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Improved secretion of *Candida antarctica* lipase B with its native signal peptide in *Pichia pastoris*

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ABSTRACT

Secretion efficiency of the 85-amino acid *Sacchromyces cerevisiae* alpha signal peptide and the 25-amino acid *Candida antarctica* lipase B signal (nsB) peptide were compared. Three reporter proteins used for the study are *C. antarctica* lipase A (CalA), lipase B (CalB) and hGMCSF. The copy number of recombinant α-CalB and nsB-CalB clones was determined by qPCR and clones with equivalent gene copies were used for comparative analysis. About threefold increased CalB production corresponding to an activity of 480 U ml^{-1} was obtained with its native signal peptide, whereas with the alpha signal peptide the maximum activity was 160 U ml^{-1}. Also, CalB was secreted as a mature protein with native N-terminus when fused to its own signal peptide, while unprocessed CalB with N-terminal extension was detected with the alpha signal peptide. Real time PCR analysis of CalB strains indicated that the difference in protein expression was not at the transcriptional level. The nsB signal sequence was also effective in secreting CalA enzyme and its secretion efficiency was on par with the alpha signal sequence. Further, hGMCSF fused inframe with the nsB signal peptide was also efficiently secreted into the medium. These results indicate that the nsB signal peptide can be a better alternative to alpha signal peptide for heterologous protein expression in *Pichia pastoris*.

1. Introduction

Pichia pastoris has emerged as a highly productive protein expression system with many successful commercial applications [1]. In addition to being a simple and fast growing host, availability of a wide range of vectors has made *P. pastoris* a versatile protein production tool. Being a single celled eukaryote, *P. pastoris* is equipped with a secretory pathway that has efficient mechanisms for protein folding, processing and other post-translational modifications.

The choice of leader peptide is one of the most important factors in optimising protein secretion in *P. pastoris*. *Sacchromyces cerevisiae* alpha factor prepro signal peptide, killer toxin signal peptide, invertase signal peptide and *P. pastoris* acid phosphatase signal peptide are some of the secretory signals used in *P. pastoris* but the most successful to date is the alpha factor signal peptide. This 85 amino acid leader sequence comprises of a 19 amino acid pre-sequence followed by a 66 amino acid pro-sequence [2]. Extensive research has been done to improve the secretory potential of alpha signal sequence by directed evolution, synthetic constructs and codon optimization in *S. cerevisiae* [3–5]. However, maximum and efficient secretion is not achieved for all proteins with the alpha signal sequence. For instance, in the case of insulin precursor, processing of the alpha factor fusion construct was found to be partial in its natural host *S. cerevisiae* whereas the fusion protein was completely processed and secreted in the heterogeneous host *P. pastoris* [6]. Thus, the degree of functionality varies between signal sequences for expression of different proteins in different host systems.

Although *P. pastoris* is flexible in recognition of signal sequences, maturation and secretion of the protein expressed is unpredictable. In some cases such as the *Bacillus* sp. alkaline β-mannanase, human procolipase and IFN-α2b only minimal or null expression was achieved with their respective native leader sequences [7–9]. Thus, it is clear that signal sequence based expression optimisation cannot be applied universally to all proteins and all expression systems but requires individual testing with many signal peptides. In view of this perspective, a short signal sequence is economical and advantageous as it can be directly fused to the coding sequence of the gene of interest in a single primer.

Candida antarctica lipase B (CalB) is synthesized as a preproprotein with a 25 amino acid secretory component consisting of: 18 amino acid signal peptide and 7 amino acid propeptide.

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Heterogenous leader peptides differing with respect to their origin, amino acid composition and length have directed CalB secretion in different host systems [10–12]. Attempts to express CalB in *E. coli* with its native signal peptide were not fruitful as processing of the signal and propeptide was not achieved [13]. In *P. pastoris*, expression of CalB and CalB fusion proteins has been reported with the alpha signal peptide [14] but not with its native signal peptide. Also, the secretion capacity of this short signal peptide with respect to non-native proteins has not been tested so far.

The purpose of this study is to investigate the secretion capacity of CalB native signal peptide (nsB) for expression of industrially important *C. antarctica* lipase A (CalA) and CalB. The two-way molecular approach of expression optimisation for these enzymes should facilitate their enhanced production at the industrial scale. Further, the applicability of nsB signal peptide for expression of non-native protein such as the human Granulocyte Macrophage Colony Stimulating Factor (hGMCSF) is described.

2. Materials and methods

2.1. Strains, plasmids and media

The yeast strain *C. antarctica* JCM 10318 was purchased from Japan Collection of Microorganisms, RIKEN BioResource Centre. Plasmids pPICZαB and pPICZB were maintained and propagated in *E. coli* host TOP10F' (Invitrogen) in low salt Luria-Bertani medium. *Pichia* host GS115 from Invitrogen was used as the expression host. YPD and YPG (1% yeast extract, 2% peptone, 2% dextrose or 2% glycerol) media were used for *P. pastoris* growth. Buffered methanol-complex medium (BMMY – 1% yeast extract, 2% peptone, 100 mM potassium phosphate; pH 6.0, 1.34% YNB, 4×10^{-5}% biotin, 0.5–1% methanol) was used for expression studies. Tributyrin agar medium (1% yeast extract, 2% peptone, 1% tributyrin, 1.5% agar, 0.5% methanol) was used for preliminary screening of lipase clones. All chemicals used were biological reagent grade.

2.2. Construction of recombinant expression plasmids

C. antarctica JCM 10318 strain was cultivated in 3 ml YPD medium at 24 °C. Genomic DNA was extracted from the culture ($O.D_{600}$ 5) by the method of Ausubel [15]. Primers specific for mature CalA [16] and CalB (GenBank accession number: EU915210.1) genes were used to amplify the coding regions from genomic DNA. PCR was carried out using high fidelity Taq DNA polymerase (Finnzymes) and the thermal cycle parameters are: one cycle of 5 min at 95 °C, followed by 30 cycles of 1 min at 95 °C, 1 min at 58 °C for CalB or 61 °C for CalA, 1.30 min at 72 °C, with a final elongation step of 7 min at 72 °C.

2.2.1. α-Signal sequence clones

The amplified genes were cloned directionally using XhoI and XbaI restriction sites in the vector pPICZαB. Both the vector and the genes were double-digested and purified. Ligation reaction was set in the ratio of 3:1 (insert:vector) and the *E. coli* transformants were selected at the zeocin concentration of 25 μg/ml. The recombinant plasmids confirmed by PCR and restriction analysis were designated as α-CalA and α-CalB.

2.2.2. nsB signal sequence clones

For construction of recombinant plasmids with the nsB signal sequence, gene-specific fusion forward primers were designed comprising the 75 bp nsB signal sequence, 18–22 initial nucleotides of the mature gene and EcoRI restriction site. Therefore, PCR amplification with the nsB gene-specific forward and reverse primers results in fusion of the gene of interest with the nsB signal sequence. The hGMCSF gene was amplified from pPICZB/hGMCSF plasmid previously constructed in our lab [17]. All the three fused gene fragments were cloned in pPICZB vector using EcoRI and XbaI restriction sites and the resultant constructs were designated as nsB-CalA, nsB-CalB and nsB-hGMCSF. The primers used for cloning are listed in Table 1.

2.3. Transformation of P. pastoris and screening for lipase activity

To enable gene integration at the AOX1 locus, the recombinant plasmids were linearized with SacI enzyme and purified. Ten to twelve nanograms of each linearized plasmid was electroporated into *P. pastoris* His^- strain GS115 using BTX Electroporator (1500 V, 250 Ω, 50 μF). Electroporation was carried out in a 0.2 cm cuvette according to the standard protocol. After pulsing, the cells were resuspended in 1 M sorbitol and incubated for 3 h at 28 °C. The cells were collected by centrifugation ($2200 \times g$), resuspended in YPD medium and incubated overnight at 28 °C. Finally, the cells were plated on YPD agar containing 0.1 mg ml^{-1}, 0.5 mg ml^{-1} and 1 mg ml^{-1} zeocin. In the case of hGMCSF clones, 0.25 mg ml^{-1} zeocin was used for selection of transformants. The recombinant colonies obtained after 3 days were confirmed by PCR using gene-specific primers. Screening of *Pichia* transformants for secretory expression of functional lipases was done by stabbing the clones on tributyrin agar plates containing 0.5% methanol. Lipase secreting clones were identified by appearance of a clear zone around the colony caused by hydrolysis of tributyrin by lipase.

2.4. Real time PCR

2.4.1. Copy number determination

Genomic DNA extracted from recombinant CalA and CalB *Pichia* strains was used as template for real time PCR analysis. Quantitative real time PCR (qPCR) was carried out with Eppendorf Realplex instrument on the basis of SYBR Green chemistry. Since pAOX1 is the common region in all the recombinant constructs, it was used as the target for amplification. *P. pastoris* host GS115 harbours a single-copy AOX1 gene; therefore it was used as the calibrator to normalize data [18]. All the samples were assayed in 20 μl volume in triplicates.

2.4.2. Relative expression analysis

Total RNA was extracted from yeast cells using RNeasy Mini kit (Qiagen). The isolated RNA was treated with DNase enzyme (Fermentas) as per the recommended protocol and purified by acid phenol:choloroform (5:1) extraction and precipitated with ice cold absolute ethanol. RNA quality was tested in 1.5% agarose gel and the $A_{260/280}$ ratio was determined using Nanodrop (Thermo Scientific). cDNA was prepared with 1 μg treated RNA using Superscript III reverse transcriptase (Invitrogen). qPCR analysis was carried out in StepOne Real Time PCR System (Applied Biosystems) using Power SYBR™ Master mix. Each reaction mix (10 μl) contained 1 X SYBR master mix, 250 nM forward and reverse primers and 1 μl 1:10 diluted cDNA template.

Melting curve analysis was included after every run to verify the specificity of amplicons. ARG4 and ACT1 targets were used as endogenous controls for copy number and gene expression experiments respectively. The experimental values were analysed by relative quantification using the method of Livak [19].

2.5. Shake flasks expression studies

Preinoculum was prepared by inoculating 50 μl glycerol stock in 3 ml YPG medium. The tubes were incubated at 28 °C for 24 h and the grown culture was inoculated in 50 ml YPG medium such that the initial $O.D_{600}$ is ~0.5. When the culture reached mid-log phase, the cells were harvested by centrifugation and resuspended in the induction medium BMMY. Methanol was added to a final concentration of 0.5% on the first day and later maintained at 1%. Expression samples were collected every 24 h with subsequent methanol addition for 6 days. The collected samples were centrifuged at $3500 \times g$ for 8 min and the supernatant and cell pellet fractions were stored separately at −80 °C for further analysis. For expression at 20 °C, the culture was incubated at 28 °C during the growth phase and then shifted to 20 °C during induction. All the expression studies were carried out in duplicates.

2.6. Reactor studies

Scale-up expression for α-CalB2 and nsB-CalB17 cultures was carried out in 7 l reactor (New Brunswick Scientific) with a working volume of 4 l. Pre-inoculum was grown in 3 ml YPG medium and then transferred to 200 ml modified BSM medium ($CaSO_4 \cdot 2H_2O$, 0.48 gl^{-1}; K_2SO_4, 9.1 gl^{-1}; $MgSO_4 \cdot 7H_2O$, 7.46 gl^{-1}; KOH, 2.06 gl^{-1}; glycerol, 40 gl^{-1}; H_3PO_4, 26.7 ml l^{-1}). The pH was adjusted to 4.8 with ammonia solution after addition of 0.04% histidine and 4.5 ml l^{-1} PTM ($CuSO_4 \cdot 5H_2O$, 6.0 gl^{-1}; NaI, 0.08 gl^{-1}; $MnSO_4 \cdot H_2O$, 3 gl^{-1}; $Na_2MoO_4 \cdot 2H_2O$, 0.2 gl^{-1}; H_3BO_3, 0.02 gl^{-1}; $CoCl_2$, 0.5 gl^{-1}; $ZnCl_2$, 20 gl^{-1}; $FeSO_4 \cdot 7H_2O$, 65 gl^{-1}; biotin, 0.2 gl^{-1}; concentrated H_2SO_4, 0.5 ml l^{-1}). In the reactor, initial batch growth was supported in the modified minimal medium with 40% glycerol. The growth conditions were maintained at 28 °C, 400 rpm and 1 vvm air throughout the cultivation process. Dissolved oxygen (DO) based feeding method was followed for the subsequent stages. After glycerol exhaustion (~24 h) as indicated by a sudden rise in DO level, glycerol feeding was started and continued for a period of 8 h at the rate of 4 ml l^{-1} h^{-1} during which time the DO level was maintained at 10%. After the transition phase, DO-based methanol pulsing was started and maintained for 160 h. Samples were collected periodically for analysis.

2.7. Lipase assay

Lipase activity was determined by pH stat assay. The assay was done using the automatic titration instrument Radiometer according to manufacture's instruction. The reaction mixture comprised of 10 ml substrate solution containing 10% tributyrin (Fluka) emulsified in distilled water with 2% gum arabic (stabilizer) using sonicator. Sodium hydroxide solution at a desired concentration, usually 50 mM, was filled in the buffer reservoir. The substrate solution was initially equilibrated to pH 7. Once the enzyme is added to solution, the reaction starts and the titrant NaOH is automatically added to maintain pH 7. Lipase activity was calculated from the volume of NaOH consumed as a function of time and expressed as tributyrin lipase unit (U). One tributyrin unit is defined as the amount of enzyme that liberates 1 μmol fatty acid per minute under the assay conditions.