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# Alleviation of Proteolytic Sensitivity To Enhance Recombinant Lipase Production in *Escherichia coli*[▽]

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**Two amino acids, Leu149 and Val223, were identified as proteolytically sensitive when *Pseudozyma antarctica* lipase (PalB) was heterologously expressed in *Escherichia coli*. The functional expression was enhanced using the double mutant for cultivation. However, the recombinant protein production was still limited by PalB misfolding, which was resolved by DsbA coexpression.**

Though *Escherichia coli* retains popularity as a host for recombinant protein production, it has technical limitations for expressing eukaryotic proteins. Host/protein incompatibility is critical, since eukaryotic proteins heterologously expressed in *E. coli* are recognized as a foreign object that can induce heat shock responses and drive protease-mediated degradation. *E. coli* contains several proteases situated in various intracellular compartments, including the cytoplasm, periplasm, inner membrane, and outer membrane (7, 9). While these proteases play a pivotal role in maintaining cell physiology by clearing up abnormal proteins (4), they can potentially degrade recombinant proteins (6). Nevertheless, the mechanisms associated with protein degradation and protein substrate specificity are incompletely understood.

Since the proteolysis of recombinant proteins is a common issue, approaches to overcome this limitation have been extensively explored. These include using protease-deficient mutants as an expression host (10, 18), lowering the cultivation temperature (3), coexpressing folding factors (19, 20), applying protein fusion technology (2, 8), eliminating protease cleavage sites (5, 15), and modifying target protein hydrophobicity (11). While genetic elimination of proteolytically sensitive sequences without impacting the target protein's bioactivity appears to be technically attractive, there is no effective prediction or identification of potential protease cleavage sites. Proteolytically sensitive sequences might not be specific to certain proteases. Various factors affecting the folding, solubility, steric hindrance, and binding of the substrate protein, e.g., neighboring domains or amino acids, can contribute to the sensitivity of proteolytic sites, making sequence manipulation for proteolysis alleviation more difficult.

Herein, the recombinant protein's sensitivity toward intracellular proteolysis was genetically alleviated to enhance the functional expression of PalB, an industrial enzyme with justified applications (1), in *E. coli* and to understand the relationship between proteolytic specificity and substrate protein sequence. Since there are three intramolecular disulfide bonds whose formation can potentially affect PalB structure and bioactivity, PalB expression in the oxidative periplasm of *E. coli* was explored (20, 22). However, the expressed PalB protein was extremely sensitive to intracellular proteolysis, resulting in complete degradation. To overcome this problem, PalB mutant variants that were stable against proteolysis were derived (Table 1).

The results for functional expression of various PalB variants are summarized in Table 2 and Fig. 1. Neither PalB activity nor PalB-related polypeptide was detectable for the BL21(DE3) (pETG) culture sample, suggesting that the expressed gene product was degraded. Error-prone PCR (ep-PCR) was conducted using pETG as the template and LB-P10/LB-P11 as the primers. Potential mutants were screened on tributyrin agar plates. A colony developed a large halo, and the harboring plasmid (i.e., pETGM-2) was purified for sequencing. Two mutations, Leu149Val and Val223Ile, were identified in the expressed PalB mutant (i.e., M-2). High PalB activity was obtained for the BL21(DE3) (pETGM-2) culture sample. However, a PalB-related polypeptide was detected in both the soluble and insoluble fractions, implying potential protein misfolding. It was previously reported that the functional expression of PalB could be limited by disulfide bond formation associated with folding (21). It was intriguing to observe the structural effect of these two simple mutations on improving PalB stabilization and functional expression.

Site-directed mutagenesis was conducted to introduce various single mutations of Leu149 and Val223 for a further understanding of the structural effect. Note that only hydrophobic amino acid residues were selected for the replacement to avoid any major structural disturbances. Among the five single mutations on Leu149, only Leu149Val and Leu149Ile were associated with a slight improvement in functional expression, and Leu149Val was more effective than Leu149Ile, whereas the others behaved similarly to the wild type. Among the five single mutations at Val223, only Val223Phe, Val223Gly, and Val223Ile showed a slight improvement in functional expression, with Val223Ile as the most effective one, whereas the others showed no improvement. The results suggest that Leu149 and Val223 are two amino acids critically affecting the proteolytic susceptibility of PalB in *E. coli*, with a synergistic effect on PalB stabilization from Leu149Val and Val223Ile mutations.

Several amino acids were identified as being involved in the

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TABLE 1. Strains, plasmids, and oligonucleotides

| Strain, plasmid, or oligonucleotide | Relevant genotype or DNA sequence[a] |
|---|---|
| **Strain** | |
| BL21(DE3) | $F^-$ *ompT dcm lon hsdS*$_B$ ($r_B^-$ $m_B^-$) *gal* λ(DE3[*lacI ind1 sam*7 *nin*5 *lacUV5-T7* gene 1]) |
| **Plasmids** | |
| pARDsbA (20) | $P_{araB}$::*dsbA*, Ori (pACYC184), $Cm^r$ |
| pARDsbC (20) | $P_{araB}$::*dsbA*, Ori (pACYC184), $Cm^r$ |
| pETG (20) | $P_{T7}$*pelB*::*palB*, Ori (pBR322), $Ap^r$ |
| pETGM-2 | pETG derivative containing two mutations of Leu149Val and Val223Ile in *palB* (by ep-PCR with pETG and LB-P10/LB-P11) |
| pETGM-3 | pETG derivative containing mutation of Leu149Gly in *palB* (by SDM[b] with pETG and P104/P114) |
| pETGM-4 | pETG derivative containing mutation of Leu149Val in *palB* (by SDM with pETG and P103/P113) |
| pETGM-5 | pETG derivative containing mutation of Leu149Ala in *palB* (by SDM with pETG and P105/P115) |
| pETGM-6 | pETG derivative containing mutation of Leu149Met in *palB* (by SDM with pETG and P106/P116) |
| pETGM-7 | pETG derivative containing mutation of Leu149Ile in *palB* (by SDM with pETG and P107/P117) |
| pETGM-8 | pETG derivative containing mutation of Val223Phe in *palB* (by SDM with pETG and P112/P122) |
| pETGM-9 | pETG derivative containing mutation of Val223Met in *palB* (by SDM with pETG and P111/P121) |
| pETGM-10 | pETG derivative containing mutation of Val223Ala in *palB* (by SDM with pETG and P110/P120) |
| pETGM-11 | pETG derivative containing mutation of Val223Gly in *palB* (by SDM with pETG and P109/P119) |
| pETGM-12 | pETG derivative containing mutation of Val223Ile in *palB* (by SDM with pETGM-2 and P136/P137, i.e., by reverting the mutation of Val149 to Leu in pETGM-2) |
| **Oligonucleotides** | |
| LB-P10 | 5′-GG*CCATGG*GTCTACCTTCCGGTTCGG-3′ |
| LB-P11 | 5′-*CTGAATTC*TCAGGGGGTGACGATGCCGGAGCAGG-3′ |
| P103 | 5′-CCTCTCGATGCA***GTC***GCGGTTAGTGCACCC-3′ |
| P113 | 5′-GGGTGCACTAACCGC***GAC***TGCATCGAGAGG-3′ |
| P104 | 5′-CCTCTCGATGCA***GGC***GCCGTTAGTGCACCC-3′ |
| P114 | 5′-GGGTGCACTAAC*GGC**GCC***TGCATCGAGAGG-3′ |
| P105 | 5′-CCTCTCGATGCA***GCCGCGG***TTAGTGCACCC-3′ |
| P115 | 5′-GGGTGCACTAA*CCGC**GGC***TGCATCGAGAGG-3′ |
| P106 | 5′-CCTCTCGATG*CC**A**TGG*CGGTTAGTGCACCC-3′ |
| P116 | 5′-GGGTGCACTAACCG*CC**A**TG*GCATCGAGAGG-3′ |
| P107 | 5′-CCTCTCGATG*CG**A**TC*GCGGTTAGTGCACCC-3′ |
| P117 | 5′-GGGTGCACTAACCG*CGATC*GCATCGAGAGG-3′ |
| P109 | 5′-GGGCCGCTGTTC***GGG***ATCGACCATGCAGGC-3′ |
| P119 | 5′-GCCTGCATGGTCGAT***CCC***GAACAGCGGCCC-3′ |
| P110 | 5′-GGGCCGCTGTTC***GCG***ATCGACCATGCAGGC-3′ |
| P120 | 5′-GCCTGCATGGT*CGAT**CG**C*GAACAGCGGCCC-3′ |
| P111 | 5′-GGGCCGCTGTTC***A*TG***ATCGACCATGCAGGC-3′ |
| P121 | 5′-GCCTGCATGGTCGAT***CA*T***GAACAGCGGCCC-3′ |
| P112 | 5′-GGGCCGCTGTTC***TTT***ATCGACCATGCAGGC-3′ |
| P122 | 5′-GCCTGCATGGTCGAT***AAA***GAACAGCGGCCC-3′ |
| P136 | 5′-CCTCTCGATGCA***CTG***GCGGTTAGTGCACCC-3′ |
| P137 | 5′-GGGTGCACTAACCGC***CAG***TGCATCGAGAGG-3′ |

[a] For oligonucleotides, italic indicates mutated nucleotides, underlining indicates restriction site (silent mutation), and bold indicates a mutation codon.
[b] SDM, site-directed mutagenesis.

catalytic mechanism (17), such as Ser107-Asp189-His226, forming the catalytic triad, and Thr42 and Gly108, acting as the hydrogen bond donors in the oxyanion hole of the active site. However, Leu149 is located at the entrance of the partially formed lid of the protein structure, whereas Val223 is situated near one of the catalytic triad residues, His226, in the active site. The substitutions at Leu149 and/or Val223 with a PalB stabilization effect potentially convert the overall protein conformation into a less proteolytically sensitive form without changing the enzyme kinetics or activity. It was interesting to observe a similarly positive effect upon replacing Val223 with either a bulky Phe or a small Gly, implying that the molecular size of this amino acid residue was not critical for alleviation of the proteolytic sensitivity. Note that PalB-related polypeptide was present in the insoluble fraction for all the PalB mutants with improved functional expression, suggesting another expression hurdle of protein misfolding.

To overcome the protein misfolding, coexpression of two periplasmic folding factors, DsbA and DsbC, was explored, and the cultivation results are summarized in Fig. 2. DsbA coexpression significantly boosted functional expression, with specific PalB activity of more than twofold that of the control culture and a slight reduction of insoluble PalB. However, the improving effect associated with DsbC coexpression was minimal. The results suggest that the initiation but not isomerization for disulfide bond formation in the periplasm became limiting. The chaperone activity associated with DsbA could assist the folding of the expressed PalB double mutant.

While many studies based on rational mutagenesis, directed evolution, and gene shuffling are conducted to derive PalB mutants with improved enzyme properties, such as thermostability, bioactivity, or enantioselectivity (12–14, 16), this is an approach to improve protein manufacturing by deriving the variants with less proteolytic susceptibility. Though it has been

TABLE 2. Cultivation performance for production of PalB mutant variants[a]

| Plasmid | Cell density ($OD_{600}$)[b] | | Activity (U/liter/$OD_{600}$)[c] | Mutation in PalB |
|---|---|---|---|---|
| | w/o IPTG | w/IPTG | | |
| pETG | 3.1 ± 0.0 | 3.3 ± 0.1 | ND | None |
| pETGM-2 | 3.4 ± 0.1 | 3.3 ± 0.0 | 155 | Leu149Val, Val223Ile |
| pETGM-3 | 3.2 ± 0.1 | 3.3 ± 0.0 | ND | Leu149Gly |
| pETGM-4 | 3.4 ± 0.1 | 3.6 ± 0.1 | 60 | Leu149Val |
| pETGM-5 | 3.3 ± 0.1 | 3.5 ± 0.1 | ND | Leu149Ala |
| pETGM-6 | 2.7 ± 0.1 | 3.0 ± 0.1 | ND | Leu149Met |
| pETGM-7 | 3.5 ± 0.0 | 3.2 ± 0.0 | 38 | Leu149Ile |
| pETGM-8 | 3.0 ± 0.0 | 3.6 ± 0.0 | 50 | Val223Phe |
| pETGM-9 | 2.9 ± 0.1 | 2.8 ± 0.0 | ND | Val223Met |
| pETGM-10 | 3.4 ± 0.1 | 3.3 ± 0.1 | ND | Val223Ala |
| pETGM-11 | 3.5 ± 0.0 | 3.5 ± 0.1 | 30 | Val223Gly |
| pETGM-12 | 3.6 ± 0.1 | 3.5 ± 0.1 | 57 | Val223Ile |

[a] BL21(DE3) was used as an expression host to harbor various plasmids. PalB enzyme assay was conducted at 37°C and pH 8.0 using a pH stat with olive oil as a substrate. One unit of enzyme activity is defined as the amount of enzyme required to liberate one μmole of fatty acid per min.

[b] $OD_{600}$, optical density at 600 nm; w/o and w/IPTG, without and with isopropyl-β-D-thiogalactopyranoside, respectively.

[c] These activities represent IPTG-induced cultures. No activity was detected for all control cultures without IPTG induction. ND, not detected.

perceived that the proteolytic specificity can be intrinsically determined by the protein substrate sequence, experimental demonstration of this is rarely reported. This study complements the lack of such experimental demonstration by showing the proteolytic specificity and sensitivity of PalB heterologously expressed in *E. coli* can be drastically altered by simple amino acid substitutions. It also demonstrates the application of molecular manipulation to enhance recombinant protein production in *E. coli*.

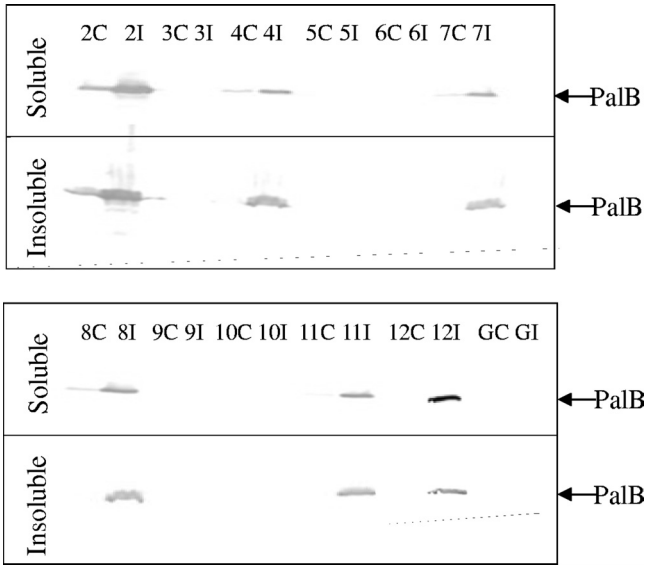


FIG. 1. Western blotting analysis of the culture samples for the expression of various PalB mutants. Both soluble and insoluble fractions are shown. The number ($n$ = 2 to 12) represents the PalB mutant (M-$n$) with the use of BL21(DE3) harboring the expression plasmid pETGM-$n$, summarized in Tables 1 and 2. "G" represents the control experiment using BL21(DE3) harboring pETG. "C" and "I" represent the cultures without and with IPTG induction, respectively.

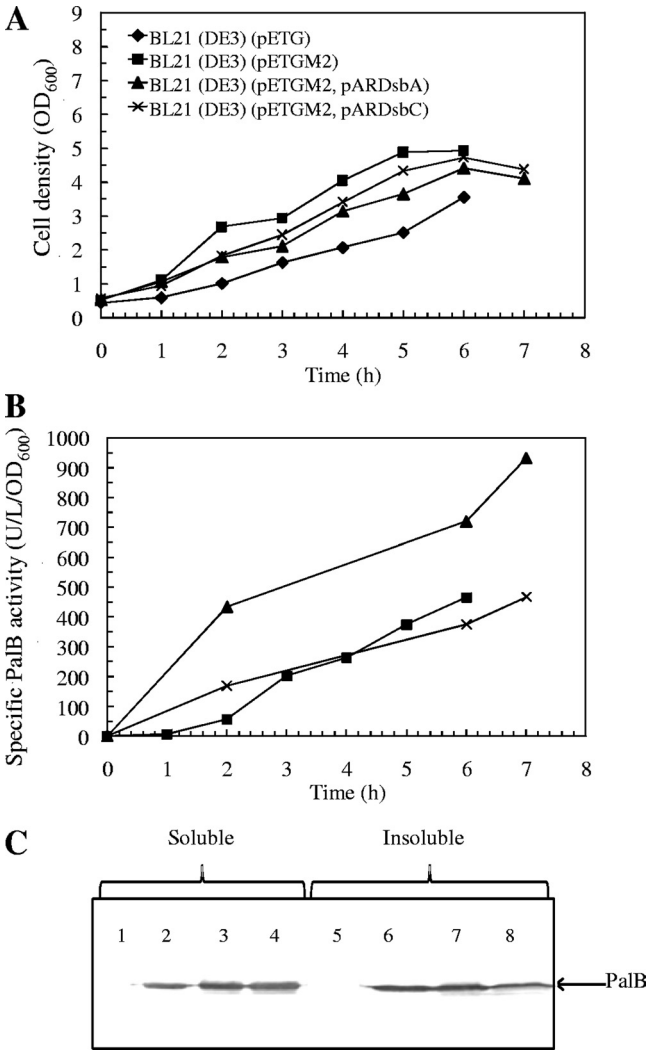


FIG. 2. Effect of DsbA or DsbC coexpression on the functional expression of the PalB double mutant. Cultivations were performed in a bioreactor containing 1 liter LB medium and operated at pH 7.0, 28°C, and 650 rpm. The cultures were induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside and/or 0.2 g/liter arabinose at a ~0.5 optical density at 600 nm ($OD_{600}$) cell density. (A) Time profiles of cell density; (B) time profiles of the specific PalB activity [note that there was no detectable activity for BL21(DE3) (pETG) culture samples]; (C) Western blotting analysis of the final samples of the four cultures. Both soluble and insoluble fractions are shown. Lanes 1 and 5, BL21(DE3) (pETG); lanes 2 and 6, BL21(DE3) (pETGM-2); lane 3 and 7, BL21(DE3) (pETGM-2, pARDsbC); lanes 4 and 8, BL21(DE3) (pETGM-2, pARDsbA).

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