance the productivity of recombinant rhPH-20.

Reduced ROS level by *ScHAC1* overexpression

Reactive oxygen species (ROS) are a group of substances that undermine cell viability and bring negative effects to recombinant protein secretion. Overexpressing rhPH-20 and Srp54p led to a significant 5.83-fold enhancement in intracellular ROS level, and co-expressing *ScHAC1* reduced ROS level by 62.9% compared to the strain OE54 (Fig. 6a). The transcriptional level of three antioxidant enzymes, *SOD1*, *SOD2*, and catalase genes showed an obvious decrease (Fig. 6b). This might indicate that overexpression of *ScHAC1* leads to relieved oxidative stress.

Though ROS was reduced with *HAC1* overexpression, the relation between ROS reduction and rhPH-20 secretion needs to be validated: in a study reported by Huang et al. overexpressing Yap1p did not help to improve leech hyaluronidase secretion. H_2O_2 in low concentration was introduced into the medium for shake-flask fermentation. The intracellular ROS level of strain OE54-ScHAC1 was elevated compared to the control group after exogenous H_2O_2 was added into the broth and cultured for 3 days (Fig. 6c). Exogenously introduced ROS did not display an obvious effect on biomass accumulation, whereas secreted rhPH-20 activity decreased with the increase of H_2O_2 concentration (Fig. 6d). Together, these results indicate that ROS reduction by ScHac1p overexpression might exert a protective effect on recombinant protein secretion.

Fermentation optimization and scale-up fermentation of optimized strain

After constructing the strain OE54-ScHAC1 with improved rhPH-20 secretion efficiency, we then set out to further elevate its productivity by fermentation optimization. Three parameters, glycerol concentration, culture temperature, and inoculum concentration were optimized sequentially on shake-flask level, and results are shown in Fig. 7. According to the results, the optimal rhPH-20 production was achieved in a modified BMGY medium containing 40-g/L glycerol, with an inoculum concentration of 10%, under 20 °C after 96 h cultivation. Under the optimized conditions, the highest 4.99 U/mL of rhPH-20 secretion was achieved (Fig. 7).

Based on fermentation optimization results, fed-batch fermentation was performed in a 5-L fermenter. As shown in Fig. 8, by supplementing glycerol, the amount of secreted rhPH-20 increased steadily until reaching a peak at 120 h,

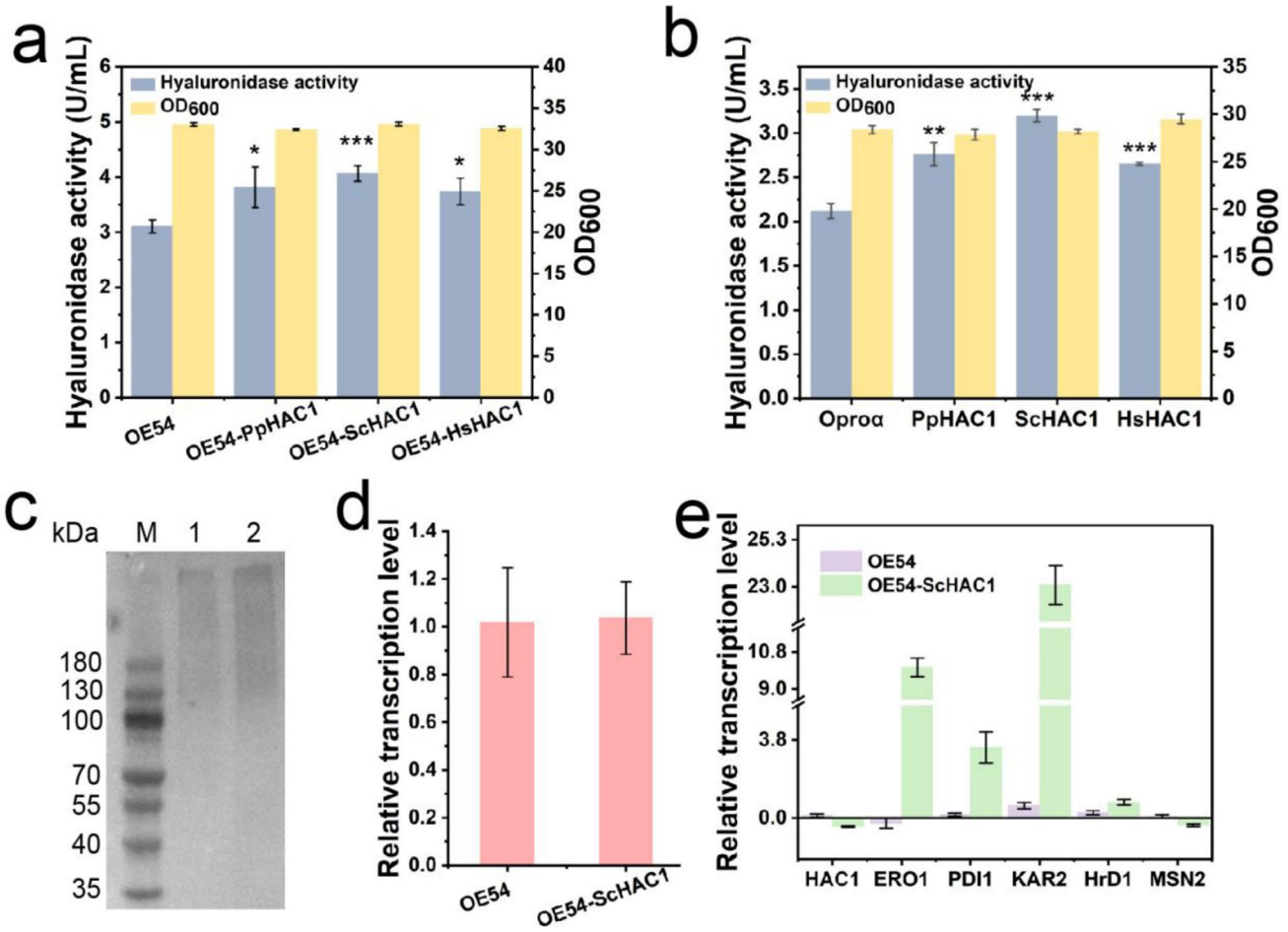

Fig. 5 Effect of *HAC1* overexpression on rhPH-20 secretion and UPR. **a** Secreted rhPH-20 activity and biomass of each *HAC1* overexpressing strain constructed based on strain OE54. **b** Secreted rhPH-20 activity and biomass of *HAC1* overexpressing strains constructed based on strain Oproα. **c** Western blot analysis of secreted rhPH-20 by strain GS115/pGCZα-PH20 (lane 1) and OE54-ScHAC1 (lane 2). Lane M: molecular mass marker. **d** Transcriptional analysis of rhPH-20 expression level before and after *ScHAC1* expression. **e** Transcriptional analysis of UPR-related genes before and after *ScHAC1* overexpression. Statistical significance was analyzed employing Student's *t* test

with the highest activity of 19.82 U/mL. This result indicates a 4.9-fold increase in secreted rhPH-20 compared to the shake-flask level before fermentation optimization, suggesting the strain after secretion pathway optimization was promising in scale-up production of rhPH-20. The constitutive rhPH-20 expression level reported here is promising compared to existing studies: under the same definition of enzyme activity, the expression level of constitutive rhPH-20 expressing strain reported by Chen et al. yielded 2 U/mL in YPD medium (Chen et al. 2016).

Discussion

Pichia pastoris is a yeast host that has been widely adopted to produce recombinant proteins. It possesses a high capability to secret heterologous proteins (Yang and Zhang 2018) while only secret endogenous proteins at very low concentrations (Duan et al. 2019). Here, by implementing combined strategies to engineer two ER-related secretion pathways, namely translocation and the UPR, we constructed a recombinant *P. pastoris* strain with an enhanced secretion capacity of rhPH-20.

rhPH-20 is a protein with multiple N-glycosylation sites. It goes through glycosylation in eukaryotic cells, which leads to a shift in molecular mass that has been observed when expressed in Hela cells and *P. pastoris* (Arming et al. 1997; Chen et al. 2016). rhPH-20 expressed in *P. pastoris* is hyper-glycosylated, which is suggested by a widely distributed apparent molecular mass in western blot analysis (Fig. 2b and (Pang et al. 2022)). This is because of the hyper-mannosylation mechanism in yeast hosts: According to the literature, about 20% of glycans in *P. pastoris* are represented in hyper-mannosylation form (Blanchard et al.

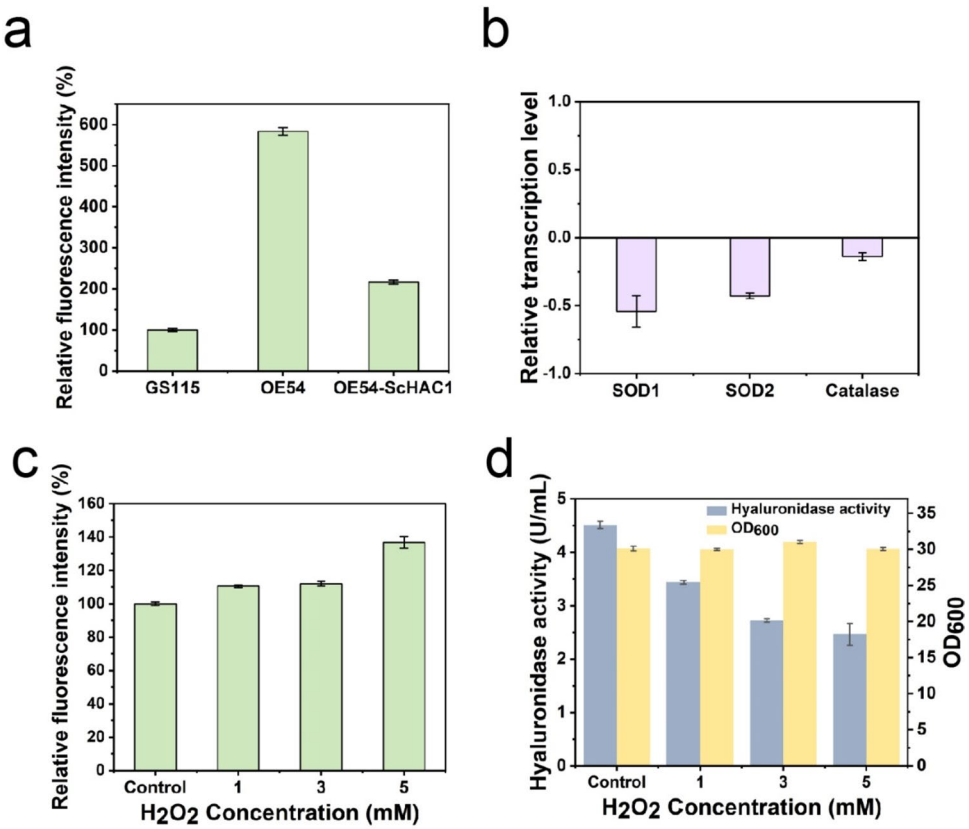

Fig. 6 Effect of *ScHAC1* overexpression on ROS level. **a** ROS level before and after overexpressing *ScHAC1*. **b** Transcription analysis of oxidative stress-responsive genes. **c** ROS level of OE54-ScHAC1 strain after adding H_2O_2 into the broth to different concentrations and cultured for 72 h. **d** Secreted rhPH-20 activity and biomass yield of strain OE54-ScHAC1 after adding H_2O_2

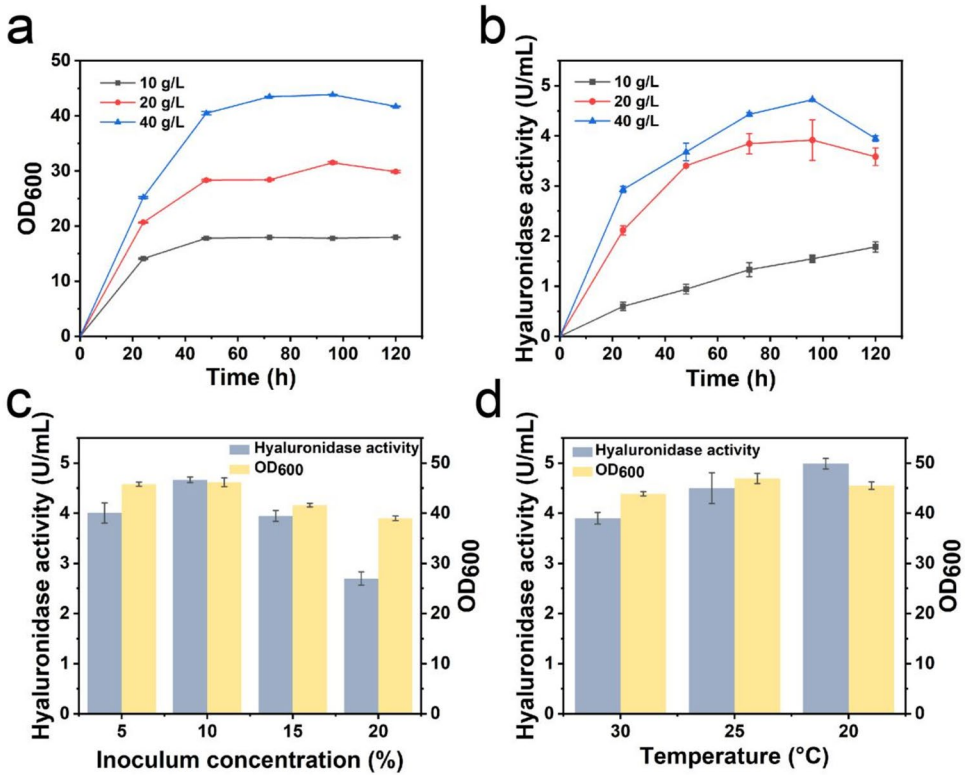

Fig. 7 Fermentation optimization of strain OE54-ScHAC1. **a** Effect of glycerol concentration on cell growth. **b** Effect of glycerol concentration on rhPH-20 secretion. **c** Effect of inoculum concentration on biomass and rhPH-20 secretion at 96 h. **d** Effect of temperature on biomass and rhPH-20 secretion at 96 h

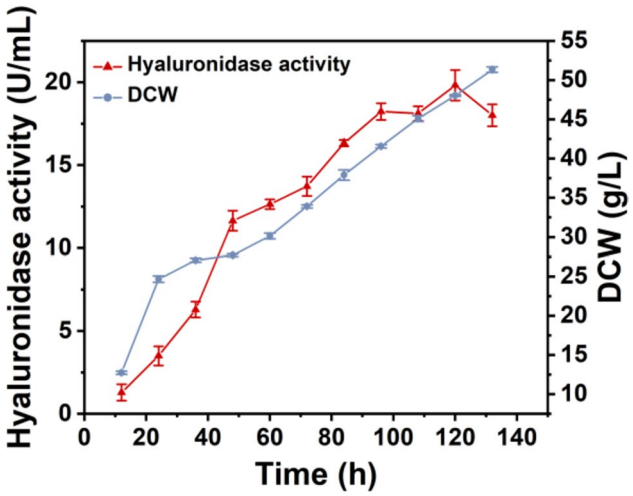

Fig. 8 Time course of dry cell weight and hyaluronidase activity in the fed-batch fermentation of strain OE54-ScHAC1

2007; Lei et al. 2013), which contributes to an increase in the protein's molecular mass (Hess et al. 2021). Considering this difference in glycosylation, future studies could be carried out to investigate the glycosylation profile of rhPH-20 in *P. pastoris*, and the influence of hyper-mannosylation on rhPH-20 activity, by comparing the wild-type host GS115 to the *P. pastoris* hosts with humanized glycosylation (Laukens et al. 2015).

Optimizing SPs has been a widely adopted strategy for improving recombinant protein secretion in yeast hosts. In *Pichia pastoris*, MFα is a commonly used SP for high-level protein secretion, but its efficiency in guiding proteins into secretion pathways may differ when expressing different proteins (Duan et al. 2019; Fitzgerald and Glick 2014). Here, by screening 9 SPs from different origins or constructed by different engineering approaches, we found that a hybrid SP, ost1-proα, can lead to enhanced rhPH-20 secretion efficiency. In this SP, the ost1 sequence was proved to guide the protein into co-translational translocation pathway to enter the ER lumen (Willer et al. 2008), while the pre-sequence of MFα guides the protein into posttranslational translocation. This result, together with existing studies, might show the importance of selecting suitable translocation pathways for protein secretion (Besada-Lombana and Da Silva 2019; Fitzgerald and Glick 2014). The msp, previously described by Liu et al. is an engineered SP with high hydrophobicity (Liu et al. 2005). The improvement in rhPH-20 secretion led by msp might also be explained by a switch in translocation mechanisms, as studies have suggested that SPs with higher hydrophobicity tend to interact with SRP complex and guide protein into co-translational translocation (Liu et al. 2022; Pool 2005). The native SP of rhPH-20 was also proved to guide its secretion, but with a lower secretion level. This might be explained by that the SP cannot be efficiently recognized by the *P. pastoris* host, thereby preventing the efficient routing of rhPH-20 into the secretion pathway (Zahrl et al. 2022).

ER translocation is indispensable for newly synthesized protein to enter ER and undergo proper glycosylation, folding, and further enter the secretion pathway by vesicle transportation (Walter et al. 1982). Though existing studies in CHO cells, *S. cerevisiae* have suggested the co-translational translocation process as a potential bottleneck in recombinant protein secretion (Le Fourn et al. 2014; Tang et al. 2015), engineering the co-translational translocation pathway has been fewer reported in *P. pastoris*. In this study, by overexpressing components of the SPR complex, we achieved the highest 48% improvement in rhPH-20 secretion. For the first time, these results might indicate the abundance of SRP in the co-translational translocation pathway is another bottleneck in protein secretion in *P. pastoris*. Meanwhile, literatures suggest that the limited number of SRP receptor (SR) complex might be a rate-limiting factor in protein secretion in Hela cells and 293 T cells, which was supported by overexpressing SR in low elongation arrest level cells helps to rescue the translocation into ER (Lakkaraju et al. 2008). However, overexpressing the complex itself in strains without this deficiency did not show to further improve protein translocation (Lakkaraju et al. 2008). Consistent with this finding, overexpression of the SR complex alone did not improve rhPH-20 secretion efficiency in *P. pastoris*. Also, overexpressing the translocon complex (Sec61), which has been found to promote immunoglobulin secretion by more than 50% in CHO cells, did not improve rhPH-20 secretion in *P. pastoris* (Le Fourn et al. 2014).

In yeast hosts, several studies have highlighted the effectiveness of enhancing translocation to improve protein yield (Table 2). With an optimized combined overexpression strategy, the yield can be significantly higher when expressing single genes alone (Table 2). Thus, combined overexpression of co-translational translocation constituents can be very promising for further improvement of protein secretion in yeast in future studies.

Proper folding and posttranslational modification are crucial for protein secretion (Liu et al. 2022). Overexpressing Hac1p, the transcriptional factor of UPR, has been validated as an effective strategy to improve protein processing in ER and thus, their secretion (Raschmanová et al. 2021). In this study, overexpressing Hac1p homologous from *S. cerevisiae* and *H. sapiens* and endogenous Hac1p were all confirmed to improve rhPH-20 secretion in *P. pastoris*, though with different adaptability in the engineered strain (Bankefa et al. 2018), and overexpressing the ScHac1p alleviated ER stress and cellular stress. The upregulated UPR-related genes (*KAR2*, *ERO1*, *PDI1*, *HRD1*) suggested the activation of UPR on the transcription level. A previous work by Lin et al. reported

Table 2 Studies that report strengthening translocation to improve protein secretion

Host	Target protein	Translocation pathway	Optimal overexpression target(s)	SP	Improvement in protein secretion	Reference
P. pastoris	rhPH-20	Co-translational	Srp54p	ost1-proα	48%	This study
P. pastoris	Fab	Post-translational	Kar2p	MFα	50%	(Zahrl et al. 2022)
S. cerevisiae	α-amylase	Co-translational	Srp54p	Yap3	103%	(Tang et al. 2015)
Yarrowia lipolytica	YFP	Post-translational	Ssa8p	SP1	47%	(Korpys-Wozniak et al. 2021)
P. pastoris	Fab	Post-translational	Ssa1p + Ydj1p + Snl1 + Kar2p + Sil1p	MFα	377%	(Zahrl et al. 2022)
P. pastoris	lipase	Post-translational	Ydj1p + Ssa1p	MFα	150%	(Samuel et al. 2013)

a proteomic study of *P. pastoris* before and after Hac1p overexpression, and they observed an 84.6% and 196.2% increase in Pdi1p and Kar2p abundance, as well as an increased abundance of chaperones (Lin et al. 2013). Combined, these results suggest that overexpression of Hac1p leads to upregulation of UPR-related proteins on both transcription and protein abundance levels.

Reducing ROS levels is a demonstrated way to improve active secretion expression of proteins in yeast hosts (Hu et al. 2022). By overexpressing the transcription factor Yap1p to downregulate ROS level, twofold enhanced secretion of trypsinogen was achieved in *P. pastoris* (Delic et al. 2014). It has been reported that overexpression of the *HAC1* gene leads to a reduced ROS level in yeast, which is consistent with our results (Wang et al. 2021). We also discovered a significant decrease in rhPH-20 secretion after supplementing H_2O_2, ROS precursor, which might indicate ScHac1p overexpression exerts a protective effect on rhPH-20 secretion by reducing ROS level. However, while several studies have also shown that Hac1p overexpression can reduce ROS levels and relieve oxidative stress in *P. pastoris* (Graf et al. 2008; Wang et al. 2021), how the ROS level was downregulated by Hac1p overexpression is still unclear. Therefore, future investigations are needed to explore the exact mechanism of ROS reduction in Hac1p-overexpressing yeasts.

To sum up, in this study, the constitutive secretory expression of rhPH-20 was realized in *P. pastoris* GS115 under the control of P_{GCW14}. Redirecting the rhPH-20 into co-translational translocation and overexpressing SRP component Srp54p improved its efficiency in ER entry, resulting in the improved secretory expression; Triggering the UPR by overexpressing ScHac1p from *S. cerevisiae* strengthened the posttranslational modification process, relieved ER stress and reduced cellular ROS level, contributed to further improved rhPH-20 secretion. The highest hyaluronidase activity of 19.82 U/mL was finally achieved in a 5-L fermenter. Our work might lay the foundations for the cost-effective production of rhPH-20, and the combined ER pathways engineering strategy could be applied to improve the secretion of other recombinant proteins in *P. pastoris* hosts.

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Author contribution YZ, JG, and JS conceived and designed the research. YZ and JJ conducted the experiments. YZ and JJ analyzed the data. YZ, JG, and ZX wrote the manuscript. All authors read and proved the final manuscript.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethical approval This article does not contain any studies with human participants performed by any of the authors.

Conflict of interest The authors declare no competing interests.

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