1 **Extracellular production of *Pseudozyma* (*Candida*) *antarctica* lipase B with genuine**
2 **primary sequence in recombinant *Escherichia coli***

4 Ayana Ujiie, Hideo Nakano and Yugo Iwasaki*

6 Laboratory of Molecular Biotechnology, Graduate School of Bioagricultural Sciences,
7 Nagoya University
8 Furo-cho, Chikusa-ku, Nagoya 464-8601, Japan

10 *Corresponding author: PHONE: +81-52-789-4143, FAX: +81-52-789-4145

11 *E-mail:* iwasaki@agr.nagoya-u.ac.jp

13 Running title: Production of CALB in recombinant *E. coli*

1 **ABSTRACT** An *Escherichia coli* expression system was established to produce
2 recombinant extracellular *Pseudozyma* (*Candida*) *antarctica* lipase B (CALB). With the
3 aim of producing the genuine CALB without additional amino acid residues, the mature
4 portion of the CALB gene was fused seamlessly to a *pelB* signal sequence and
5 expressed in *E. coli* BL21 (DE3) using the pET system. Inducing gene expression at
6 low temperature (20°C) was crucial for the production of active CALB; higher
7 temperatures caused inclusion body formation. Prolonged induction for 48 h at 20°C
8 allowed for the enzyme to be released into the culture medium, with more than half of
9 the activity detected in the culture supernatant. A catalytically inactive CALB mutant
10 (S105A) protein was similarly released, suggesting that the lipid-hydrolyzing activity of
11 the enzyme was not the reason for the release. The CALB production level was further
12 improved by optimizing the culture medium. Under the optimized conditions, the
13 CALB in the culture supernatant amounted to 550 mg/L. The recombinant CALB was
14 purified from the culture supernatant, yielding 5.67 mg of purified CALB from 50 mL
15 of culture. N-terminal sequencing and ESI-MS analyses showed proper removal of the
16 *pelB* signal sequence and the correct molecular weight of the protein, respectively,
17 confirming the structural integrity of the recombinant CALB. The kinetic parameters
18 towards *p*-nitrophenylbutyrate and the enantiomeric selectivity on
19 *rac*-1-phenylethylacetate of the recombinant CALB were consistent with those of the
20 authentic CALB. This is the first example of *E. coli*-based extracellular production of a
21 CALB enzyme without extra amino acid residues.

22

23 **KEYWORDS:** recombinant *Escherichia coli*; extracellular production; *Pseudozyma*
24 (*Candida*) *antarctica* lipase B; *pelB* signal sequence

1 INTRODUCTION

2 *Pseudozyma* (*Candida*) *antarctica* lipase B (CALB) is one of the most widely used
3 lipases in the world, due to its excellent properties such as high stereoselectivity and
4 stability (1). Further improving the properties of CALB by protein engineering
5 approaches is therefore an attractive and challenging goal for many researchers.
6 Eukaryotic microorganisms, such as *Aspergillus oryzae* (2), *Pichia pastoris* (3–7),
7 *Saccharomyces cerevisiae* (8) and *Yarrowia lipolytica* (9), are often used as recombinant
8 protein expression hosts, and these eukaryotic host systems have been extensively
9 utilized for protein engineering of the lipase (8, 10–15).

10 However, *Escherichia coli* is still an attractive option as the host organism for
11 recombinant CALB expression because of the ease of its genetic manipulation, the
12 availability of various host/vector systems, and its fast growth. To date, many research
13 groups have attempted to express CALB in the cytoplasm (16–20), the periplasm (4,
14 21–24) or the culture medium (25) of recombinant *E. coli* cells, and have applied these
15 systems to protein engineering studies (26–30). Among these expression systems, one
16 remarkable achievement is the work by Seo et al., who expressed CALB as a fusion
17 protein with a “solubility enhancer” (e.g., malate dehydrogenase) and obtained the
18 active enzyme from the cytoplasm with a yield as high as 0.1 g of purified CALB per
19 300 mL culture (17). Another outstanding work is that of Kim et al., in which the CALB
20 protein was fused to the *pelB* signal sequence and a five-aspartate tag (D5-tag), and,
21 when expressed, generated extracellular enzyme with yields of 31 mg/L in batch culture
22 and 1.9 g/L in fed-batch culture (25).

23 All the *E. coli*-based expression systems reported so far, however, generated CALB
24 with additional amino acid residues at the N- and/or C-termini; the recombinant CALB

1 proteins prepared are therefore not “genuine” ones with respect to the primary
2 sequences. For example, the method by Kim et al. generates CALB with two amino acid
3 residues followed by the D5-tag at the N-terminus (25), whereas the method by Seo et
4 al. makes an enzyme fused with the solubility enhancer protein at the N-terminus and a
5 His₆-tag at the C-terminus (17). Although these extra residues may not affect the
6 catalytic properties of the recombinant enzymes, it is favorable, in a strict sense, to use
7 the enzyme with the *bona fide* amino acid sequence for the evaluation of
8 structure-activity relationships in protein engineering studies. The additional residues
9 can be removed from the target protein using a sequence-specific protease, such as
10 factor Xa, thrombin, or enterokinase, but this requires extra time and cost. During our
11 ongoing research project for protein engineering of CALB, we decided to establish a
12 simple expression platform for a version of CALB with a *bona fide* amino acid
13 sequence to facilitate precise analyses of the structure-activity relationship of variants of
14 the enzyme.

15 In this paper we focused on the preparation of genuine CALB directly, and
16 established a simple *E. coli*-based expression system of CALB. We demonstrate that
17 expression of the mature portion of the CALB gene fused seamlessly with a *pelB* signal
18 sequence allows for extracellular production of the CALB enzyme with no extra
19 residues, and that simple shaking-flask cultivation followed by a conventional
20 purification procedure yields large amounts of the enzyme.

21

22 MATERIALS AND METHODS

23 *Materials*

Oligonucleotides were from Greiner Bio One (Japan). *p*-Nitrophenylbutyrate (pNPB) was from SIGMA. Pyridine-2-azo-*p*-dimethylaniline cephalosporin (PADAC) was from Calbiochem (USA). *o*-Nitrophenyl- β -galactopyranoside (ONPG) and *rac*-1-phenylethanol were purchased from Wako Pure Chemical (Japan). (*R*)- and (*S*)-1-phenylethanol were from Tokyo Chemical Industries (Japan). *rac*-, (*R*)- and (*S*)-1-phenylethylacetate (PEA) were prepared by acetylating the corresponding 1-phenylethanol in dry pyridine/acetic anhydride, followed by purification using a silica gel column. CALB-L, a commercial CALB preparation was a gift from Novozymes Japan, and was used as the authentic enzyme after purification as described later.

Construction of plasmids

Figure 1 shows the structure of pET22b-CALB used for CALB expression. The plasmid contains the mature portion of the CALB gene fused with the *pelB* signal sequence at its N-terminus between the *Nde*I and the *Hind*III sites of pET22b (+) (Novagen). The gene was custom-synthesized by GeneScript (USA) based on the amino acid sequence of CALB from *C. antarctica* LF058 (31), and was codon-optimized by taking into account the codon usage of the *E. coli* B strain (<http://www.kazusa.or.jp/codon/>). The sequence of the synthetic gene was registered with the DNA Data Bank of Japan (DDBJ) under accession number LC026014.

Another plasmid for expression of the S105A mutant of CALB (in which Ser 105, the catalytic residue, was replaced with Ala) was constructed as follows. The whole sequence of the pET22b-CALB was amplified by inverse PCR using two phosphorylated primers, CALB-S105A-Fw: 5'-GCCCCAGGGTGGTCTGGTGGCGC (the GCC codon for Ala is underlined) and CALB-S105A-Rv:

1 5'-CCAGGTCAGCACCGGCAGTT, introducing the S105A mutation. After
2 purification, the amplified DNA fragment was self-circularized, resulting in plasmid
3 pET22b-CALB(S105A). DNA sequencing confirmed that the plasmid contained the
4 desired mutation (AGC to GCC).

5 These two plasmids code for the seamlessly fused protein consisting of *pelB*
6 signal-CALB (or its S105A mutant) without any additional sequence, under the control
7 of T7-*lac* promoter, and were introduced to the host strain *E. coli* BL21 (DE3) for the
8 expression experiments.

9 10 ***Culture media***

11 For cultivation of the recombinant *E. coli* strains, the following culture media were
12 used: Luria-Bertani (LB; 1% tryptone, 0.5% yeast extract, 1% NaCl), 2xYT (1.6%
13 tryptone, 1% yeast extract, 0.5% NaCl), Super Broth (SB; 3.2% tryptone, 2% yeast
14 extract, 0.5% NaCl) and Terrific Broth (TB; 1.2% tryptone, 2.4% yeast extract, 0.5%
15 glycerol, 17 mM KH₂PO₄, 72 mM K₂HPO₄). Ampicillin (Ap) was used as a selection
16 antibiotic at 100 µg/mL.

17 18 ***Expression of the recombinant CALB***

19 Thirty microliters of an overnight culture of the recombinant strain of *E. coli* BL21
20 (DE3) harboring pET22b-CALB was inoculated into 3 mL of fresh liquid medium (*i.e.*,
21 LB, 2xYT, SB or TB) supplemented with 100 µg/ml Ap and cultivated aerobically at
22 37°C for 3 h. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to give a final
23 concentration of 0.1 mM, and the cultivation continued at 37°C, 30°C, or 20°C for 24,
24 48, or 72 h (the detailed induction times and temperatures are described in the Results

section). The culture was centrifuged to separate the supernatant and the cell pellet. The cell pellet was resuspended in 0.3 mL (one tenth of the original culture volume) of 20 mM Tris-HCl buffer (pH 8.0), disrupted by sonication, and centrifuged, and the supernatant containing cellular soluble proteins was recovered. The precipitate containing cellular insoluble proteins was resuspended in 0.3 mL of 20 mM Tris-HCl buffer (pH 8.0). The fractions thus obtained (*i.e.*, the culture supernatant, the cellular soluble fraction and the cellular insoluble fraction) were used for further analyses.

Clear zone-forming assay on agar media

The *E. coli* cells harboring pET22b-CALB and pET22b-CALB (S105A) were grown overnight on a 90-mm diameter LB agar plate with 100 µg/ml Ap. Twenty milliliters of 0.8% soft agar containing 40 mM Tris-HCl (pH 8.0), 1% tributyrin (Wako), 0.8% Triton X-100, 1 mM IPTG, and 100 µg/ml Ap was overlaid onto the colonies. After incubating the plate at 20°C over night, a clear zone was observed around the colonies.

Purification of the recombinant CALB from the E. coli culture

The recombinant *E. coli* strain harboring pET22b-CALB was cultivated, and expression of the target gene was induced at 20°C for 48 h in 100 mL of TB using a 500-mL Sakaguchi-flask. The cells were removed by centrifugation, and the culture supernatant (90 mL) was recovered. We used half of the culture supernatant (*i.e.*, 45 mL) for purification. The supernatant was concentrated to approximately 4 mL with a centrifugal ultrafiltration device (Amicon Ultra-4, molecular weight cut off 10 kDa, Merck-Millipore). The concentrate was dialyzed against 20 mM Tris-HCl buffer (pH

1 8.0). The dialysate was applied to a Resource Q (6 mL) column (GE Healthcare) and
2 eluted with the same buffer at a flow rate of 1 mL/min. The target protein was recovered
3 in the flow-through fraction, whereas most of the impurities bound to the column. The
4 flow-through fraction (6 mL) containing the target protein was mixed with 1.142 mL of
5 5 M ammonium acetate to give a final concentration of 0.8 M, and then loaded onto a
6 hydrophobic interaction column (Hi Prep 16/10 Butyl FF, GE Healthcare) equilibrated
7 with 20 mM Tris-HCl (pH 8.0) containing 0.8 M ammonium acetate, **essentially as**
8 **described elsewhere (2, 3)**. The column was eluted at a flow rate of 2.0 mL/min with a
9 linear gradient of 0.8 M to 0 M ammonium acetate in 20 mM Tris-HCl (pH 8.0). The
10 fractions containing the target protein were pooled, dialyzed against 20 mM Tris-HCl
11 (pH 8.0), and used for the subsequent experiments. **The commercial CALB was purified**
12 **similarly, but without the ultrafiltration step.**

13

14 ***N-terminal amino acid sequencing of the purified CALB***

15 The purified CALB was separated on SDS-PAGE and transferred to a
16 polyvinylidenedifluoride membrane (SequeBlott membrane, BioRad). After washing the
17 membrane with 10 mM H₃BO₃-NaOH buffer (pH 8.0) containing 25 mM NaCl, the
18 membrane was stained with Coomassie Brilliant Blue, and then washed with 50%
19 methanol. The band of the target protein was excised and subjected to amino acid
20 sequence analysis (Procise 491HT, Applied Biosystems).

21

22 ***Determination of molecular weight of the purified CALB***

23 The molecular weight of the recombinant CALB was determined by electrospray
24 ionization mass spectrometry (ESI-MS) analysis. The purified CALB was dialyzed

thoroughly against ultra-pure water. One hundred microliters of the dialysate containing 0.13 mg/mL of protein was mixed with 900 μ L of 50% methanol containing 5% acetic acid to make a final protein concentration of 13 μ g/mL. Ten microliters of the sample was injected into an ESI-MS instrument (LCMS-2010EV, Shimadzu) in flow-injection mode with a mobile phase of 50% methanol containing 5% acetic acid at a flow rate of 10 μ L/min. The ESI-MS was operated in the positive ion mode with mass-to-charge ratio (m/z) of 700–2000. A series of multiply charged ion peaks was obtained. The actual molecular weight of the target protein was calculated by deconvoluting these ion peaks manually according to Strupat (32).

Assays

Lipase activity was measured using pNPB as substrate. In a cuvette, 5 μ L of enzyme sample was mixed with 475 μ L of 21 mM Tris-HCl (pH 8.0), 0.526% Triton X-100. The hydrolysis reaction was initiated by adding 25 μ L of 20 mM pNPB solution in 2-propanol. The final composition of the reaction mixture was 20 mM Tris-HCl (pH 8.0), 0.5% Triton X-100, 1 mM pNPB and 5% 2-propanol. The progress of the hydrolysis was monitored spectrophotometrically at 410 nm at 25°C for 3 min. One unit of hydrolytic activity was defined as the amount of enzyme that liberates one μ mol of *p*-nitrophenol in one min, which was calculated using an absorption coefficient of 16200 M⁻¹ cm⁻¹. Kinetic analysis of the lipase was performed similarly with varying substrate concentrations (0.1~5 mM pNPB). The substrate-versus-velocity plots were fitted to the Michaelis-Menten equation by a non-linear regression algorithm using *Prism* software (Graph Pad, USA) to calculate the kinetic constants.

1 β -lactamase (BLA) activity was measured using PADAC as substrate. A 300- μ L
2 substrate solution consisting of 298 μ L of 50 mM sodium phosphate buffer (pH 7.0) and
3 2 μ L of 2 mM PADAC in dimethylsulfoxide was placed in a cuvette. Five microliters of
4 enzyme sample were added to the substrate solution and the decrease in absorbance at
5 570 nm was monitored at 25°C.

6 β -Galactosidase (β -Gal) activity was assayed using ONPG as substrate. Ten
7 microliters of the enzyme sample was added to 140 μ L of the substrate solution
8 containing 0.1 M sodium phosphate buffer (pH 7.0) and 1 mM ONPG, and incubated at
9 37°C for 30 min. After stopping the reaction with 50 μ L of 1 M Na₂CO₃, the absorbance
10 at 420 nm was measured spectrophotometrically.

11 Protein concentration was measured by the method of Bradford (33) using a
12 Bio-Rad Protein Assay Kit with bovine serum albumin as a standard.

13

14 ***Kinetic resolution of rac-1-PEA***

15 A reaction mixture consisting of 890 μ L of 50 mM potassium phosphate buffer (pH
16 7.0), 70 μ L of 0.36 M *rac*-1-PEA in acetonitrile, and 50 μ L of 60 μ g/mL purified CALB
17 was incubated at 37°C. Portions (100 μ L) of the mixture were withdrawn intermittently,
18 mixed with 100 μ L of *n*-hexane, and centrifuged to obtain phase separation. The
19 *n*-hexane layer containing the product and the unreacted substrate was recovered.
20 Anhydrous sodium sulfate (approx. 20 mg) was added to the solution and the sample
21 was kept in a freezer (-20 °C) until analysis.

22 Enantiomeric purities of the generated alcohol and the unreacted ester were
23 analyzed using an HPLC system (Prominence, Shimadzu) with a chiral stationary phase
24 column (Chiralcel OF, 4.6 mm x 250 mm, 10 μ m particle size, Daicel Chemical). The

column was eluted at a flow rate of 1 mL/min with *n*-hexane/2-propanol (99/1) for 10 min, and then with *n*-hexane/2-propanol (95/5) for a further 10 min. Peaks were detected spectrophotometrically at 254 nm using a photodiode array detector (SPD20A, Shimadzu). The retention times of (*R*)-ester, (*S*)-ester, (*R*)-alcohol, and (*S*)-alcohol were 5.4, 5.9, 16.0, and 17.8 min, respectively, which were confirmed by injecting each of the compounds. The enantiomeric ratio (*E*) was calculated from enantiomeric excess values of the remaining esters and the generated alcohols according to Chen et al. (34).

RESULTS

Expression of CALB

Figure 2 shows the expression of CALB by the recombinant strain in 3 mL LB medium at 37, 30 and 20°C. The induction temperature was very important for the expression of soluble CALB; induction at 37°C or 30°C yielded target protein exclusively in the intracellular insoluble fraction (Fig. 2A), whereas lowering the temperature to 20°C enabled expression in a soluble form (Fig 2C). At 20°C, a portion of the expressed CALB was detected in the culture supernatant. The activity of the expressed CALB proteins was confirmed by measuring the enzyme activities in the culture supernatant and the cellular soluble fractions (Fig. 2D). Prolonged induction for 48 or 72 h increased the amount of CALB activity in the culture supernatant. As induction at 20°C for 48 h gave the highest level of extracellular activity, we chose these conditions for the subsequent experiments.

Comparison of the wild-type and the activity-less mutant

1 Since a considerable amount of active CALB was detected in the extracellular
2 fraction, we wondered whether the excretion of the enzyme was caused by cell lysis
3 resulting from the catalytic activity of the lipase, which may hydrolyze the membrane
4 phospholipids of the host cells. Figure 3 compares the expression of wild-type (WT)
5 CALB and its activity-less mutant (S105A). SDS-PAGE analysis (Fig. 3A) indicated
6 that the S105A mutant protein was also excreted into the culture supernatant at similar
7 levels as the WT protein. The lack of activity in the S105A mutant was confirmed by
8 quantifying the lipase activity (Fig. 3B), and by the clear zone-forming assay on agar
9 plates (Fig. 3C). These results indicated that the excretion of CALB does not depend on
10 its catalytic activity.

11

12 ***Improvement of CALB production***

13 Next, we tried to improve the CALB production. We tested four different culture
14 media, all of which are conventional ones used for *E. coli* in laboratory experiments. An
15 overnight culture (30 μ L) of the recombinant cells grown in LB media at 37°C was
16 inoculated into each of the four different culture media (3 mL), and grown at 37°C for 3
17 h. IPTG was added, the induction continued at 20°C with shaking for 48 h, and the
18 enzyme production analyzed by SDS-PAGE and lipase assay. As shown in Figure 4,
19 culture media that supported better cell growth yielded better total (intra- and
20 extracellular) CALB production (Fig. 4A and B). Plotting the lipase activity against the
21 cell density for each medium revealed that the intracellular activity was proportional to
22 the cell density with a good correlation, indicating that the amount of intracellular
23 activity per cell was constant regardless of the culture medium (Fig. 4C). In contrast,

1 such a correlation was not observed between the extracellular activity and the cell
2 density.

3 Analysis of the distribution of BLA (a periplasmic enzyme) and β -Gal (a cytosolic
4 enzyme) activities in the intra- and the extracellular fractions revealed that 85% of the
5 β -Gal remained in the cell, whereas 90% of the BLA was in the extracellular fraction
6 (Fig. 4D). This suggests that the excretion of the CALB (66% of which was in the
7 extracellular fraction) was caused by a leak from the periplasm, and not by cell lysis. As
8 the use of TB medium resulted in the highest level of extracellular activity, the
9 subsequent experiments were performed using TB.

11 ***Purification of CALB***

12 The CALB protein was purified from the culture supernatant of the recombinant
13 strain. As we did not use any affinity tags, the purification was done by conventional
14 biochemical techniques. It is reported that CALB does not absorb to anion exchange
15 resins, because it is electrically almost neutral at pH 8.0, despite a theoretical isoelectric
16 point of 6.0 (35). Therefore, the use of an anion exchange column (Resource Q) was
17 very effective, enabling the recovery of the target protein in the flow-through fraction
18 while leaving most of the impurities absorbed onto the column (Fig. 5, lane 3). After
19 hydrophobic interaction column chromatography, the enzyme preparation gave a single
20 band on SDS-PAGE with a molecular mass of 33 kDa (Fig. 5, lane 4). From a 50 mL
21 culture (approximately 45 mL of culture supernatant), the three-step purification
22 procedure yielded as much as 5.67 mg of purified CALB, with a recovery of 23.0%
23 (Table 1).

1 The purified CALB showed specific activity of 15.9 U/mg (Table 1), which
2 allowed us to estimate the amount of CALB in the crude culture supernatant. It was
3 calculated that approximately 550 mg of CALB per liter of supernatant was produced
4 by this simple shaking-flask culture system.

6 ***Catalytic and structural integrity of the purified CALB***

7 Table 2 shows the kinetic constants of CALB purified from the recombinant *E. coli*
8 and the authentic enzyme, on the hydrolysis of pNPB. The K_M and the k_{cat} values of the
9 CALB from *E. coli* were in agreement with those of the authentic enzyme, though the
10 enzyme from *E. coli* showed somewhat higher k_{cat} and lower K_M . Also shown in Table 2
11 are the parameters on the kinetic resolution of *rac*-1-PEA. Both of the two enzymes
12 showed excellent enantioselectivity towards the (*R*)-ester with *E* values of >200, and
13 45% of the substrate conversion after 6 h-reaction, consistent with the values reported
14 elsewhere (22). These results confirmed that the recombinant CALB had the catalytic
15 ability comparable to that of the authentic one.

16 The first five N-terminal amino acids of the purified CALB were determined to be
17 Leu-Pro-Ser-Gly-Ser, which are identical to those of the mature CALB, indicating that
18 the *pelB* signal sequence was correctly removed and that the recombinant CALB had
19 the *bona fide* N-terminus. ESI-MS analysis of the purified CALB yielded a series of
20 multiply charged ion signals, for which the charge states were assigned (Fig. S1).
21 Deconvoluting these ion peaks indicated the molecular weight of the recombinant
22 CALB to be 33021±21, which agreed well with the theoretical value of 33022. These
23 results confirmed that the CALB prepared from the recombinant *E. coli* had the correct
24 primary sequence with no additional residues.

1 **DISCUSSION**

2 In this paper, we have demonstrated extracellular production of CALB from a
3 recombinant strain of *E. coli*. The recombinant CALB could be recovered from the
4 culture supernatant and purified by a simple process, yielding as much as 5.67 mg of
5 purified CALB per 50 mL of culture. The amount of CALB in the crude culture
6 supernatant was estimated to be ~550 mg/L, which is one of the highest reported for the
7 *E. coli*-based CALB production systems utilizing shaking-flask cultivation. **Although**
8 **the specific activity dropped after the hydrophobic interaction chromatography, the**
9 **value of the final preparation (15.9 U/mg) seems reasonable, because the reported**
10 **specific activity values for the authentic CALB in the preceding papers (9, 21, 25) are in**
11 **the range of 10.5~20.6 U/mg. Rather, the activity after Resource Q (28.6 U/mg) seems**
12 **too high, but the reason for this is unknown.**

13 The N-terminal sequence analysis and the molecular weight measurement
14 confirmed the integrity of the structure; the recombinant enzyme had the correct
15 primary sequence without any extra residues. In addition, the specific activity toward
16 hydrolysis of pNPB and the enantioselectivity of kinetic resolution of *rac*-1-PEA were
17 consistent with the reported values. To our knowledge, this is the first example of
18 CALB production with a *bona fide* amino acid sequence by a recombinant *E. coli*.

19 It was a surprise for us that CALB could be produced so readily without any
20 special “tricks”, like the ones described in 11 previous papers for *E. coli*-based CALB
21 production (4, 16–25). Our expression system features the following four points: 1) the
22 *pelB* signal sequence *directly* fused to the mature portion of CALB; 2) a
23 codon-optimized gene; 3) a low induction temperature; and 4) a good culture medium

1 (i.e., TB). Although each of these points is not a novel idea, the combination of them
2 resulted in highly successful CALB production.

3 The use of the *pelB* signal sequence for secretory expression is one major reason
4 for the successful production. CALB has three disulfide bonds that are important for
5 folding of the protein into the correct tertiary structure (30). The *pelB* signal enabled the
6 translocation of the CALB into the periplasm, where the protein could be correctly
7 folded with the formation of the disulfide bridges. The correct folding was also
8 attributed to the low induction temperature, as higher temperatures caused protein
9 aggregation (Fig. 2). In fact, the use of *pelB* signal for the CALB expression was
10 reported in four previous papers (4, 21, 23, 25), but the productivity in those reports was
11 not as high as we achieved here. In those four reports, unlike our system, the N-termini
12 of the target proteins retained extra residues, even after removal of the signal peptide
13 upon translocation. We speculate that the presence of such extra N-terminal residues
14 might negatively influence the efficiency of CALB production.

15 Generally, *E. coli* does not secrete large amounts of proteins into the culture
16 medium, but some recombinant proteins can be secreted into the medium (36, 37). In
17 our system, the correct removal of the *pelB* signal from the target protein suggested that
18 the protein synthesized in the cytoplasm was first exported across the inner membrane
19 to the periplasm by a signal peptide-dependent mechanism (38). Judging from the
20 distribution of β -Gal and BLA activities mainly in the intra- and extracellular fractions,
21 respectively (Fig. 4D), the export of the CALB into the culture medium from the
22 periplasm was caused by a breakage of the periplasm, but not by cell lysis. The catalytic
23 activity of the lipase was not necessary for excretion of the enzyme, since the

1 activity-less S105A mutant CALB was excreted in similar fashion as the WT enzyme
2 (Fig. 3).

3 The intracellular CALB activity, *i.e.*, per cell unit of mass, was constant regardless
4 of the cell density, but the extracellular activities per cell number were variable and
5 depended on the culture medium used (Fig. 4C). We speculate that the *E. coli* cells may
6 have a certain threshold for the amount of overexpressed protein that is retainable inside
7 the periplasm. When the amount of the accumulated protein exceeds the threshold, the
8 surplus might “seep out” from the periplasm into the culture medium with breakage of
9 the outer periplasmic membrane.

10 It should be noted that the CALB expression did not cause cell death; the
11 recombinant cells that expressed CALB on the agar plate (Fig. 2C) could be picked up,
12 re-grown on another LB-Ap plate, and induced again to express the enzyme. This will
13 be advantageous when performing colony-based high-throughput screening, as it would
14 not require the use of colony replica plating prior to screening, unlike the situation in
15 our previous work on extracellular production of a *Streptomyces* phospholipase A₂ in *E.*
16 *coli* (39).

17 Optimization of a recombinant protein’s codons for the host’s codon usage is often
18 effective for increasing recombinant protein synthesis, especially when the target gene
19 contains rare codons (40-42). The mature portion of the native CALB gene from *C.*
20 *antarctica* strain LF058 contains two AGG (Arg), three CGA (Arg), one CTA (Leu), two
21 GGA (Gly), and 17 CCC (Pro) codons (31), which are known as rare codons. The
22 CALB gene used in this study was codon-optimized when synthesized to alleviate the
23 rare codon problem. Although we did not compare the synthetic gene with the native
24 version, the use of the synthetic gene may have contributed to the efficient production

1 of CALB. Jung and Park also used a synthetic CALB gene to produce the enzyme, and
2 reported yields of 1 mg/L for WT CALB and 3.3 mg/L for a mutated CALB (22).

3 TB was the best medium among the four different media tested. One reason for TB
4 being the best is that it contained potassium phosphate, which worked both as a
5 phosphate source and as a pH buffer, supporting the good cell growth. The use of TB
6 greatly enhanced the CALB production, but it did not improve the specific activity (as a
7 measure of purity) of the CALB in the culture supernatant. A simple comparison among
8 lanes "C" of each culture medium in Figure 4A demonstrates that culture media giving
9 thicker CALB bands also give stronger bands of impurities. This is because the
10 excretion of the target protein to the medium is caused by a non-specific leak from the
11 periplasmic, but not by any target-specific mechanism, as proved by the distribution
12 analysis of BLA (Fig. 4D).

13 Finally, we summarize the *E. coli*-based CALB expression systems reported to date
14 in Table S1. It should be noted that our expression system, in which no additional
15 residues remain in the target, is indeed very effective. As the pure CALB can be readily
16 prepared in multi-milligram quantities from a shaking-flask culture, the system should
17 facilitate protein-engineering studies of this enzyme. It also seems promising as a
18 system for large-scale production of the enzyme by means of advanced fermentation
19 engineering methods, such as high cell density cultivation.

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1 **FIGURE LEGENDS**

2 **Figure 1. Structure of CALB-expressing plasmid**

3 (A) Schematic diagram of the expression cassette of pET22b-CALB. Only the part
4 between the promoter and the terminator is shown. The abbreviations are as follows:
5 T7P, T7 promoter; *lac O*, lac operator; rbs, ribosome binding site; T7T, T7 terminator.
6 (B) Nucleotide sequences of *pelB* signal-CALB fusion gene with the corresponding
7 amino acid sequence. The *pelB* signal sequence, *Nde* I, and *Hind* III recognition sites
8 are underlined. The catalytic Ser105 residue is boxed. Another plasmid,
9 pET22b-CALB(S105A), is a derivative of pET22b-CALB with a mutation for Ser105
10 (AGC) to Ala (GCC), as shown in parentheses.

11

12 **Figure 2. Effects of temperature and induction time on CALB expression**

13 (A–C) SDS-PAGE analysis. The expression of CALB was induced at 37°C (A), 30°C
14 (B) and 20°C (C) for the indicated times. Ten-μl equivalents of the culture supernatant
15 (lanes “C”), cellular soluble fraction (lanes “S”) and cellular insoluble fraction (lanes
16 “I”) were analyzed on a 12.5% polyacrylamide gel along with the molecular size marker
17 (lanes “M”). Protein bands were visualized with Coomassie Brilliant Blue R-250. The
18 arrows indicate the positions of CALB on the gels. (D) Quantification of the lipase
19 activity in the culture supernatants (“C”) and in the cellular soluble fractions (“S”) using
20 pNPB as the substrate. The activity shown is an average from duplicate measurements
21 of each sample, and error bar represent deviation from the average.

22

23 **Figure 3. Comparison of the extracellular production of WT CALB and the S105A** 24 **mutant**

1 (A and B) The expression of WT and S105A mutant CALBs was induced at 20°C for
2 48 h, and the proteins and the lipase activity were analyzed by SDS-PAGE (A) and by
3 enzyme assay (B), respectively. The symbols are the same as in the legend of Figure 2.
4 The activity shown is an average from duplicate measurements of each sample, and
5 error bar represents deviation from the average. (C) Clear zone-forming assay for the
6 recombinant strains expressing the WT and S105A mutant CALBs.

7

8 **Figure 4. Optimization of culture medium for CALB production**

9 (A and B) The expression of CALB was induced at 20°C for 48 h using different culture
10 media as indicated. (A) SDS-PAGE analysis of each of the fractions (10-μL culture
11 equivalents). (B) The lipase activity assay (open columns), together with the cell density,
12 reached at the end of cultivation (closed circles). The average values of duplicate
13 measurements from two independent cultures are shown with error bars representing
14 standard deviation. (C) Plot of the lipase activity versus the cell density, showing the
15 proportional relationship between the cell density and the intracellular activity (closed
16 squares), and the lack of correlation between the cell density and the extracellular
17 activity (open squares). The dotted line is the regression line between the cell density
18 and the intracellular activity with the correlation coefficient, $r = 0.989$. (D) Distribution
19 of marker enzymes between the intra- and extracellular fractions. Enzyme activities of
20 the culture supernatant (black columns) and the cellular soluble (white columns)
21 fractions prepared from two independent cultures induced in TB at 20°C for 48 h were
22 analyzed. The samples were measured in duplicate and the mean values are used for the
23 calculation of distribution. Data shown are average values from the two culture samples
24 with error bars representing deviation from the average.

1 **Figure 5. Purification of CALB from recombinant *E. coli***
2 Protein samples in each of the purification steps were analyzed by SDS-PAGE. Lane M,
3 molecular size marker; lane 1, culture supernatant; lane 2, after ultrafiltration; lane 3,
4 after Resource Q chromatography; lane 4, after HiPrep Butyl FF chromatography. The
5 position of CALB is indicated with an arrow.

Table 1. Summary of purification of CALB from recombinant *E. coli* ^{a)}

Purification Step	Total Activity (U)	Total Protein (mg)	Specific activity (U/mg)	Activity yield (%)	Purification (-)
Culture Supernatant	392	32.0	12.3	100	1.00
Ultra Filtration	328	30.0	10.9	83.7	0.89
Resource Q	281	9.82	28.6	71.7	2.33
Hi Prep Butyl FF	90.0	5.67	15.9	23.0	1.29

a) From 50 mL culture (45 mL culture supernatant)

Table 2. Catalytic parameters of CALBs from the recombinant *E. coli* and the commercial preparation

Enzyme source	Hydrolysis of <i>p</i> NPB		Kinetic resolution of <i>rac</i> -PEA		
	K_M (mM)	k_{cat} (min ⁻¹)	Reaction time (h)	Conversion (%)	<i>E</i>
Recombinant <i>E. coli</i>	0.80 ± 0.21	1020 ± 92	6	45.4	>200, (<i>R</i>)-selective
Commercial (CALB-L)	1.26 ± 0.26	823 ± 67	6	44.6	>200, (<i>R</i>)-selective

Fig. 1

A

B

Nde I

CATATGAAATACCTGCTGCCGACCGCTGCTGCTGGTCTGCTGCTCCTCGCTGCCCAGCCGGCGATGGCCCTGCCGAGCGGTAGCGATCCGGCGTTTAGCCAGCCGAAAAGCGTTCTGGAT
 M K Y L L P T A A A G L L L L A A O P A M A L P S G S D P A F S Q P K S V L D
 |--> *pelB* signal sequence |--> mature CALB

GCAGGCCTCACGTGTCAGGGTGCGAGCCCGAGCAGCGTTAGCAAACCGATTCTGCTGGTTCGGGTACGGGTACGACCGGTCCGCAGAGCTTTGATAGCAATTGGATTCCGCTGAGCACG
 A G L T C Q G A S P S S V S K P I L L V P G T G T T G P Q S F D S N W I P L S T

CAACTCGGCTATACCCCGTGTGGATTAGCCCGCCGCCGTTTATGCTGAATGATACCCAGGTGAATACCGAATATATGGTGAATGCGATTACCGCGCTGTATGCGGGTAGCGGTAATAAT
 Q L G Y T P C W I S P P P F M L N D T Q V N T E Y M V N A I T A L Y A G S G N N

(GCG)

AAACTGCCGGTGCTGACCTGGAGCCAGGGTGGTCTGGTGGCGCAGTGGGGCCTGACCTTTTTTCCGAGCATTCGTAGCAAAGTGGATCGTCTGATGGCGTTTGCGCCGGATTATAAAGGC
 K L P V L T W S Q G G L V A Q W G L T F F P S I R S K V D R L M A F A P D Y K G
 (A)

ACCGTGCTGGCGGGTCCGCTGGATGCGCTGGCGGTGAGCGCGCCGAGCGTGTGGCAGCAGACCACCGGTAGCGCGCTGACCACCGCGCTGCGTAATGCGGGTGGTCTGACCCAGATTGTG
 T V L A G P L D A L A V S A P S V W Q Q T T G S A L T T A L R N A G G L T Q I V

CCGACCACCAATCTGTATAGCGCGACCGATGAAATTGTGCAGCCGCAGGTGAGCAATAGCCCGCTGGATTTCGAGCTATCTGTTTAATGGTAAAAACGTTCAAGCACAGGCGGTGTGTGGT
 P T T N L Y S A T D E I V Q P Q V S N S P L D S S Y L F N G K N V Q A Q A V C G

CCGCTGTTTGTGATTGATCATGCGGGTAGCCTGACCAGCCAGTTTAGCTATGTGGTGGGTCGTTCCGGCACTGCGTAGCACCACCGGCCAGGCGCGTAGCGCGGATTATGGCATTACCGAT
 P L F V I D H A G S L T S Q F S Y V V G R S A L R S T T G Q A R S A D Y G I T D

TGTAATCCGCTGCCGGCGAATGATCTGACCCCGGAACAGAAAGTTGCCGCTGCTGCCCTGCTGGCTCCAGCGGCGGCAGCCATTGTGGCGGGTCCGAAACAGAATTGTGAACCGGATCTG
 C N P L P A N D L T P E Q K V A A A A L L A P A A A A I V A G P K Q N C E P D L

Hind III

ATGCCGTATGCGCGTCCGTTTGCGGTGGGTAAACGTACCTGTAGCGGTATTGTGACCCCGTAATAAGCTT
 M P Y A R P F A V G K R T C S G I V T P end

Fig. 2

Fig. 3

Fig. 4

Fig. 5