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Efficient extracellular expression of transpeptidase sortase A in *Pichia pastoris*

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ABSTRACT

In order to achieve efficient extracellular expression of Sortase A (SrtA), various strategies in *Pichia pastoris* system were applied in this study. Among different constructed recombinant strains, the SMD1168 strain integrated 5.7 copies of *srtA* gene under control of AOX1 promoter was proved to be the best strain for the extracellular SrtA expression. After the optimization of fermentation conditions (induction 72 h at 28 °C, initial pH 6.0, supplemented with 1.5% methanol), the highest yield and activity of extracellular SrtA reached 97.8 mg/L and 131.9 U/mL at the shake-flask level, respectively. This is the first report on the efficient secretory expression of SrtA in *P. pastoris* and the yield of SrtA is the maximum compared with previous reports. In addition, the transpeptidation activity of extracellular SrtA was confirmed by the successful immobilization of enhanced green fluorescent protein (EGFP) onto Gly_3-polystyrene beads.

1. Introduction

Sortase A (SrtA, EC: 3.4.22.70) is a transpeptidase found in *Staphylococcus aureus* [1]. It anchors surface proteins with a specific C-terminal sorting signal (LPXTG, X stand for any amino acid except cysteine) to cell wall by cleaving the amide bond between threonine and glycine and transferring the thioester intermediate to the amino group of pentaglycine cross-bridges [2]. Due to its site-specific transpeptidation reaction, SrtA has been widely used as a ligation tool in the fusion of protein domains with their preserved functionality [3], post-translational modification of bionanoparticles [4], peptides and protein cyclization [5], preparation of complex glycoconjugates [6] and live cell labelling [7]. Especially, SrtA-mediated ligation was applied to synthesize antibody-drug conjugates as highly promising biotherapeutics [8]. Furthermore, efforts to evolve SrtA by site-mutations have generated variants of SrtA with improved kinetics, which were applied in antibody and protein conjugation reactions [9,10]. Thus, SrtA has great potential applications in biotechnology industry.

So far, expression of SrtA has long been limited in intracellular expression in *Escherichia coli* by using various commercial plasmids, including pET23b (Novagen) [6], pQE30 (Qiagen) [11] and pBAD (Invitrogen) [12] etc.. Although these strategies could produce SrtA for the laboratory use, the expression level of SrtA maintained at low level with yields ranging from several milligrams to maximum 76.9 mg/L [13]. Disadvantages of these intracellular expression strategies, including cumbrous cell disruption and time-consuming downstream purification process, obstructed its future industrial application. In addition, compared with the extensive applications of SrtA, few endeavors were made to improve or optimize the expression level of this important enzyme. Therefore, new strategy for the efficient extracellular expression of SrtA is highly demanded.

In the last two decades, the methylotrophic *Pichia pastoris* has proven to be an excellent host for the production of a variety of heterologous proteins in both academia and industry. This expression system featured with ease genetic manipulation, high cell densities using inexpensive medium and post-translational modification. In addition, various *P. pastoris* hosts, such as GS115 (Mut^+ phenotype), SMD1168 (Mut^+ phenotype, defective in the vacuole peptidase A) and KM71 (Mut^S phenotype) etc. [14], and promoters (including the methanol-induced AOX1 promoter and constitutive GAP promoter etc.) [15] were available for screening the specific expression requirement. In particular, with the

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successful α-factor signal peptide from *Saccharomyces cerevisiae* [16], many large-scale extracellular production of recombinant proteins was achieved at high levels in *P. pastoris* [17]. This α-factor signal peptide can efficiently mediate the secretion of heterologous proteins through endoplasmic reticulum, Golgi apparatus and plasma membrane [18]. Furthermore, target protein was harvested from the culture medium directly without cell-disruption or refolding operation, which greatly simplify the downstream purification steps [19]. Therefore, the *P. pastoris* system has a potential for the efficient extracellular expression of SrtA.

In this study, extracellular expression of a truncated version of SrtA (Δ59-SrtA with good solubility and the same transpeptidation activity with full-length SrtA) [20] in *P. pastoris* was investigated. By adopting various strategies (different hosts, promoters and gene copy numbers) for protein expression in *P. pastoris*, a series of recombinant engineered strains were constructed and the extracellular expression of SrtA was firstly achieved. Based on SDS-page analysis and activity assay, a suitable strain to secret expression of SrtA was obtained. Then, the expression level of SrtA was further enhanced by the optimization of fermentation conditions. Finally, the transpeptidation activity of secreted SrtA was confirmed by immobilization of enhanced green fluorescent protein (EGFP) onto glycine-modified polystyrene beads.

2. Materials and methods

2.1. Strain, plasmid and media

The *P. pastoris* host strain GS115 (*his4*, Mut$^+$, His-, *aox1*$^+$, *aox2*$^+$), SMD1168 (*his4*, *pep4*, Mut$^+$, His-, *aox1*$^+$, *aox2*$^+$) and KM71 (*his4*, Mut^S, His-, *aox1*-, *aox2*$^+$) and plasmid pPIC9k were purchased from Invitrogen (Carlsbad, USA). The *E. coli* JM109 (Novagen, Madison, WI) was used as the host for cloning and DNA sequencing. *E. coli* strain was cultured in LB medium (Tryptone 10 g/L, Yeast extract 5 g/L, NaCl 10 g/L) with 100 μg/mL ampicillin. *P. pastoris* strains were grown in YPD medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose), BMGY medium (13.4 g/L YNB without amino acids, 20 g/L peptone, 10 g/L yeast extract, 10 g/L glycerol and 100 mM potassium phosphate) or BMMY medium (13.4 g/L YNB without amino acids, 20 g/L peptone, 10 g/L yeast extract, 10 g/L methanol and 100 mM potassium phosphate) according to the manufacturer's instructions (Invitrogen, Carlsbad, USA). The MD medium plate (13.4 g/L YNB without amino acids, 20 g/L glucose, 0.4 mg/L biotin) and YPD-G418 medium plate (YPD medium with a final concentration of 1.0, 2.0 and 4.0 g/L geneticin) were used to select the His$^+$ transformants and *srtA* multicopy transformants, respectively. As for shaking-flask SrtA expression, 12 mL/L trace elements solution PTM1 (0.2 g/L $Na_2MoO_4 \cdot 2H_20$, 3.0 g/L $MnSO_4 \cdot H_20$, 6.0 g/L $CuSO4 \cdot 5H_20$, 65.0 g/L $FeSO_4 \cdot 7H_20$, 0.5 g/L $CoCl_2$, 20.0 g/L $ZnCl_2$, 0.08 g/L NaI, 0.02 g/L H_3BO_3, 0.2 g/L biotin and 5.0 mL/L H_2SO_4) in pure methanol was supplemented into BMMY medium every 24 h.

2.2. Expression of SrtA in P. pastoris

The genome of *S. aureus* (ATCC 35556) was extracted by Genomic Extraction Kit (Qiagen, Valencia, CA) according to the manufacturer's instruction and applied as the template for amplifying Δ59-*srtA* (forward primer: CCG GAA TTC CAA GCT AAA CCT CAA ATT CCG; reverse primer: ATA AGA ATG CGG CCG CTT AGT GGT GGT GAT GAT GAT GTT TGA CTT CTG TAG CTA CAA AGAT; restrict enzyme sites are underlined). The Δ59-*srtA* fragment were digested and inserted into the *Eco*R I/*Not* I site of pPIC9k. The plasmid (pPIC9k-Δ59-*srtA*) was transferred into *E. coli* JM109 cell and confirmed by DNA sequencing. As for the plasmid (pPIC9kGAP-Δ59-*srtA*), the GAP promoter was amplified from genome of *P. pastoris* GS115 (forward primer: CCG GAG CTC TTT TTG TAG AAA TGT CTT GGT; reverse primer: CGC GGA TCC ATA GTT GTT CAA TTG ATT GAA ATA GG; restrict enzyme sites are underlined) and replaced the original AOX1 promoter at *Sac* I/*Bam*H I site of pPIC9k-Δ59-*srtA*. The correct pPIC9k-Δ59-*srtA* and pPIC9kGAP-Δ59-*srtA* plasmid were linearized at *Sal* I site and transformed into *P. pastoris* GS115, SMD1168 or KM71 cells, respectively.

2.3. Determination of srtA copy number by quantitative real-time PCR (qPCR)

Genomic DNA from selected *P. pastoris* colonies were isolated by Genomic Extraction Kit (Qiagen, Valencia, CA) and quantified by Nanodrop ND-2000 spectrophotometer (Thermo Scientific). To detect *srtA* copy number, qPCR was performed using SYBR Premix Ex *Taq*™ Kit (Takara) and the primer sets were designed by Beacon Designer 7.0 software (*srtA* forward primer: CCT CAA ATT CCG AAA GAT AAA; *srtA* reverse primer: GTC AAT GAA AGT GTG TCC TG; *ACT1* forward primer: CTC CAA TGA ACC CAA AGT CCA AC; *ACT1* reverse primer: GAC AAA ACG GCC TGA ATA GAA AC). The qPCR reactions were conducted using a LightCycler 480 II Real-time PCR instrument (Roche Applied Science, Mannheim, Germany) and were carried out in final volumes of 20 μL with 10 μL of SYBR Green I Master, 0.8 μL forward and reverse primers, 1 μL diluted genomic DNA and 7.4 μL ddH_2O. The program for qPCR experiment was: pre-incubation at 95 °C for 30 s; 40 cycles of amplification step 95 °C for 5 s and 60 °C for 20 s; a cooling step at 50 °C for 30 s; a melting curve analysis with a temperature gradient of 0.3 °C/s from 50 to 95 °C. The copy number of *srtA* gene in each strain was estimated by previous $2^{-\Delta\Delta C}{}_T$ method [21] normalized to the endogenous reference *ACT1* gene (encoding actin). All experiments were performed in biological triplicates with an independent measurement of each sample and the mean values were used for further calculations. Analysis of variance (ANOVA) was used to evaluate the difference of *srtA* copy number in different colonies. Data were analyzed using software SPSS 18.0 by Duncan's multiple range test.

2.4. Purification of SrtA

The SrtA fermentation supernatant and Ni-NTA agarose (Qiagen) were mixed and loaded into a gravity flow column. The mixture was kept at 4 °C for 4 h and then washed with a linear gradient of imidazole (10–40 mM) to eliminate contaminating proteins. The C-terminal His_6-tagged SrtA was eluted from the column by 500 mM imidazole and desalted by an Amicon Ultra 3 K device (Millipore). The concentration of purified SrtA were determined by the Bradford method [22].

2.5. SDS-page

The expression of SrtA in various recombinant strains were checked by NuPAGE™ 12% Bis-Tris precast protein gels with pre-stained protein standard (Thermo Scientific). The bands were visualized with coomassie brilliant blue R250.

2.6. SrtA activity assay

The specific substrate of SrtA (Dabcyl-QALPETGEE-Edans) was obtained from GL biochem Ltd. (Shanghai, China). The SrtA activity arrays were performed in 200 μL volume of 50 mM Tris-HCl buffer (150 mM NaCl, 10 mM $CaCl_2$, 0.5 mg substrate, pH 7.8) and 10 μL fermentation supernatant. The reactions were carried on at 37 °C for 1 h by use of a Synergy H4 hybrid microplate reader (BioTek) and the fluorescence intensity (FI) was detected with 350 nm for excitation and 495 nm for recordings. One unit of SrtA activity was