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Functional expression of trypsin from *Streptomyces griseus* by *Pichia pastoris*

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Abstract In the present study, the genes encoding trypsinogen and active trypsin from *Streptomyces griseus* were both cloned and expressed in the methylotrophic yeast *Pichia pastoris* with the α-factor secretion signal under the control of the alcohol oxidase promoter. The mature trypsin was successfully accumulated extracellularly in soluble form with a maximum amidase activity of 6.6 U ml^{-1} (batch cultivation with flask cultivation) or 14.4 U ml^{-1} (fed-batch cultivation with a 3-l fermentor). In contrast, the recombinant trypsinogen formed inclusion bodies and no activity was detected. Replacement of the trypsin propeptide Ala-Pro-Asn-Pro confirmed that its physiological function was as a repressor of activity. More importantly, our results proved that the propeptide inhibited the activity of trypsinogen after its successful folding.

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Introduction

Trypsin (EC 3.4.21.4) is a sort of serine protease which has potential applications in leather bating, food processing, pharmacy, clinical diagnoses, and biochemical tests [32]. To date, trypsin and trypsin-like proteases have been isolated from a number of mammalian sources, such as bovine, dogfish, starfish, horseshoe crab, shrimp, sea pansy, and silkworms [25, 31, 35]. In particular, the bovine trypsin, which plays an important role in the activation cascade of pancreatic enzymes, has been thoroughly studied for its commercial application. Besides, trypsin also occurs in certain microorganisms, including several species of *Streptomyces* [31]. *Streptomyces griseus*, a type genus of *Streptomyces*, has been well studied and widely used for the production of many kinds of secondary metabolites (such as streptomycin and actinorhodin) [4, 23] and various proteases (especially *S. griseus* trypsin, SGT) [14]. Actually, SGT was successfully isolated from a mixture of enzymes and proteins derived from the extracellular filtrate of its cultures in the 1970s [32].

In nature, the mammal trypsin is expressed and secreted as trypsinogen (an inactive form of trypsin) by the pancreas. Activation of trypsinogen involves the removal of the Val-(-Asp-)$_4$-Lys fragment and formation of an active site, which is catalyzed by enterokinase [30]. Similarly, previous studies proved that trypsin is also initially produced as trypsinogen in *S. griseus* and consists of three parts: an amino terminal signal (32 amino acid residues), a propeptide (4 amino acid residues) and the active trypsin (Fig. 1) [17]. Furthermore, it was elucidated that the active

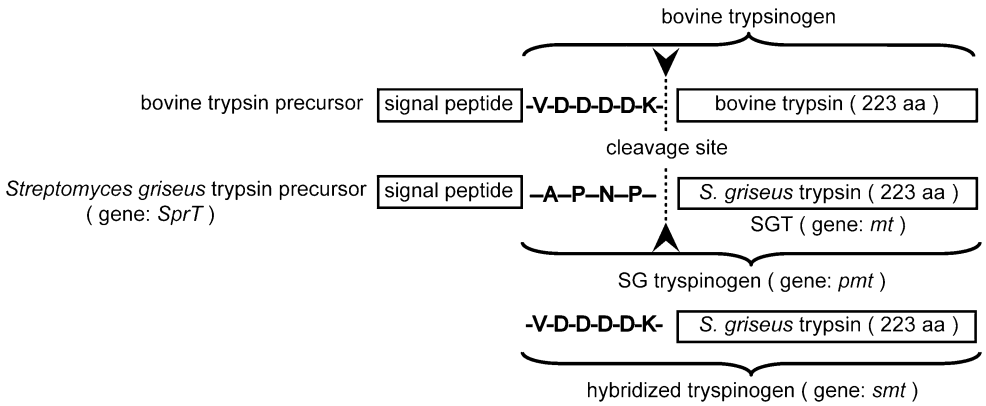

Fig. 1 Comparison of the structure of trypsin precursor from bovine and *Streptomyces griseus*. *SprT* gene encoding for SGT precursor, *pmt* fragment encoding for SG trypsinogen, *mt* fragment encoding for SGT, *smt* fragment encoding for bovine trypsinogen propeptide with SGT. Cleavage site between propeptide and active trypsin (*arrowheads*)

SGT is composed of 223 amino acid residues with a computed molecular weight of 23.1 kDa [24, 25]. Although there are six disulfide bonds present in bovine trypsinogen whereas only three disulfide bonds are present in SGT [19, 26], SGT has a remarkable homology with bovine trypsin in amino acid sequence, the active site, substrate binding region, and substrate specificity [25, 26, 28]. Therefore, it was accepted that SGT is one exception to the distinction between other *Streptomyces* enzymes and mammalian enzymes [28].

In the past, trypsin was mainly extracted from porcine or bovine pancreas, but this conventional method could not meet the high demand, and also contamination with pathogens associated with the donor organism was a great concern. With the development of molecular biotechnology, much attention has been paid to the microbial production of recombinant trypsin. The genes encoding trypsin from rat and human were cloned and expressed with the *Escherichia coli* system [13, 33]. However, the production of active enzyme secreted by the *E. coli* periplasm was very low. Most portions of protein appeared in the form of intracellular inclusion bodies [38]. Although the bovine trypsinogen was successfully expressed heterologously in *Pichia pastoris* [11], the yield of the recombinant trypsinogen was low. Moreover, to activate trypsinogen, enterokinase must be added artificially. Recently, the bovine trypsin gene was also studied in *Lactococcus lactis* [37] and transgenic rice cells [18]. Nevertheless, the amount of active recombinant trypsin was still very low even after codons were optimized according to their bias. Taken together, it was concluded that the production of active mammalian trypsin with a microbial expression system appeared to be an unachievable goal. As a result, attention has turned to microbial trypsin genes.

Because of its wide application and biosafety, *Streptomyces* sp. was considered to be an important source of trypsin [25, 31]. Using a synthetic oligonucleotide probe, Kim et al. [17] successfully cloned and characterized the *sprT* gene encoding SGT precursor from *S. griseus*. Subsequently, *sprU* gene was also identified and proved to be an SGT precursor encoding gene [23]. Further studies suggested that in *S. griseus*, *sprT* was the main gene responsible for SGT activity and complicated regulation [16, 22]. After optimizing the overexpression system, Chi et al. [3] successfully overexpressed *sprT* from *S. griseus* in *S. lividans*. Although active SGT has been overexpressed by *Streptomyces* sp., the fermentation period was relatively long and many other proteases were secreted which caused contamination during the fermentation. Consequently, other alternative heterologous expression systems appeared to be necessary. The methylotrophic yeast *P. pastoris*, because of its many advantages, such as the simplicity of cultivation and its well-characterised genetic manipulation, has been extensively used for producing foreign proteins at high level, especially for secreted proteins which require glycosylation or disulfide bond formation [2].

The aim of the present study was to clone and analyze the genes which encode the trypsinogen and active trypsin with the chromosome-integrated vector pPIC9k in *P. pastoris*. At the same time, we expected to prepare a comparatively robust recombinant *P. pastoris* for producing active trypsin extracellularly. In addition, although it is accepted that the propeptide might play an essential role in keeping the trypsinogen inactive, the physiological function of the propeptide is still unclear. Consequently, to obtain some insight into the biofunction and mechanism of the propeptide, we will replace the propeptide of the SG trypsinogen with the similar bovine trypsinogen propeptide (Fig. 1) and analyze its effect on SGT. To our knowledge, this is the first study producing active trypsin extracellularly with recombinant *P. pastoris* in one step.

Materials and methods

Construction of expression vectors

Streptomyces griseus ATCC 10137^{TM} (purchased from the American Type Culture Collection, USA) was provided as a template for *sprT* gene cloning. *E. coli* JM109 and

plasmid pET-20b(+) purchased from Invitrogen were used for gene cloning and mutation. *Pichia pastoris* GS115 and the chromosome-integrated vector pPIC9k were purchased from Invitrogen and used for the construction of recombinant strains. Specifically, the genes encoding the *S. griseus* trypsinogen and the active SGT are defined as *pmt* and *mt*, respectively. *smt*, which encodes a hybridized trypsinogen, was defined as the gene (bovine trypsinogen propeptide with *S. griseus* active trypsin).

Sequence assembly and nucleotide sequence were analyzed using the Vector NTI advance 11.5 software (Invitrogen). The amino acid sequence was aligned using the BLASTp program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the ClustalW program. The signal peptide was predicted by Signal P (http://www.cbs.dtu.dk/services/SignalP/).

To subclone *sprT* into the chromosome-integrated vector pPIC9k, two *Sal*I restriction sites in the *sprT* gene have to be mutated. *SprT* was first amplified using primers SprT-5′ and SprT-3′ (Table 1) and cloned into pET-20(+). Subsequently, with specific primers jSalA-5′ and jSalA-3′, and jSalB-5′ and jSalB-3′, respectively (Table 1), the two *Sal*I sites were mutated synonymously by site-specific mutagenesis using the TaKaRa MutanBEST Kit. Using the above cloned plasmid with mutant gene *SprT* as the template, the genes *pmt*, *mt*, and *smt* were amplified using jpmt-5′and jpmt-3′, jmt-5′ and jmt-3′, and jsmt-5′ and jsmt-3′, respectively (Table 1). Then, the purified fragments were digested with *Eco*RI and *Not*I and subcloned into vector pPIC9k which was digested with the same enzymes (Table 1). After being transformed into *E. coli* JM109, transformants (pPIC9k-*pmt*, pPIC9k-*mt*, and pPIC9k-*smt*) were selected on LB plates containing 50 μg ml^{-1} ampicillin. The plasmids pPIC9k-*pmt*, pPIC9k-*mt*, and pPIC9k-*smt* were successively identified by PCR with the corresponding primers as shown in Table 1, analysis with restriction enzymes, and DNA sequencing to confirm the absence of mutations and the integrity of the expression cassettes.

Transformation in *P. pastoris*

For preparing competent cells of *P. pastoris* GS115, the cells were cultivated in 50 ml YPD (10 g l^{-1} yeast extract, 20 g l^{-1} peptone, and 20 g l^{-1} dextrose) and harvested when an optical density at 600 nm (OD_{600}) of 1.3 was obtained. Then, the cells were washed and concentrated 1,000-fold in ice-cold 1 M sorbitol. Subsequently, 10 μg linear DNA was added into 80 μl concentrate of competent cells, and the mixture was precooled and then transferred to ice-cold electroporation cuvettes (0.2 cm). After the mixtures had incubated on ice for 5 min, transformation was performed with a Gene Pulser X-cell II electroporation system (Bio-Rad, Mississauga, ON, Canada) equipped with a Pulse Controller Plus accessory according to the instruction manual. The voltage was set to 1.5 kV (resistor, 200 Ω; capacitor, 25 μF; time, 4.0–5.0 ms). Once electrotransformation was finished, all cells were plated onto solid MD plates (13.4 g l^{-1} YNB, 4×10^{-3} g l^{-1} biotin, 20 g l^{-1} glucose, 20 g l^{-1} agar). Plates with transformants were wrapped in aluminum foil and incubated at 30 °C for 3–4 days. Then all the transformants were inoculated onto solid YPD (10 g l^{-1} yeast extract, 20 g l^{-1} peptone, 20 g l^{-1} dextrose, and 20 g l^{-1} agar) plates containing 0.25, 1.0, 2.0, 3.0, and 4.0 mg ml^{-1} G418 with the replica plating method. After incubation, the colonies were transferred to the plate with 4.0 mg ml^{-1} G418 to confirm integration.

Confirmation of integration and determination of Mut phenotype

There are three phenotypes of *P. pastoris* host strains with regard to methanol utilization. The Mut^+, methanol utilization plus phenotype, grows on methanol at the wild-type rate that requires a high feeding rate of methanol for induction in large-scale fermentations [2]. The Mut^s, methanol utilization slow phenotype, has a disruption in the AOX1 gene. Since the cells must rely on the weaker AOX2 for methanol metabolism, a slower growing and slower methanol utilization strain is produced. The Mut^-, methanol utilization minus phenotype, is unable to grow on methanol, because this type of strain has both AOX genes deleted. The phenotypes of the Mut^s and Mut^- strains utilized methanol comparatively slowly, which was beneficial to induction; however, their growth rate was relatively low. Therefore, the phenotype of Mut^+ was applied in this study.

In the phenotype verification of the transformants integrated with gene *pmt*, *mt*, and *smt* by PCR using AOX primer-5′ and AOX primer-3′ [20], the Mut^+ recombinants are detected in two bands with one pair of primers due to single cross-over recombination, one band representing the AOX gene of the host which is about 2,200 bp, and the other representing the target gene (*pmt*, *mt*, or *smt*) plus the homologous arm which is about 1,000 bp; Mut^s recombinants obtained using the AOX primer-5′ and AOX primer-3′ only have one band at about 1,000 bp because of double cross-over recombination. The genomic DNA of *P. pastoris* GS115 was used as a template. PCR amplification was performed with Taq DNA polymerase (SC0010, BBI).

Expression assays of recombinant trypsin and trypsinogen in *P. pastoris* recombinants

At least two clones from each construction in the presence of different concentrations of G418 were selected for time

Table 1 Strains, plasmids, and oligonucleotides used in this study

Strains and plasmids	Genotype and characteristics	References
Strains		
S. griseus ATCC 10137™	*Streptomyces griseus* subsp. *griseus*	[17]
E. coli JM109	F′*traD36 proA*$^+$*B*$^+$ *lacIq* Δ(*lacZ*)*M15*/Δ(*lac-proAB*) *glnV44e14-gyrA96 recA1 relA1 endA1 thi hsdR17*	Lab stock
P. pastoris GS115	*his4* Mut$^+$ His$^-$ (*aox1*$^+$, *aox2*$^+$)	[7]
P. pastoris GS115-9k	*his4* Mut$^+$ His$^+$ (*aox1*$^+$, *aox2*$^+$), pPIC9k transformants	This study
P. pastoris GS115-*pmt*	Mut$^+$ His$^+$(*aox1*$^+$, *aox2*$^+$), pPIC9k-*pmt* transformants	This study
P. pastoris GS115-*mt*	Mut$^+$ His$^+$(*aox1*$^+$, *aox2*$^+$), pPIC9k-*mt* transformants	This study
P. pastoris GS115-*smt*	Mut$^+$ His$^+$(*aox1*$^+$, *aox2*$^+$), pPIC9k-*smt* transformants	This study
Plasmids		
pET-20b(+)	*Amp*^{r} T7 promoter	Invitrogen
pPIC9k	*HIS4 Amp*^{r} or *Kan*^{r}	[7]
pPIC9k-*pmt*	*HIS4 Amp*^{r} or *Kan*^{r}	This study
pPIC9k-*mt*	*HIS4 Amp*^{r} or *Kan*^{r}	This study
pPIC9k-*smt*	*HIS4 Amp*^{r} or *Kan*^{r}	This study
Oligonucleotides (5′ → 3′)		
SprT-5′	CCGGAATTCGTGAAGCACTTCCTGCGTGCG	This study
SprT-3′	CCGCTCGAGTCAGAGCGTGCGGGCGG	This study
jSalA-5′	GGCGTCGTTGATCTCCAGTCG	This study
jSalA-3′	GCCGGTGGCGGTGATCG	This study
jSalB-5′	GGCGTTGATACCTGCCAGGGT	This study
jSalB-3′	ACCGGTGTCGGGGTATCCG	This study
jpmt-5′	CCGGAATTCGCCCCCAACCCCGTCGT	This study
jpmt-3′	ATAAGAATGCGGCCGCTCAGAGCGTGCGGGCGG	This study
jmt-5′	CCGGAATTCGTCGTCGGCGGAACCCG	This study
jmt-3′	ATAAGAATGCGGCCGCTCAGAGCGTGCGGGCGG	This study
jsmt-5′	CCGGAATTCGTTGATGATGATGATAAGGTCGTCGGCGGAACCCG	This study
jsmt-3′	ATAAGAATGCGGCCGCTCAGAGCGTGCGGGCGG	This study
AOX primer-5′	GACTGGTTCCAATTGACAAGC	This study
AOX primer-3′	TCCTACAGTCTTACGGTAAACGG	This study

The restriction enzyme sites are underlined

course expression assays, which were conducted by growing His$^+$ Mut$^+$ clones from fresh individual colonies in 30 ml BMGY (10 g l^{-1} yeast extract, 20 g l^{-1} peptone, 100 mM potassium phosphate, pH 6, 13.4 g l^{-1} YNB, 4 × 10^{-3} g l^{-1} biotin, 10 g l^{-1} glycerol) medium at 30 °C and 200 rpm for 18–24 h. The grown cells were harvested by centrifugation (10,000×*g*, 10 min, 4 °C) and resuspended in BMMY (same as BMGY substituting glycerol with 10 g l^{-1} methanol) to an OD_{600} = 1. Induction was carried out in 250-ml baffled flasks incubated at 250 rpm and 30 °C for 120 h [34]. The cultures were fed with pure methanol to a final concentration of 10 g l^{-1} at 24-h intervals, and 1-ml samples were taken at different times during the expression. The OD_{600} was determined using a spectrophotometer (Biospe-1601; Shimadzu Co., Kyoto, Japan). Yeast pellets and supernatants were stored separately at −80 °C.

Recombinant proteins purification and SDS-PAGE

The fermentation cultures of SG trypsinogen, SGT, and hybridized SG trypsinogen were collected and centrifugated at 10,000 rpm for 10 min and then filtered through a 0.22-μm PVDF filter. The sample was loaded onto a Hitrap benzamidine FF column (Φ1.6 × 2.5 cm, GE Healthcare, catalog number 17-5144-01) which was equilibrated with buffer A (50 mM Tris–HCl, pH 7.4, 0.5 M NaCl). The concentrated proteins were eluted with buffer B (50 mM glycine, 10 mM HCl, pH 3.0) and fractions displaying trypsin activity were collected. Then the column was eluted with 6 M guanidine chloride solution at a flow rate of 1.5 ml min^{-1}. The protein concentration was determined by the Bradford Kit (P0006, Beyotime, China).

The purified proteins were separately subjected to a 12 % sodium dodecyl sulfate polyacrylamide gel

electrophoresis (SDS-PAGE) on a vertical mini gel apparatus (Bio-Rad) at 150 V for 1 h. Protein bands were stained with Coomassie Brilliant Blue R-250 solution (Tiangen Biotech, China). Molecular weight marker was purchased from Fermentas (Thermo Fisher Scientific Inc).

Activation of recombinant hybridized trypsinogen

The transformants producing recombinant hybridized trypsinogen (bovine trypsinogen propeptide with *S. griseus* active trypsin) were screened, verified, and cultivated as described above and the supernatant was collected for purification. Then portions (90 µl) of purified hybridized trypsinogen were mixed with 0.4 µl of 200 IU recombinant bovine enterokinase (RP031, Sangon, China) and incubated at 25 °C for 16 h.

Measurement of trypsin activity

In nature, trypsin cleaves the peptides on the C-terminal side of lysine and arginine amino acid residues. The rate of hydrolysis is slower if an acidic residue is on either side of the cleavage site and no cleavage occurs if a proline residue is on the carboxyl side of the cleavage site [35]. As trypsin has the ability to hydrolyze ester and amide linkages of several synthetic substrates, trypsin activity can be defined as amidase or esterase activity.

The amidase activity of the sample was estimated using N_α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) [3] as a substrate. Briefly, samples (100 µl) were mixed with 800 µl of assay buffer (50 mM Tris–HCl, pH 8.0; 0.02 M $CaCl_2$ at 37 °C) and 100 µl of 0.1 M BAPNA. The change in absorbance at 410 nm was then monitored in a Shimadzu UV-2450 PC spectrophotometer. One BAPNA unit (U ml^{-1}) of trypsin was defined as the amount of enzyme required to produce an absorbance increase of 0.1 under the above conditions. The amidase activity was calculated according to Eq. (1):

$$\text{BAPNA unit}\left(\text{U ml}^{-1}\right) = \frac{\Delta A_{410}/\text{min} \times \text{df}}{0.1} \quad (1)$$

where df is the dilution factor.

The esterase activity of the sample was estimated using N_α-benzoyl-L-arginine ethyl ester hydrochloride (BAEE) as a substrate [9]. Samples (200 µl) were immediately mixed with 3 ml of assay buffer (67 mM sodium phosphate buffer pH 7.6 at 25 °C containing 0.25 mM BAEE). The change in absorbance at 253 nm was then monitored in a Shimadzu UV-2450 PC spectrophotometer. One BAEE unit (U ml^{-1}) will produce a ΔA_{253} of 0.001 per minute with BAEE as the substrate at pH 7.6 at 25 °C in a reaction volume of 3.2 ml. The esterase activity was calculated according to Eq. (2):

$$\text{BAEE unit}\left(\text{U ml}^{-1}\right) = \frac{\Delta A_{253}/\text{min} \times \text{df}}{0.001 \times 0.2} \quad (2)$$

where df is the dilution factor.

Trypsin production in shake flask culture

Pichia pastoris GS115-*mt*55, which displayed the highest trypsin activity, was selected and grown in 30 ml BMGY medium (10 g l^{-1} yeast extract, 20 g l^{-1} peptone, 100 mM potassium phosphate, pH 6, 13.4 g l^{-1} YNB, 4×10^{-3} g l^{-1} biotin, and 10 g l^{-1} glycerol). After cultivating for 24 h at 30 °C and 250 rpm, cells were harvested by centrifugation and resuspended in a 250-ml shake flask containing 30 ml BMMY medium (10 g l^{-1} glycerol was replaced with 10 g l^{-1} methanol in BMGY medium) for induction. To efficiently induce the AOX1 promoter, methanol was added every 24 h and cultivation was maintained for 144 h according to a previous report [34]. Both extracellular and intracellular trypsin amidase activity and biomass were measured with an interval of 24 h. The OD was determined at 600 nm in a spectrophotometer (Biospe-1601; Shimadzu Co., Kyoto, Japan). Yeast pellets and supernatants were stored separately at −80 °C.

Production of recombinant trypsin by fed-batch fermentation

After cultivating in a 500-ml shake flask containing 50 ml YPD (20 g l^{-1} glucose, 20 g l^{-1} peptone, 10 g l^{-1} yeast extract) at 30 °C, 200 rpm for 24 h, 10 % (v/v) of the culture was inoculated into a 3-l fermentor (LiFlus GM BioTRON, Korea) with 800 ml basal salts medium (40 g l^{-1} glycerol, 18 g l^{-1} K_2SO_4, 4.13 g l^{-1} KOH, 14.9 g l^{-1} $MgSO_4{\cdot}7H_2O$, 27 ml l^{-1} H_3PO_4, 0.93 g l^{-1} $CaSO_4$) plus 4.4 ml l^{-1} trace mineral solution (PTM1: 6 g l^{-1} $CuSO_4{\cdot}5H_2O$, 0.09 g l^{-1} KI, 3 g l^{-1} $MnSO_4{\cdot}H_2O$, 0.02 g l^{-1} H_3BO_3, 0.2 g l^{-1} $MoNa_2O_4{\cdot}2H_2O$, 0.5 g l^{-1} $CoCl_2$, 20 g l^{-1} $ZnCl_2$, 65 g l^{-1} $FeSO_4{\cdot}7H_2O$, 0.2 g l^{-1} biotin, 5.0 ml l^{-1} H_2SO_4). The pH of the medium was adjusted and controlled at 5.5 with the addition of 25 % ammonium hydroxide and 30 % phosphoric acid. Fermentation was operated at 30 °C and the dissolved oxygen level was maintained above 30 % of air saturation by a cascaded control of agitation rate (500–1,000 rpm) and aeration rate (2–5 l min^{-1}). During cell growth phase, cells were allowed to grow until the glycerol was exhausted which was indicated by an increase of dissolved oxygen (DO) level. The feeding medium, containing 500 g l^{-1} glycerol and 12 ml l^{-1} PTM1 solution, was pumped into the fermentor according to a predetermined protocol. After the glycerol was consumed, pure methanol containing 12 ml l^{-1} PTM1 solution was fed to start the induction

phase. At the same time, the temperature was reduced to 22 °C. The methanol concentration was maintained at 18 g l^{-1} by the methanol online control station (FC2002, East China University of Science and Technology).

High cell density could be attained via fed-batch culture through a glycerol feeding strategy. In order to simplify the exponential fed-batch, the feeding rate was increased every 1 h and calculated according to Eq. (3) [36].

$$F(t) = \frac{\mu_{\text{set}}}{Y_{\text{X/S}}(S_{\text{F}} - S)} X_0 V_0 \exp(\mu_{\text{set}} t) \quad (3)$$

where t is defined as the cultivation time after initiation of glycerol exponential feeding (h). X_0 and V_0 are the initial cell concentration (g l^{-1}) and culture volume (l) at the beginning of glycerol feeding, respectively. $Y_{\text{X/S}}$ is the yield of biomass on glycerol (g g^{-1}) and is estimated from a prior batch culture. S_{F} is the glycerol concentration (g l^{-1}) in the feeding medium, and S is the glycerol concentration (g l^{-1}) in the fermentation broth. The maximum specific growth rate $\mu_{\max}$ is 0.22 h^{-1}, close to that reported elsewhere [10], and the specific growth rate μ_{set} (h^{-1}) was assigned to be 80 % of $\mu_{\max}$.

Estimation of glycerol concentration, biomass, and intracellular alcohol oxidase activity

During the fed-batch fermentation, residual glycerol concentration was measured by GPO-POD glycerol enzymatic rapid-test kits purchased from Sigma Aldrich (China). For dry cell weight (DCW) analysis, samples were collected every 12 h. A 10-ml sample was centrifuged in a pre-weighed centrifuge tube at 10,000*g* and 4 °C for 10 min. The tubes containing the pellets were dried to constant weight at 80 °C and then measured. At the same time, the supernatant was preserved for further determination.

For intracellular alcohol oxidase activity analysis, another 1-ml sample was centrifugated under the above conditions. The pellets were washed twice with 50 mM phosphate buffer (pH 7.0) and resuspended in the same buffer and disrupted by high pressure homogenization (Constant Cell Disruption Systems). Cell debris was removed by centrifugation and the supernatant was collected as a cell-free extract. The intracellular AOX activity was assayed by measuring H_2O_2 produced during oxidation of methanol. The standard assay mixture contained 100 μM phosphate buffer (pH 7.0), 4.3 μM phenol, 15 IU peroxidase, 1 μM 4-aminoantipyrine, 400 μM methanol, and enzyme in a total volume of 3 ml. The reaction was carried out at 37 °C for 10 min and monitored by the increase in absorbance at 500 nm. One enzyme unit is defined as the amount of enzyme required for the formation of 1 μmol of H_2O_2 per min [36]. The AOX activity was calculated according to Eq. (4):

$$\text{AOX unit}\left(\text{U g}^{-1}\right) = \frac{\Delta A_{500}/\text{min} \times 30 \times 1{,}000 \times \text{df}}{12.34 \times \text{DCW}} \quad (4)$$

where df is the dilution factor; 30 means the dilution factor when 100 μl enzyme is added into a 3-ml reaction system; 1,000 means that 1 ml was collected from 1 l of cell culture supernatant; 12.34 is the absorbance constant of 4-aminoantipyrine; DCW is the dry cell weight of the sample.

Demonstration of functional property of the recombinant SGT

The demonstration of the biomedical function of the purified recombinant SGT was done with digestion of B16 mouse melanoma cells. For comparison, B16 mouse melanoma cells were digested with the same concentration of commercial bovine trypsin (T0458, Sangon, China). During this digestion, the cell morphology of B16 mouse melanoma cells was observed using an inverted microscope (ECLIPSE TE2000-S, Nikon, Japan).

Results

Construction and verification of recombinant *P. pastoris*

Using the genome of *S. griseus* ATCC 10137 as a template, the genes encoding for trypsinogen and trypsin were cloned into plasmid pPIC9k under the control of the AOX1 gene promoter with the α-factor secretion signal from *Saccharomyces cerevisiae*. After the recombinant plasmids pPIC9k-*pmt*, pPIC9k-*mt*, and pPIC9k-*smt* were transformed into *P. pastoris* GS115 by electrotransformation, recombinant *P. pastoris* strains were successfully screened for phenotype His^+ Mut^+ and resistance to different concentrations of G418 (Figs. 1, 4a). In general, high copy integration was consistent with high resistance toward G418. As a result, we then chose the most promising recombinant strains *P. pastoris* GS115-*pmt*105, *P. pastoris* GS115-*mt*55, and *P. pastoris* GS115-*smt*46 for further study. Meanwhile, sequencing of the amplified fragments also confirmed the success of integration (data not shown).

Effect of the propeptide on folding and activity of trypsin

To analyze the effect of the leader sequence on the folding and activity of trypsin, the recombinants constructed were initially assessed with shake flask cultivation. As shown in Fig. 2, virtually no trypsin activity was detected in all the transformants integrated with the SG trypsinogen gene *pmt*.

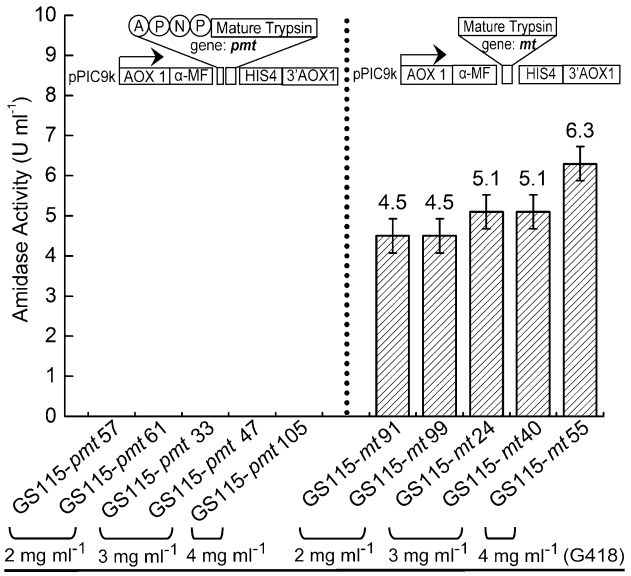

Fig. 2 Comparison of trypsin amidase activity between recombinants GS115-*pmt* and GS115-*mt*. All the recombinants were cultivated in baffled shake flasks (30 ml/250 ml) induced by 10 g l^{-1} methanol at 30 °C and 250 rpm

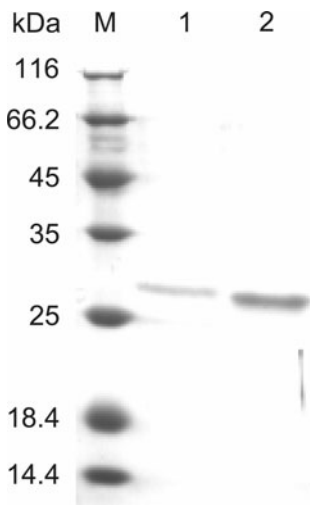

Fig. 3 SDS-PAGE analysis distribution of the purified trypsinogen and trypsin. Purified recombinant SG trypsinogen and recombinant SGT from *P. pastoris* culture supernatant (10 µl) were loaded onto the gel. *M* molecular mass markers, *lane 1* purified recombinant SG trypsinogen from GS115-*pmt*105 culture supernatant (extracellular), *lane 2* purified recombinant SGT from GS115-*mt*55 culture supernatant (extracellular)

In contrast, all the transformants integrated with the mature trypsin gene *mt* exhibited activity; strain *P. pastoris* GS115-*mt*55 obtained in the presence of 4 mg l^{-1} G418 showed the highest amidase activity (6.3 ± 0.4 U ml^{-1}), which suggested a higher copy number *mt* gene. Moreover, we successfully purified both recombinant SG trypsinogen and SGT from the respective extracellular supernatant (Fig. 3).

To further confirm the effect of propeptide, the recombinant strain *P. pastoris* GS115-*smt*46 was analyzed with strain *P. pastoris* GS115-9k as control. Similar to the result discussed above, although the hybridized trypsinogen was overexpressed successfully and secreted into the extracellular medium (Fig. 4a), no activity was detected without the additional enterokinase (Fig. 4b). Surprisingly, after digestion with enterokinase, the extracellular supernatant of strain *P. pastoris* GS115-*smt*46 showed an obvious trypsin activity (Fig. 4b). Apparently, the hybridized *S. griseus* trypsinogen can be successfully activated by removal of the propeptide. As shown in Fig. 4b, the highest final recovery of the trypsin amidase activity (1.38 ± 0.08 U ml^{-1}) was obtained at 72 h. More importantly, activation of the hybridized trypsinogen by removal of the propeptide from bovine trypsinogen confirmed that the propeptide from *S. griseus* was similar to that from bovine trypsinogen.

Characterization of recombinant *P. pastoris* GS115-*mt*55

According to the screening results, *P. pastoris* GS115-*mt*55 was confirmed to be the best strain and we therefore further characterized the strain in flask cultivation with *P. pastoris* GS115-9k as the control strain. As shown in Fig. 5, extracellular active trypsin secreted by *P. pastoris* GS115-*mt*55 was increased consistent with cell growth. When cultivated up to 120 h, an average level of amidase activity was achieved (6.3 ± 0.3 U ml^{-1}). However, no amidase activity was detected in the extracellular supernatant of the control strain of *P. pastoris* GS115-9k. Both *P. pastoris* GS115-*mt*55 and *P. pastoris* GS115-9k showed a comparatively small detectable amidase activity intracellularly, which might due to the background expression of *P. pastoris* GS115 [27]. In addition, the cell growth curves of *P. pastoris* GS115-*mt*55 and *P. pastoris* GS115-9k were also evaluated. Compared with the control strain *P. pastoris* GS115-9k, the recombinant *P. pastoris* GS115-*mt*55 accumulated less biomass, which might be due to the burden of overexpression of trypsin or the toxicity of the overexpressed trypsin [11].

Production of active trypsin by fed-batch fermentation

To evaluate the further capacity of the recombinant *P. pastoris* GS115-*mt*55, fed-batch fermentation with methanol as the sole carbon source was carried out. As shown in Fig. 6, the operation process was composed of three parts. Apparently, before induction, no trypsin was accumulated in the culture. When methanol was added as an inducer, the recombinant trypsin activity increased substantially and a maximum amidase activity of 14.4 ± 0.4 U ml^{-1} occurred

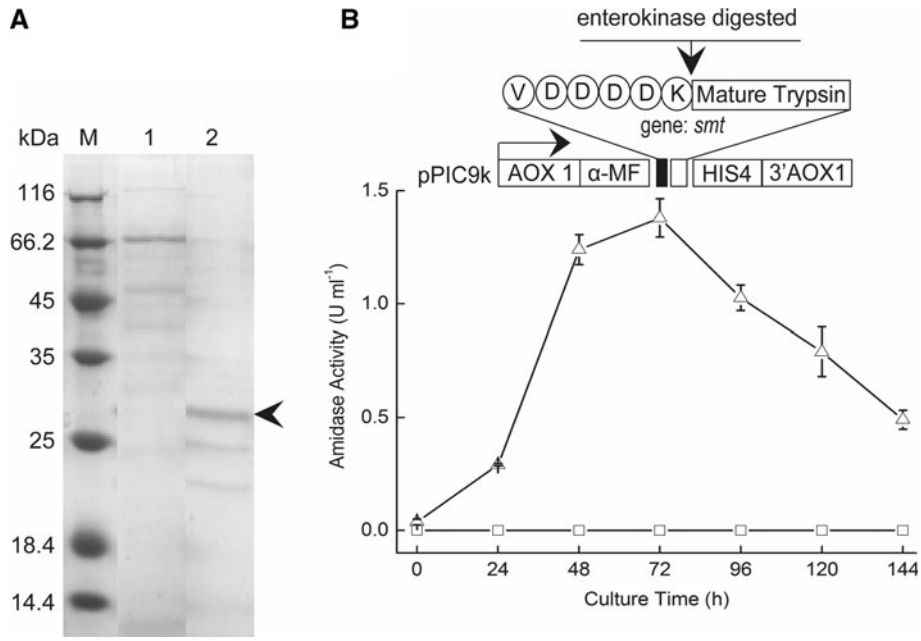

Fig. 4 SDS-PAGE and activity assay of recombinant hybridized trypsinogen (bovine trypsinogen propeptide with SGT). **a** *P. pastoris* culture supernatant (10 µl) was loaded onto the gel. *M* molecular mass markers, *lane 1* GS115-9k (extracellular), *lane 2* purified recombinant hybridized trypsinogen from GS115-*smt*46 culture supernatant (extracellular); *P. pastoris* GS115 recombinants were grown to near saturation ($OD_{600} = 20$) at 30 °C in 30 ml of glycerol-based yeast medium for 2 days. Cells were harvested by centrifugation and resuspended in 30 ml of the same medium with 1 % (v/v) methanol instead of glycerol and incubated for 5 days. **b** 90 µl supernatant of recombinant *P. pastoris* GS115-*smt*46 was digested with 200 IU recombinant bovine enterokinase at 25 °C in 25 mM Tris–HCl, 50 mM NaCl, 2 mM $CaCl_2$, pH 8.0, for 16 h. Undigested (*squares*), digested (*triangles*). Trypsin activity was assessed with the substrate α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) at 37 °C

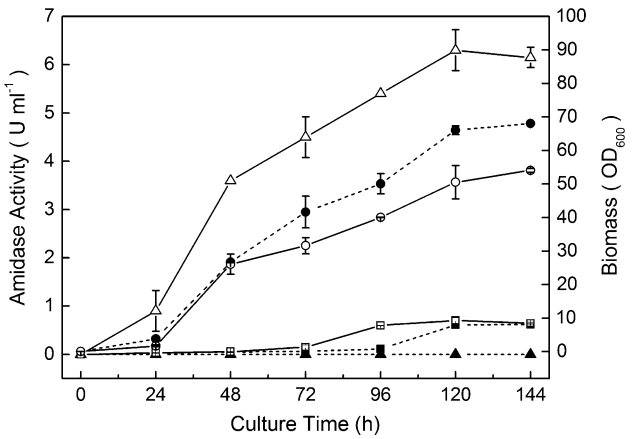

Fig. 5 Characterization of the recombinant *P. pastoris* GS115-*mt*55. Extracellular trypsin amidase of GS115-9k (*filled triangles*), extracellular trypsin amidase of GS115-*mt*55 (*open triangles*), intracellular trypsin amidase of GS115-9k (*filled squares*), intracellular trypsin amidase of GS115-*mt*55 (*open squares*), OD_{600} of GS115-9k (*filled circles*), OD_{600} of GS115-*mt*55 (*open circles*). The culture conditions were 30 ml/250 ml BMMY; temperature, 30 °C; agitation speed, 250 rpm

at 84 h with a high cell dry weight (118 ± 4.8 g l^{-1}). In contrast, the activity of trypsin decreased gradually in the late stationary phase, which is likely due to the degradation of the recombinant trypsin caused by the altered environment; similar behavior was reported elsewhere [29].

When the levels of dissolved oxygen and specific AOX activity were compared [36], no AOX activity was detected. Consistent with the trypsin activity, the intracellular AOX activity increased immediately when methanol was added. These results indicated that the addition of methanol activated the AOX1 promoter, which immediately drives the expression of the downstream genes encoding for trypsin and AOX [15]. Nevertheless, along with the increase of trypsin activity, the intracellular AOX activity decreased dramatically from 98.67 ± 0.03 to 3.92 ± 0.30 U g^{-1} within 24 h (Fig. 6), which might be caused by the toxicity of the active trypsin to the host cell, especially to the metabolism of methanol [12, 29]. As expected, under the constant agitation speed and aeration rate during the induction phase, the relative DO level was dependent on the cell growth strictly but reversely.

For future application, we also analyzed the functional property of the recombinant SGT with bovine trypsin as a control. B16 mouse melanoma cells were digested with 19 µg ml^{-1} recombinant SGT (purified in this study) and the same concentration of bovine trypsin, independently. As shown in Fig. 7, there was apparently that no obvious difference between the recombinant SGT and bovine trypsin. After digesting with either bovine trypsin or the

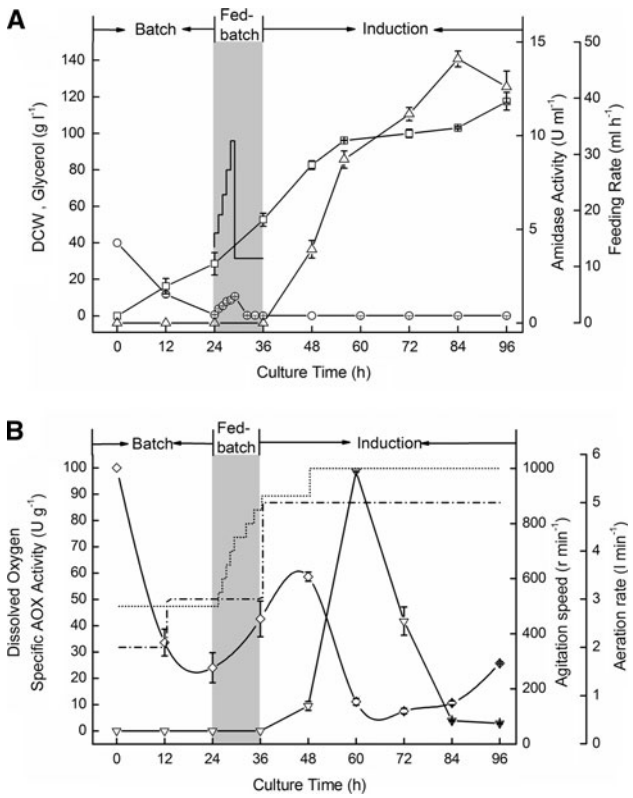

Fig. 6 Time course profile of recombinant trypsin production in a 3-l fermentor. **a** Trypsin activity (*triangles*), dry cell weight (*squares*), residual glycerol (*circles*), glycerol feeding rate (*solid line*). **b** Dissolved oxygen (*diamonds*), intracellular specific AOX activity (*triangles*), agitation speed (*dotted line*), aeration rate (*dashed-dotted line*). The culture temperature during batch and fed-batch stages was 30 °C, during the induction stage it was 22 °C. The pH was kept at 5.5 with concentrated ammonium hydroxide

recombinant SGT, the cell morphology changed from a shuttle shape to a circle shape, resulting in the distribution of the mammal cells throughout the culture supernatant. The results herein proved that the functional secreted recombinant SGT has the same biofunction as the bovine trypsin.

Comparison of the amidase and esterase of the recombinant trypsin

Although the recombinant trypsin activity from *Streptomyces* was always measured with BAEE as the substrate [9] because of its trypsin specificity and was often used as the substrate of the trypsin from mammal pancreas, it could also react with chymotrypsin [1]. Therefore, BAEE was also used as a substrate of the purified trypsin from mammal pancreas. When analyzing the recombinant trypsin with BAEE and BAPNA, both amidase and esterase activities were present. The cleavage site is an ester bond linked to the arginine residue in BAEE, whereas the cleavage site is an amide bond linked to the arginine residue in BAPNA. Compared with the substrate BAPNA, the esterase substrate BAEE has a very sensitive response to the trypsin activity (Table 2). Consequently, BAEE would be a better substrate for the trypsin activity measurement especially when the amount of trypsin is low or when the culture contains some contaminants such as substrate solvent and the endogenous subtilisin.

Discussion

Trypsin, because of its wide application and increasing demand, has been overexpressed by several systems, such as bacteriophages [5], *E. coli* [33], *L. lactis* [37], *S. lividans* [3], transgenic plant cells [18], and mammalian cells [6]. Although some of these systems have been successfully applied for trypsin studies, they are not feasible for industrial-scale application because of many problems, such as low activity, safety, and operational difficulties. For example, it was reported that the recombinant bovine trypsin formed inactive inclusion bodies in the *E. coli* system. In fact, we also attempted to overexpress the *Streptomyces* trypsin in *E. coli* but failed to detect any activity (data not shown). Compared with the *E. coli* system, the yeast *P. pastoris* system was much more suitable for recombinant protein overexpression, especially for proteins requiring glycosylation or disulfide bond formation [7]. Niles et al. [21] reported that a trypsin-like serine protease tryptase beta (EC 3.4.21.59) from human mast cells was overexpressed by the *P. pastoris* system.

Recently, the recombinant bovine trypsin was successfully overexpressed by *L. lactis* and transgenic rice cells, separately [18, 37], but the activity of the recombinant bovine trypsin was relatively low. Traditionally, the trypsinogen produced has to be digested and activated by enterokinase. In the present study, we successfully constructed a *P. pastoris* system for active SGT directly. To facilitate purification, α-factor secretion signal was applied for the recombinant SGT secretion. Eventually, an extracellular trypsin activity (14.4 ± 0.4 U ml^{-1}, Fig. 6) was achieved, which indicated that the *P. pastoris* system was suitable for the heterogeneous production of microbial-resourced trypsin. The results also suggested that the α-factor secretion signal used was efficient for trypsin secretion. In addition, we successfully purified the trypsinogen and the active SGT (Fig. 3). Interestingly, SDS-PAGE results for trypsinogen and active SGT showed that both the bands were bigger than the deduced molecular weight of the trypsin, possibly because of their post-translational modification, such as glycosylation [8].

Previous studies of amino acid sequences of both bovine and *Streptomyces* trypsinogen showed that they were

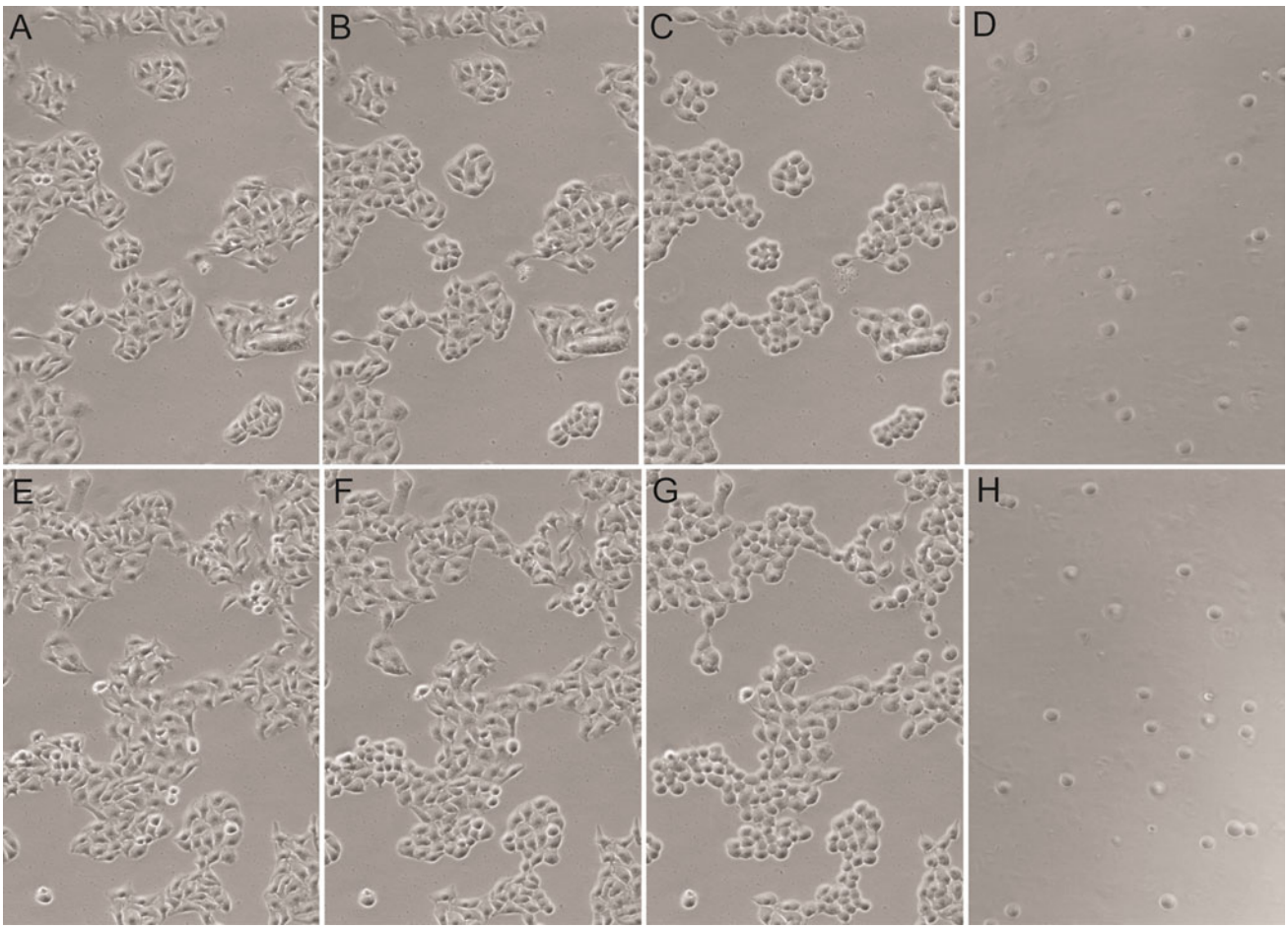

Fig. 7 Comparison of the biofunction of bovine trypsin and recombinant SGT. **a** Cell morphology before the digestion of bovine trypsin. **b** Cell morphology after the digestion of bovine trypsin for 5 min. **c** Cell morphology after the digestion of bovine trypsin for 10 min. **d** Supernatant cell morphology after the digestion with bovine trypsin. **e** Cell morphology before the digestion with recombinant SGT. **f** Cell morphology after the digestion with recombinant SGT for 5 min. **g** Cell morphology after the digestion with recombinant SGT for 10 min. **h** Supernatant cell morphology after the digestion with recombinant SGT

Table 2 Comparison of recombinant trypsin amidase and esterase activity

Sample	Concentrated fold (v/v)	Amidase activity (U ml^{-1})	Esterase activity (U ml^{-1})
1	2.1	7.5 ± 0.4	418.4 ± 52.7
2	2.5	12.9 ± 0.4	994.5 ± 102.5
3	4.8	30.7 ± 0.1	3,797.5 ± 74.25

Sample 1 flask culture supernatant with 3.6 U ml^{-1} amidase activity was concentrated from 5 to 2.38 ml by ultrafiltration; *sample 2* flask culture supernatant with 6.3 U ml^{-1} amidase activity was concentrated from 5 to 1.98 ml by ultrafiltration, *sample 3* fermentor culture with 14.7 U ml^{-1} amidase activity supernatant was concentrated from 1,200 to 250 ml by ultrafiltration

composed of two parts: propeptide and active trypsin [17]. The physiological function and activation mechanism of the bovine trypsin propeptide were clear. In native *S. griseus*, the propeptide of trypsinogen has to be cleaved for its activation. In contrast, the physiological function and activation mechanism of the SG trypsinogen propeptide were not clear because of its structural similarity to both bovine trypsin and subtilisin [28]. Herein, the results of SDS-PAGE and enzyme activity analysis showed that even without any activity, trypsinogen was soluble and could be secreted into the extracellular medium. This suggested that the propeptide played a key role in inhibiting the trypsin activity. In addition, the results of the recombinant hybridized trypsin analysis showed that after digestion with enterokinase, the activity of the hybridized trypsinogen was recovered. Obviously, taken together, the results demonstrated that inhibition of the propeptide to SGT occurred after its successful folding but was not involved in its folding process.

In conclusion, we successfully accomplished functional expression of secreted SGT by the *P. pastoris* system. At the same time, biofunction of the recombinant SGT was

demonstrated. Furthermore, we confirmed a previous hypothesis that the physiological function of the propeptide from *S. griseus* is as an inhibitor of trypsinogen, which is similar to role of the propeptide from bovine. More importantly, our results demonstrated the inhibition mechanism of the propeptide. After successfully being translated and folded, the propeptide inhibited the activity of trypsinogen by affecting its conformation.

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