Process Biochemistry 50 (2015) 2137–2143

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Improvement in the secretory expression of recombinant *Candida rugosa* lipase in *Pichia pastoris*

Ting-Chun Kuo [a], Jei-Fu Shaw [b,**], Guan-Chiun Lee [a,*]

[a] Department of Life Science, National Taiwan Normal University, Taipei, Taiwan
[b] Department of Biological Science and Technology, I-Shou University, Kaohsiung, Taiwan

ARTICLE INFO

Article history:
Received 20 February 2015
Received in revised form 24 August 2015
Accepted 11 September 2015
Available online 14 September 2015

Keywords:
Lipase
Multi-copy strains
Temperature
Secretion
Pichia pastoris
Candida rugosa

ABSTRACT

The yeast *Candida rugosa* can secrete a mixture of lipase isoenzymes (Lips), which have been widely applied in industry. Eight Lip genes (*LIP1* to *LIP8*) have been identified and are expressed in *Pichia pastoris*. However, the expression level was not sufficient for economical industrial application. In this study, two combined processes of antibiotic selection and low-temperature culture efficiently elicited a high-level secretion of recombinant Lip2 in *P. pastoris*. The *LIP2* gene copy number of the *Pichia* transformants was increased by sequential selections at gradually increasing Zeocin concentrations. After the first selection at 500 μg/mL of Zeocin, three clones (500-clones) with 2.4-fold to 5.8-fold improvement in Lip2 secretion were identified from 105 survival clones through lipase activity screening. Although the maximum number of *LIP2* gene copy was four among these three 500-clones, the lipase secretion of the four-copy clone was not higher than that of the three-copy clone. The effects of multiple gene copy number and low culture temperature resulted in a maximal 32-fold increase in Lip2 secretion. This method could be applied to other Lip isoforms to enhance their yields in *P. pastoris*.

1. Introduction

Lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) is an important group of versatile enzymes in the field of biotechnology. In addition to the hydrolysis of triacylglycerols, lipase can also catalyze the synthesis of esters and the conversion of racemic mixtures to enantiomerically pure compounds. Because of its diverse functions, lipase has been extensively used in the detergent, food, pharmaceutical, cosmetic, leather, paper industries, etc. [1]. Among the most commonly used industrial lipases is *Candida rugosa* lipase (CRL). However, the isoenzymatic profiles of commercial crude CRL samples are different and may be affected by fermentation conditions [2,3]. Five lipase isoenzymes have been biochemically characterized [4], and the purified isoenzymes exhibit different substrate specificities and thermal stabilities [5–8]. Thus, commercial crude CRL used as a biocatalyst usually results in inconsistency [9].

The similarities in the biochemical properties and the differential expression levels of the five lipase isoforms make it difficult to purify individual isoenzyme directly from culture of *C. rugosa* on a preparative scale for industrial applications. Thus, the expression of individual isoenzymes by genetic engineering is the more practical solution. Currently, at least eight lipase genes, named *LIP1* to *LIP8*, have been identified in *C. rugosa* [10–13]. We successfully expressed and characterized recombinant Lip isoenzymes (Lip1–Lip5) in *P. pastoris*. These recombinant isoenzymes exhibit distinct substrate preferences and catalytic activities [14–18]. Different expression amounts of recombinant Lip1 to Lip5 have been detected in *P. pastoris*; however, these amounts are still considered to be uneconomical for industrial applications.

A frequently used method to improve the protein expression in *P. pastoris* is to increase the copy number of the foreign gene by selecting resistant transformants immediately after transformation on high-level antibiotics (e.g., Zeocin or G418) [19,20]. However, there is only a rare part of highly drug-resistant colonies which are derived from an increased number of vector copies and thus produce a high level of recombinant protein [21]. Hence, Sunga et al. developed post-transformational vector amplification (PTVA) to obtain a high proportion of multiple vector copy strains that are selected by subjecting strains transformed with only one or a few vector copies, long after transformation, to further selection at high levels of drug rather than immediate selection on high levels of drug [22]. However, recent studies have shown that protein production is not always related to the gene copy number, particularly with

* Corresponding author at: Department of Life Science, National Taiwan Normal University, Taipei 11677, Taiwan. Fax: +886 2 2931 2904.
** Corresponding author. Fax: +886 7 657 7051.
E-mail addresses: Shawjf@isu.edu.tw (J.-F. Shaw), gclee@ntnu.edu.tw (G.-C. Lee).

http://dx.doi.org/10.1016/j.procbio.2015.09.013
1359-5113/

respect to extracellular proteins [23]. Moreover, the productivity of a secreted protein may reach a plateau, and further increases in the copy number may decrease the productivity [24].

To improve the Lip2 expression in *P. pastoris*, we previously showed that codon optimization of the Lip2-coding sequence and designing the expression construct without an additional N-terminal peptide can slightly improve the secretory level of Lip2 [25]. To further improve the production of Lip2 in *P. pastoris*, the present study applied PTVA process for a screening-accompanied selection strategy to obtain high-expression multi-copy strains, and a low culture temperature was used to enhance Lip2 production. In addition, the correlation of the *LIP2* gene dosage with Lip2 production was analyzed to identify the optimized *LIP2* copy number for secretory expression.

2. Materials and methods

2.1. Strains, plasmid, and media

P. pastoris strain SMD1168H (Invitrogen, Carlsbad, CA, USA), harboring the recombinant plasmid pGAPZαC-nfco*LIP2*, was used to express recombinant Lip2. The Lip2-coding sequence was inserted between the *Kpn*I and *Xba*I sites of pGAPZαC (Invitrogen) [15]. The partial sequence of the Lip2-coding sequence was codon-optimized as described previously [25]. *Escherichia coli* strain DH5α was used to propagate the recombinant plasmid and cultured in low-salt Luria-Bertani medium supplemented with 25 μg/mL Zeocin (Invitrogen). *P. pastoris* transformants were grown on a YPD (2% dextrose, 1% yeast extract, and 2% peptone) plate containing different concentrations of Zeocin as indicated in this study. Cultures were grown in shaking flasks in 10 mL glycerol medium (GM; 2% glycerol, 1% yeast extract, and 0.5% ammonium sulfate) containing 100 μg/mL Zeocin at 30 °C and 200 rpm. Glycerol medium uses the cheaper carbon source glycerol and nitrogen bases to culture *P. pastoris*. In addition, glycerol can constitutively induce the recombinant protein expression under the control of *GAP* promoter as well as glucose [26].

2.2. P. pastoris *transformation*

The plasmid (4 μg) harboring the engineered nfco*LIP2* was linearized with *Avr*II and transformed into *P. pastoris* SMD1168H by electroporation. High-voltage pulses (1.5 kV) were delivered to 100 μL sample in a 0.2 cm electrode gap cuvette using a MicroPulser™ electroporator (Bio-Rad, Hercules, CA, USA). Immediately after pulsing, 1 mL cold 1 M sorbitol was added to the cuvette. Cell suspensions were transferred to a 15 mL centrifuge tube, and 1 mL YPD medium was added. Cells were then incubated for 4 h in a shaking incubator at 100 rpm and 30 °C. Afterward, the cells were plated on YPD plates containing 100 μg/mL Zeocin to isolate Zeocin-resistant transformants.

2.3. Generation of high-expression multi-copy strains

Each single Zeocin-resistant transformant was streaked to another YPD plate to ensure that each strain was the progeny of a single cell. This colony purification was repeated thrice. To generate multi-copy strains, the purified transformants (parental clones) were suspended in sterile water and then spotted on a YPD plate containing an increased concentration of Zeocin (500 μg/mL). The 500 μg/mL Zeocin-resistant clones (500-clones) were selected. To screen the high-expression strains, the 500-clones were cultured in a 96-deepwell plate containing 0.2 mL GM with 100 μg/mL Zeocin. The 96-deepwell plates were covered with a gas-permeable heat seal (4Titude Ltd., Surrey, UK) and incubated at 30 °C at 200 rpm for 3 days. The cultures were centrifuged at 2500 × *g* for 10 min. The lipase activities of the supernatants were assayed. To increase the *LIP2* gene copies and the lipase productivity, the obtained high-expression strains were further spotted on a 2000 μg/mL Zeocin-containing YPD plate. The 2000-clones were selected and screened for the high-expression strains.

2.4. Determination of the gene copy number by quantitative real-time polymerase chain reaction (qPCR)

Genomic DNA (gDNA) from *P. pastoris* transformants was prepared using a Tissue & Cell Genomic DNA Purification Kit (GeneMark, Ltd., Taichung, Taiwan). The gDNA concentration was measured using a Nanodrop 1000 (Thermo Scientific, Wilmington, DE, USA) at 260 nm. The quality of isolated gDNA was determined by gel electrophoresis. To determine the gene copy number of the *LIP2* gene in *P. pastoris* by qPCR, the *ARG4* gene was quantified in parallel and was used as a reference gene [27]. Primer Express v3.0 software (Applied Biosystems, Foster City, CA, USA) was used to design the qPCR primers. The amplicon of the *LIP2* gene was amplified using the primer set of the forward primer 5′-GGCAGCGGCAGTGTGATCT and the reverse primer 3′-CGGCGGGTTGGAAAAGA. The *ARG4* gene was amplified by the primer set of the forward primer 5′-TCCTCCGGTGGCAGTTCTT and the reverse primer 3′-TCCATTGACTCCCGTTTTGAG. qPCR amplification was conducted in an ABI StepOne™ instrument (Applied Biosystems). The reaction was performed in a 20 μL mixture containing 10 μL 2× Maxima™ SYBR Green/ROX qPCR Master Mix (Thermo Scientific), 0.4 μL 10 μM primers, and 2 ng gDNA at 95 °C for 10 min and 40 cycles of 95 °C for 1 min and 60 °C for 15 s. Melting curve analysis was performed at a temperature gradient of 0.1 °C/s from 70 °C to 95 °C to ensure that only a specific amplification product was obtained. The relative quantification of the copy number was performed according to the $2-\Delta DCt$ method. A strain containing one copy of the *LIP2* gene according to Southern blot was used as a calibrator strain.

2.5. Extraction of total RNA and reverse transcription (RT)-qPCR analysis

Total RNA extraction was performed using the RNeasy Mini Kit (Qiagen, Hilden, Germany), and then the residual DNA was removed by the RNase-free DNase Set (Qiagen). To ensure that the RNA template did not contain DNA contamination, general PCR analysis was performed. The general PCR was performed in a 20 μL mixture containing 10 μL 2× Maxima™ SYBR Green/ROX qPCR Master Mix, 0.2 μL 10 μM *LIP2* gene primer set, and 50 ng RNA templates at 95 °C for 10 min and 35 cycles of 95 °C for 1 min and 60 °C for 15 s. The PCR product from DNA contamination was detected by gel electrophoresis. Power SYBR® Green RNA-to-CT™ 1-Step Kit (Applied Biosystems) was used to detect the mRNA expression level. The RT-PCR mixture contained 50 ng RNA template mixed with 10 μL 2× Power SYBR® Green RT-qPCR Mix, 0.16 μL 125× RT Enzyme Mix, and 0.2 μL 10 μM primer set. The reaction was carried out with the following program: 48 °C for 30 min, 95 °C for 10 min, and 40 cycles of 95 °C for 1 min and 60 °C for 15 s, and then melting curve analysis was performed as described in qPCR. The relative amount of mRNA (mRNA ratio) was determined according to the $2-\Delta\Delta Ct$ method. A strain containing one copy of the *LIP2* gene according to Southern blot was used as a calibrator strain.

2.6. Enzyme characterization

The molecular mass of recombinant Lip2 was determined by sodium dodecyl sulfate–polyacrylamide (12.5%) gel electrophoresis (SDS-PAGE). SDS-PAGE analyses were performed in a Hoefer mini-gel apparatus (Hoefer Scientific Instruments, USA).