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# Engineering of a Microbial Cell Factory for the Extracellular Production of Catalytically Active Phospholipase A$_2$ of *Streptomyces violaceoruber*

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Phospholipase A$_2$ (PLA$_2$) from *Streptomyces violaceoruber* is a lipolytic enzyme used in a wide range of industrial applications including production of lysolecithins and enzymatic degumming of edible oils. We have therefore investigated expression and secretion of PLA$_2$ in two workhorse microbes, *Pichia pastoris* and *Escherichia coli*. The PLA$_2$ was produced to an activity of 0.517 ± 0.012 U/ml in the culture broth of the recombinant *P. pastoris*. On the other hand, recombinant *E. coli* BL21 star (DE3), overexpressing the authentic PLA$_2$ (P-PLA$_2$), showed activity of 17.0 ± 1.3 U/ml in the intracellular fraction and 21.7 ± 0.7 U/ml in the culture broth. The extracellular PLA$_2$ activity obtained with the recombinant *E. coli* system was 3.2-fold higher than the corresponding value reached in a previous study, which employed recombinant *E. coli* BL21 (DE3) overexpressing codon-optimized PLA$_2$. Finally, we observed that the extracellular PLA$_2$ from the recombinant *E. coli* P-PLA$_2$ culture was able to hydrolyze 31.1 g/l of crude soybean lecithin, an industrial substrate, to a conversion yield of approximately 95%. The newly developed *E. coli*-based PLA$_2$ expression system led to extracellular production of PLA$_2$ to a productivity of 678 U/l·h, corresponding to 157-fold higher than that obtained with the *P. pastoris*-based system. This study will contribute to the extracellular production of a catalytically active PLA$_2$.

**Keywords:** Phospholipase A$_2$, *Pichia pastoris*, *Escherichia coli*, extracellular production

## Introduction

Phospholipase A$_2$ (PLA$_2$, EC 3.1.1.4) hydrolyzes the ester bond in the sn-2 position of phospholipids, producing free fatty acids and the corresponding lysophospholipids. In comparison with native lecithins, lysolecithins prepared by PLA$_2$ not only exhibit enhanced O/W emulsifying properties but also form stable emulsions under various process conditions [1]. Thus, lysolecithins are used in a wide range of industrial applications such as food, cosmetics, and pharmaceuticals [2]. In particular, PLA$_2$ can be used extensively for enzymatic degumming, a key process in the refining of vegetable and other edible oils [3].

Previous studies have been mainly focused on expression and characterization of eukaryotic secretory PLA$_2$s. While eukaryotic PLA$_2$s have been successfully expressed in yeast [4-7] and fungus [8], inclusion bodies were formed when expressed in *Escherichia coli* due to the presence of five to eight disulfide bonds [9, 10]. Nevertheless, it would be desirable to establish an *E. coli*-based PLA$_2$ expression system because eukaryotic systems are generally considered as time consuming and uneconomic in comparison to prokaryotic systems. For this reason, several research groups developed expression systems for soluble expression of the eukaryotic PLA$_2$ in *E. coli* using maltose-binding protein (MBP) [11], thioredoxin [12], and protein disulfide bond isomerase (DsbC) [13] as fusion partners. These expression systems, however, require additional steps to eliminate fusion partners and hence are not economic for practical use [14].

It has been relatively easy to express prokaryotic PLA$_2$ in *E. coli* because it has only two or zero disulfide bonds [15]. The first PLA$_2$ identified in prokaryotes was from *Streptomyces violaceoruber* A-2688, a soil bacterium [16]. It is a small protein with molecular weight of 14 kDa containing two disulfide bonds and requires Ca$^{2+}$ for catalytic activity. The PLA$_2$ from *S. violaceoruber* was successfully produced extracellularly by *P. pastoris* [17] and *E. coli* [18]. The PLA$_2$ expressed in *P. pastoris*, however, contains a part of its signal sequence at the N-terminal end of mature PLA$_2$ protein, which might alter properties of the authentic PLA$_2$.

In this study, expression and secretion levels of the authentic PLA$_2$ in *P. pastoris* and *E. coli* were compared. Since the amount of extracellular PLA$_2$ produced in *E. coli* was 8.4 times higher than that in *P. pastoris*, we sought to develop an efficient PLA$_2$ expression system in *E. coli*. To do so, effects of the following factors on extracellular

production of PLA$_2$ were systematically investigated: (1) codon optimization, (2) various host strains, and (3) attachment of aspartate tags.

## Materials and Methods

### Strains and Plasmids

*E. coli* TOP10 strain was used for genetic manipulation, and *P. pastoris* X-33, *E. coli* BL21 star (DE3), Origami 2 (DE3), BL21 (DE3), BL21 RIL (DE3), C41 (DE3), and C43 (DE3) strains were used for PLA$_2$ production. For expression of PLA$_2$ in *P. pastoris*, codon-optimized PLA$_2$ gene was cloned behind the *AOX1* promoter in plasmid pPICZαA and their transcription was induced by adding methanol. Codon optimization was carried out by using the program (https://zendto.bioneer.co.kr/codon/index.py) provided by Bioneer (Korea). For expression of PLA$_2$ in *E. coli*, the natural and codon-optimized PLA$_2$ genes were located behind the *T7* promoter in plasmid pET-26b(+) and their transcription was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG). The natural and codon-optimized PLA$_2$ genes were synthesized by Bioneer. Strains and plasmids used in this study are listed in Table 1.

### Genetic Manipulation

The natural PLA$_2$ or codon-optimized PLA$_2$ genes without native signal sequence (Fig. S1) were PCR amplified with primers of HL01 (with *Msc*I site) and HL02 (with *Xho*I site) or HL03 (with *Msc*I site) and HL04 (with *Xho*I

**Table 1. Strains and plasmids used in this study.**

| Name | Description | Reference |
|---|---|---|
| *E. coli* | | |
| *E. coli* TOP10 | F⁻ *mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK rpsL*(Str$^R$) *endA1 nupG* | Invitrogen |
| *E. coli* BL21 (DE3) | F⁻ *ompT hsd*S (rB- mB-) *dcm gal* (DE3 [*lacI lac*UV5-T7 gene 1 *ind*1 *sam*7 *nin*5]) | Novagen (Darmstadt, Germany) |
| *E. coli* BL21 star (DE3) | BL21 *rne131* (DE3) | Invitrogen |
| *E. coli* BL21 CodonPlus-RIL (DE3) | BL21 (DE3) *dcm*⁺ Tet$^R$ *endA* Hte [*argU ileY leuW* Cam$_R$] | Agilent technologies |
| *E. coli* C41 (DE3) | BL21 (DE3 *[lacI lac-T7 gene 1 ind1 sam7 nin5]*) | Lucigen (Middleton, WI, USA) |
| *E. coli* C43 (DE3) | C41 (DE3) derivative | Lucigen |
| *E. coli* Origami 2 (DE3) | *Δara–leu7697 ΔlacX74 ΔphoAPvuII phoR araD139 ahpC galE galK rpsL* F′*[lac⁺(lacI�q)pro] gor522::Tn10trxB* (Str$^R$, Tet$^R$) (DE3) | Novagen |
| SK33 | BL21 star (DE3) containing pET-26b(+) | This study |
| SK31 | BL21 star (DE3) containing pHLK01 | This study |
| SK32 | BL21 star (DE3) containing pHLK02 | This study |
| SK35 | Origami 2 (DE3) containing pHLK01 | This study |
| SK36 | BL21 (DE3) containing pHLK01 | This study |
| SK37 | BL21 RIL (DE3) containing pHLK01 | This study |
| SK38 | C41 (DE3) containing pHLK01 | This study |
| SK39 | C43 (DE3) containing pHLK01 | This study |
| SK54 | BL21 star (DE3) containing pHLK03 | This study |
| SK55 | BL21 star (DE3) containing pHLK04 | This study |
| SK56 | BL21 star(DE3) containing pHLK05 | This study |
| SK57 | BL21 star(DE3) containing pHLK06 | This study |
| SK87 | BL21 star(DE3) containing pSHK01 | This study |
| *P. pastoris* | | |
| *P. pastoris* X-33 | Wild type | Invitrogen |
| PX | X-33 containing pPICZαA | This study |
| PP | X-33 containing pMFα-PLA$_2$ | This study |
| Plasmids | | |
| pET-26b(+) | pBR322 origin, *T7* promoter, PelB signal sequence, His-tag, Kan$^R$ | Novagen |
| pHLK01 | Expression vector containing P-PLA$_2$, Kan$^R$ | This study |
| pHLK02 | Expression vector containing P-Opt. PLA$_2$, Kan$^R$ | This study |
| pHLK03 | Expression vector containing P-D3-PLA$_2$, Kan$^R$ | This study |
| pHLK04 | Expression vector containing P-D5-PLA$_2$, Kan$^R$ | This study |
| pHLK05 | Expression vector containing P-D7-PLA$_2$, Kan$^R$ | This study |
| pHLK06 | Expression vector containing P-D9-PLA$_2$, Kan$^R$ | This study |
| pSHK01 | Expression vector containing PLA$_2$, Kan$^R$ | This study |
| pPICZαA | pUC origin, *AOX1* promoter, MFα signal sequence, His-tag, Zeocin$^R$ | Invitrogen |
| pMFα-PLA$_2$ | Expression vector containing M-Opt. PLA$_2$, Zeocin$^R$ | This study |

site). After the gene amplification, PCR products were cut with *Msc*I and *Xho*I and then ligated with plasmid pET-26b(+) digested with the same enzymes to construct pHLK01 and pHLK02 (Table 1). To attach various lengths of aspartate residues at the N-terminal end of PLA$_2$, plasmid pHLK01 was amplified with the primer sets and then ligated after *Msc*I treatment. The primer sets used for amplification of the DNA fragments are as follows: HL07 (with *Msc*I site) and HL11 (with *Msc*I site) for pHLK03; HL08 (with *Msc*I site) and HL11 (with *Msc*I site) for pHLK04; HL09 (with *Msc*I site) and HL11 (with *Msc*I site) for pHLK05; HL10 (with *Msc*I site) and HL11 (with *Msc*I site) for pHLK06. Plasmid pSHK01 is identical to pHLK01 except that it does not contain the PelB signal sequence. To make this change, a DNA fragment without PelB signal sequence was amplified with primers SH05 (with *Nde*I site) and SH06 (with *Nde*I site) using pHLK01 as template. This linear DNA was digested with *Nde*I and ligated to construct pSHK01.

The codon-optimized PLA$_2$ gene for expression in *P. pastoris* was PCR-amplified with primers F_PLA$_2$ (with *Nhe*I site) and R_PLA$_2$ (with *Spe*I site). The plasmid pPICZαA was amplified with primers F_pPICZαA (with *Nhe*I site) and R_pPICZαA (with *Spe*I site). These two linear DNA fragments were digested with *Nhe*I and *Spe*I, and ligated to construct pMFα-PLA$_2$. Transformation of the cassette for overexpressing PLA$_2$ was performed using the *Pichia* EasyComp Kit (Invitrogen, USA). Plasmid pMFα-PLA$_2$ was cut with *Mss*I and transformed. Transformants were selected on YPDS medium (10 g/l yeast extract, 20 g/l peptone, 20 g/l glucose, and 182 g/l sorbitol) containing 100 μg/ml Zeocin. PCR amplification was done with primers (F_ch_AOX1p and R_ch_pPICZα) to verify positive transformants. All plasmids and the check PCR products were sequenced by automatic sequencing (Cosmogenetech, Korea). Names of recombinant PLA$_2$ gene products and schematic structures are shown in Fig. 1, and primers used for plasmid constructions and confirmations are listed in Table S1.

### Media and Culture Conditions

*P. pastoris* was pre-cultured in 100 ml BMGY medium (10 g/l yeast extract, 20 g/l peptone, 13.4 g/l yeast nitrogen base, $3 \times 10^{-4}$ g/l biotin, 10 g/l glycerol, and 100 mM potassium phosphate (pH 6.0)) at 30$^{\circ}$C and 200 rpm for 24 h. Pre-cultured cells were then inoculated into 100 ml BMMY medium containing 10 g/l yeast extract, 20 g/l peptone, 13.4 g/l yeast nitrogen base, $3 \times 10^{-4}$ g/l biotin, 5 or 10 g/l methanol, and 100 mM potassium phosphate (pH 6.0). Expression of PLA$_2$ was induced by adding methanol every 24 h at a final concentration of 5 or 10 g/l.

*E. coli* cells were pre-cultured in LB medium (5 g/l yeast extract and 10 g/l bacto-trypton) at 37$^{\circ}$C and 230 rpm for 12 h. After harvesting the cells, the cell pellets were used for inoculation. Batch fermentations were carried out in a 500 ml baffled flask containing 100 ml of Riesenberg medium [13.5 g/l KH$_2$PO$_4$, 4.0 g/l (NH$_4$)$_2$HPO$_4$, 1.7 g/l citric acid, 1.4 g/l MgSO$_4$·7H$_2$O, 10 ml/l trace element solution (10 g/l Fe(III) citrate, 2.25 g/l ZnSO$_4$·7H$_2$O, 1.0 g/l CuSO$_4$·5H$_2$O, 0.35 g/l MnSO$_4$·H$_2$O, 0.23 g/l Na$_2$B$_4$O$_7$·10H$_2$O, 0.11 g/l (NH$_4$)$_6$Mo$_7$O$_{24}$, 2.0 g/l CaCl$_2$·2H$_2$O), pH 6.8] with 20 g/l glucose. Agitation speed was maintained at 200 rpm. When OD$_{600}$ reached 0.8-1.2, 0.2 mM IPTG was added to the culture broth. After induction, cultivation was continued at 25$^{\circ}$C for an additional 24 h.

### Preparation of Protein

After IPTG induction of 24 h, the culture broth was centrifuged at 15,000 ×*g* for 10 min to collect the medium fraction. The remaining pellet was resuspended in B-PER$^{TM}$ reagent (Thermo Fisher Scientific, USA) and lysed as specified by the manufacturer. The total, soluble, and insoluble fractions of intracellular proteins were prepared as described in the previous report [19].
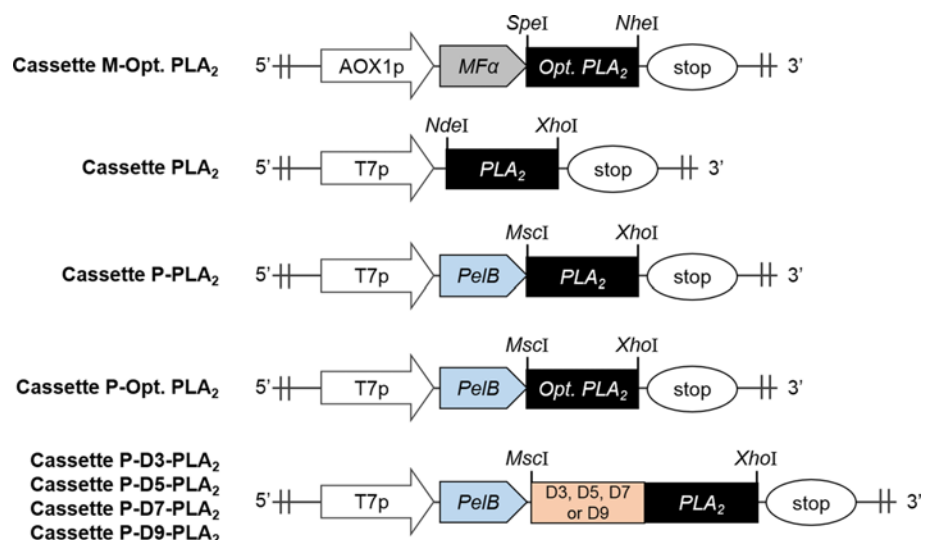


**Fig. 1. Schematic diagrams of the structures of recombinant PLA$_2$ expression cassettes.** Symbols: *AOX1* promoter (AOX1p), *T7* promoter (T7p), translational stop codon (stop), and the genes coding for the signal sequence of *S. cerevisiae* mating factor α (*MFα*), the signal sequence of pectate lyase B from *Erwinia carotovora* (*PelB*), *S. violaceoruber* phospholipase A$_2$ (*PLA$_2$*), codon-optimized PLA$_2$ (*Opt. PLA$_2$*), 3 aspartates (D3), 5 aspartates (D5), 7 aspartates (D7), and 9 aspartates (D9).

### Protein Purification

A 20 ml-scale column containing 750 μl of Ni-NTA agarose (QIAGEN, Germany) was washed with 20 ml of the His-tag binding buffer (pH 7.4) containing 20 mM NaH$_2$PO$_4$, 20 mM Na$_2$HPO$_4$, 0.5 M NaCl, and 40 mM imidazole. After 100 ml of the medium fraction prepared as described above was mixed with 300 ml of the His-tag binding buffer, the prepared mixture was loaded into the column. The proteins eluted from the column were collected, and protein concentration was determined using the Bio-Rad protein assay kit with bovine serum albumin (BSA) as the standard.

### Analysis of Protein Expression and Enzyme Assay

To visualize recombinant PLA$_2$s, the protein samples were electrophoresed in 12% sodium dodecyl sulfate–polyacrylamide gel, and were either stained using Coomassie blue or were transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; EMD Millipore, USA). The membrane was then probed with anti-6X His tag antibody (abcam, UK). After incubating the membrane with goat F(ab')2 Anti-mouse IgG (abcam) conjugated to alkaline phosphatase, the blot was developed using the BCIP/NBT chromogenic substrate solution (SurModics, Eden Prairie, USA) as specified by the manufacturer. The quantification of band intensity was carried out using the densitometry software (Total Lab 1.01; Nonlinear Dynamics Ltd.).

PLA$_2$ activity was measured using the sPLA$_2$ Assay Kit (Cayman Chemical, USA) according to the manufacturer's instructions. The absorbance change at 37°C and 414 nm of wavelength was monitored by a spectrophotometer (OPTIZEN POP, Mecasys, Korea) after addition of enzyme solution. One unit (U) of PLA$_2$ activity was defined as the amount of PLA$_2$ able to hydrolyze 1 μmol of diheptanoyl thio-phosphatidylcholine in one minute.

### Biotransformation of Soybean Lecithin

The 10 ml of reaction mixture was formulated with 0.5 mM Tris-HCl, 6 mM CaCl$_2$, 31.1 g/l crude soybean lecithin (Sigma-Aldrich, catalog number P3644) (pH 8.0), and 10% (v/v) of enzyme solution. The reaction conditions of 37°C and 400 rpm were maintained using a stirring heating mantle (LKLAB KOREA, Korea). According to the manufacturer's information, soybean lecithin consists of an average of 55% (42–63%) L-α-phosphatidylcholine and 20% (10-32%) phosphatidylethanolamine. Concentrations of fatty acids were determined by gas chromatography/mass spectrophotometry (GC/MS), as previously reported [20-22]. Fatty acids present in 500 μl of samples were extracted by mixing with 2 ml of isopropyl alcohol, 500 μl of heptane, and 50 μl of sulfuric acid. For the derivatization, 25 μl of N-methyl-N-(trimethylsilyl)trifluoroacetamide (TMS) (TCI Chemicals, Tokyo, Japan) dissolved in 75 μl of pyridine was added to 60 μl of sample solution containing lauric acid (TCI Chemicals) as the internal standard. Concentrations of TMS derivatives were determined using GC/MS (Agilent Technologies, USA) equipped with a flame ionization detector, split injection system, and nonpolar capillary column (30 m length, 0.25 mm film thickness, HP-5MS, Agilent Technologies). Column temperature was controlled by the following gradient program: 235°C for 3 min; increase at a rate of 25°C/min; 270°C for 10 min; increase at a rate of 5°C/min; 300°C for 1 min. Mass spectra and scan spectra were obtained by electron impact ionization at 70 eV and within the range of 100–600 m/z, respectively. Selected ion monitoring was used for the detection and fragmentation analysis of the reaction products.

## Results and Discussion

### Production of PLA$_2$ in *P. pastoris* X-33

To construct expression plasmid for the PLA$_2$ from *S. violaceoruber* in *P. pastoris* X-33, the codon-optimized PLA$_2$ gene without its native signal sequence was cloned into pMFα-PLA$_2$ vector under the transcriptional control of the alcohol oxidase 1 (*AOX1*) gene promoter [23] containing a signal sequence of *S. cerevisiae* mating factor α (MFα) [24]. A schematic diagram for the PLA$_2$ expression cassette in pPICZαA plasmid and its name are displayed in Fig. 1. We noted that recombinant PLA$_2$ expressed previously in *P. pastoris* (PLA$_2$-Pp) was designed to contain a part of its native signal sequences (Ala-Pro-Pro-Gln-Ala) [17] whereas these five amino acids are not present in the authentic mature PLA$_2$ and recombinant PLA$_2$ produced in other previous studies (PLA$_2$-Ec), which employed *E. coli* as a host strain (Fig. S2) [16, 18, 25]. In addition to the presence of native signal sequences, amino acid sequences of the PLA$_2$-Pp were not identical (Fig. S2) to those of the PLA$_2$-Ec because these two PLA$_2$s were originated from different *S. violaceoruber* sources: the PLA$_2$-Pp was from *S. violaceoruber* 2917 whereas the PLA$_2$-Ec was from *S. violaceoruber* A-2688. The presence of additional five amino acids at the N-terminal end of PLA$_2$-Pp resulted in a lower optimum pH of 6.0 [17] compared to the PLA$_2$-Ec, which has optimum pH of 7.3–8.3. In addition to optimum pH, this factor might alter the expression level and some properties of the enzyme as reported for lipase B from *Candida antarctica* (CalB) [26]. Therefore, the PLA$_2$-Ec, which does not have its native signal peptide, was used in this study for accurate comparison of PLA$_2$ production in *P. pastoris* and *E. coli*.

As expected, growth of the control strain containing the empty plasmid (pPICZαA) and the *P. pastoris* X-33 harboring pMFα-PLA$_2$ was virtually identical regardless of methanol concentrations (Fig. 2A), indicating that expression of PLA$_2$ in *P. pastoris* had no obvious detrimental effect on growth in general. A batch fermentation of the *P. pastoris* X-33 harboring pMFα-PLA$_2$ with intermittent addition of 1.0% methanol led to an extracellular production of PLA$_2$-Ec to an activity of 0.517 ± 0.012 U/ml in 120 h (Fig. 2B) (see the Materials and Methods for the activity assay). This value is much lower than the corresponding value (34.7 U/ml) obtained by a batch fermentation of *P. pastoris* overexpressing the PLA$_2$-Pp [17]. This is likely due to the difference of PLA$_2$ sequences and activity assay methods. While the extracellular PLA$_2$ activity with addition of 0.5% methanol was similar to that with 1.0% methanol, addition of 1.0% methanol shortened overall fermentation time from 144 h to 120 h.
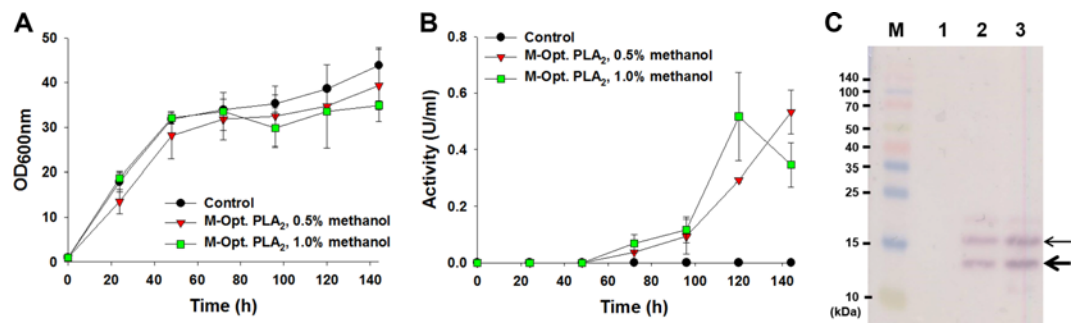
**Fig. 2. Batch production of PLA$_2$ in recombinant *P. pastoris*.** (**A** and **B**) growth curves (**A**) and extracellular PLA$_2$ activities (**B**) of *P. pastoris* X-33 harboring pPICZαA (Control) and pMFα-PLA$_2$. Batch production of PLA$_2$ was induced in duplicate by adding 0.5% (v/w) or 1.0% methanol every 24 h. The activities of crude PLA$_2$s in the extracellular fraction were measured in triplicate using diheptanoyl thio-phosphatidylcholine as a substrate. (**C**) Western blotting for His-tagged PLA$_2$ from the extracellular fraction of the recombinant *P. pastoris* strains. Lanes: M, prestained SDS-PAGE standards; 1, the control strain; 2, *P. pastoris* X-33 harboring pMFα-PLA$_2$ induced with 0.5% methanol; 2, *P. pastoris* X-33 harboring pMFα-PLA$_2$ induced with 1.0% methanol. The thin and thick arrows point to the protein bands of PLA$_2$ with and without glycosylation, respectively.

Although PLA$_2$ was difficult to identify using Coomassie blue staining, it was clearly detected by western hybridization analysis using monoclonal anti-His antibodies (Fig. 2C). A band corresponding to the 14 kDa predicted molecular mass of PLA$_2$ was visible in both 0.5% and 1.0% methanol induction conditions. A protein band of approximate molecular mass of 16 kDa was also detected (Fig. 2B), and we speculated that this protein band corresponds to glycosylated PLA$_2$. This result is consistent with a previous study showing that a part of PLA$_2$ expressed in *P. pastoris* was glycosylated as it has three putative glycosylation sites [17].

**Production of PLA$_2$ in Various *E. coli* Strains**

The authentic PLA$_2$ gene contains several rare codons for *E. coli* including Leu (CTC). This codon bias problem could be solved by codon optimization of the gene or by supplying rare-codon tRNAs. Here, we investigated effects of codon optimization of PLA$_2$ gene on its expression and secretion. The PLA$_2$ gene expression system was constructed with and without the PelB signal sequence (Fig. 1), which is involved in targeting the proteins to the periplasmic space [27, 28]. As expected, the PLA$_2$ without the signal sequence showed a basal level of lipase activity in both intracellular and extracellular fractions (Fig. 3A). On the other hand, the lipase activities increased up to $9.4 \pm 1.5$ U/ml in the intracellular fraction and $16.1 \pm 0.9$ U/ml in the culture broth of the recombinant *E. coli* BL21 star (DE3) overexpressing the authentic PLA$_2$ gene (P-PLA$_2$). This is 6.3- and 4.8-times higher than the corresponding values of the case of codon-optimized PLA$_2$ (P-Opt. PLA$_2$) (Fig. 3A). In addition to the enzyme activity assay, SDS–PAGE analysis showed the high secretion of P-PLA$_2$ in the culture medium (Fig. S3). This study and earlier studies [29, 30] suggest that a faster expression from the optimized gene could lead to higher concentration of target protein, which in turn results in degradation and/or misfolding of the protein.

Protein expression in *E. coli* BL21 (DE3), BL21 RIL (DE3), BL21 star (DE3), Origami 2 (DE3), C41 (DE3), and C43 (DE3) were analyzed by SDS-PAGE to select a host for PLA$_2$ production. The expression level of PLA$_2$ was the highest in *E. coli* BL21 star (DE3) which has a mutation in the gene encoding RNaseE (*rne*131 mutation), indicating that protection of mRNAs from RNases plays an important role in PLA$_2$ expression (Fig. S4). Therefore, the highest activities in both intracellular and extracellular fractions were obtained for recombinant *E. coli* BL21 star (DE3) overexpressing P-PLA$_2$ (Fig. 3B). PLA$_2$ activities in culture broth of *E. coli* BL21 (DE3) and BL21 RIL
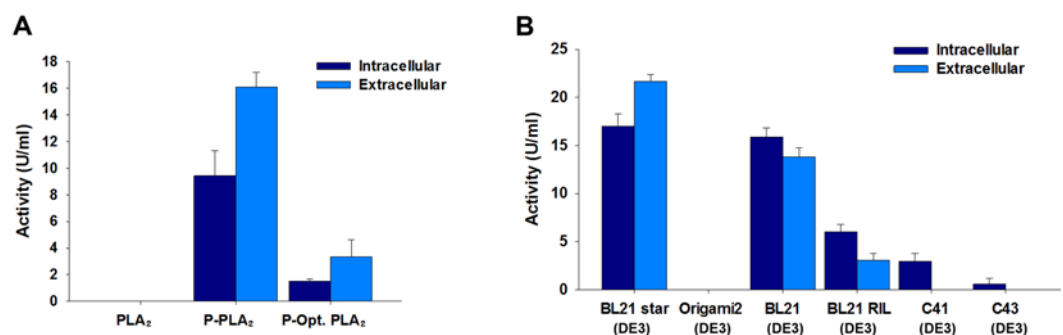


**Fig. 3. Effects of codon optimization (A) and *E. coli* host strains (B) on activities of recombinant PLA$_2$ in intracellular and extracellular fractions.** The activities of crude PLA$_2$s in the soluble and extracellular fractions (see the Materials and Methods for details) collected 24 h after IPTG induction were measured in triplicate using diheptanoyl thio-phosphatidylcholine as a substrate.
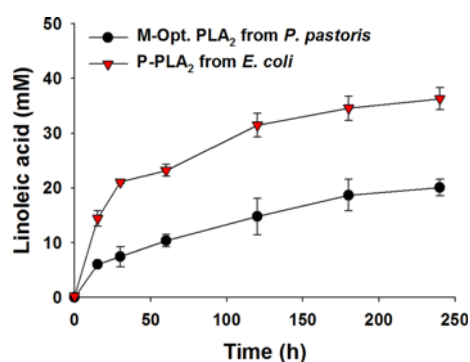
**Fig. 4. Biotransformation of crude soybean lecithin into linoleic acid and lysolecithin by recombinant PLA$_2$ collected from recombinant *P. pastoris* X-33 and *E. coli* BL21 star (DE3) overexpressing PLA$_2$.** Biotransformation was initiated by adding 10-fold concentrated extracellular crude enzyme solutions from the recombinant *P. pastoris* X-33 and *E. coli* BL21 star (DE3) to the reaction mixture consisting of 0.5 mM Tris-HCl, 6 mM CaCl$_2$, and 31.1 g/l crude soybean lecithin (pH 8.0). Results are the mean of triplicate experiments and error bars indicate standard deviations.

(DE3) exhibited 63.6 and 14.3% of extracellular activity in *E. coli* BL21 star (DE3). Thus, *E. coli* BL21 star (DE3) strain was chosen as the host of PLA$_2$ production.

We concluded from these data that alleviating codon bias by codon optimizing the PLA$_2$ gene or by supplying rare-codon tRNAs has negative effects on correct folding of PLA$_2$. This conclusion supports the hypothesis that the translation is a bottleneck in functional expression of PLA$_2$, and hence an overall delay in PLA$_2$ expression gives protein machineries more time to fold PLA$_2$ correctly.

**Hydrolysis of Soybean Lecithin by Extracellular PLA$_2$s from the Recombinant *E. coli* and *P. pastoris***

The extracellular PLA$_2$ activities of the recombinant *E. coli* P-PLA$_2$ and *P. pastoris* X-33 M-Opt. PLA$_2$ were examined by using an industrial substrate (*i.e.*, crude soybean lecithin). When the extracellular fraction of *P. pastoris* X-33 M-Opt. PLA$_2$ culture (shown in Fig. 2B) was added into the reaction medium containing 31.1 g/l of crude soybean lecithin (see the Materials and Methods for details), linoleic acid, which was the major fatty acid constituent of soybean lecithin, was produced to 20.1 mM at t = 240 min (Fig. 4). This indicated that approximately 70% of soybean lecithin was hydrolyzed into lysolecithin and linoleic acid. The extracellular fraction of *E. coli* P-PLA$_2$ culture displayed a biotransformation profile similar to that of *P. pastoris* X-33 M-Opt. PLA$_2$ culture (Fig. 4). Remarkably, linoleic acid was produced to 36.3 mM at t = 240 min (Fig. 4). This indicated that approximately 95% of soybean lecithin was converted into lysolecithin and linoleic acid. Moreover, the initial conversion rate was 2.6-fold greater than that of *P. pastoris* X-33 M-Opt. PLA$_2$ culture. Besides, the cultivation time of *E. coli* P-PLA$_2$ was significantly shorter than that of *P. pastoris* X-33 M-Opt. PLA$_2$ (32 h vs. 120 h). It was thereby assumed that the *E. coli*-based PLA$_2$ expression system would be superior to the *P. pastoris* system in terms of extracellular PLA$_2$ productivity.

**Effects of N-terminal Repeat of Aspartate Residues on Specific Activity and Expression of PLA$_2$**

We previously reported that fusion tag systems composed of the PelB signal sequence and repeated aspartate tags improved both expression and secretion of CalB and asparaginase isozyme II (AnsB) from *E. coli* [31, 32]. To investigate whether or not repeated aspartate residues would improve the secretion and activity of PLA$_2$, various lengths of aspartate residues were introduced into the N-terminal end of PLA$_2$ gene to construct the cassettes P-D3-PLA$_2$, P-D5-PLA$_2$, P-D7-PLA$_2$, and P-D9-PLA$_2$ as shown in Fig. 1. Crude PLA$_2$ enzymes present in the intracellular and extracellular fractions were subjected to SDS-PAGE (Fig. S5) and activity (Fig. 5A) analyses.
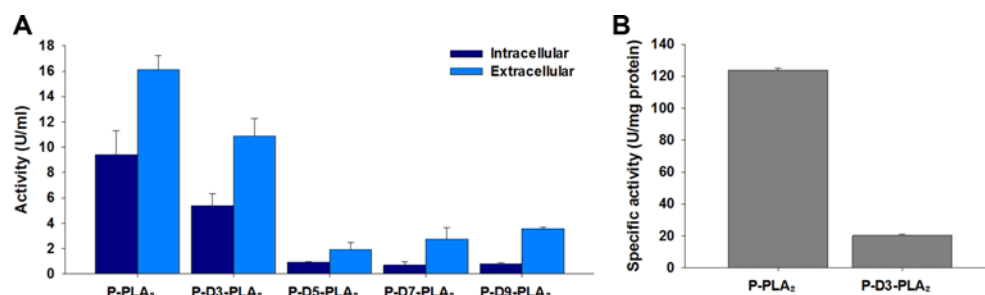


**Fig. 5. Activity assays of recombinant PLA$_2$s with various lengths of aspartate tags (A) and His-tag purified P-PLA$_2$ and P-D3-PLA$_2$ (B) to investigate the effects of aspartate tags on expression in *E. coli* and specific activity of PLA$_2$.** Results are the mean of triplicate experiments and error bars indicate standard deviations.

Among a series of repeated amino acids consisting of 3, 5, 7, or 9 aspartates, the three aspartates facilitated the secretion of $PLA_2$ and hence comparison of the band intensities from the extracellular fractions showed that the band corresponding to the P-D3-$PLA_2$ was 64% greater than P-$PLA_2$ (Fig. S5). However, the intracellular and extracellular lipase activities obtained for recombinant *E. coli* BL21 star (DE3) overexpressing P-D3-$PLA_2$ were instead 42.6% and 32.3% lower than the corresponding values obtained in the case of P-$PLA_2$ (Fig. 5A). These results suggested that the presence of three aspartate residues at the N-terminal end of $PLA_2$ might alter the specific activity of $PLA_2$. To confirm the hypothesis, $PLA_2$ and P-D3-$PLA_2$ were His-tag purified and subjected to activity assay. As expected, specific activity of P-D3-$PLA_2$ was 6.1 times lower than that of $PLA_2$ (Fig. 5B). This result is consistent with previous studies reporting that the attachment of repeated aspartates altered catalytic efficiency of CalB and $\alpha$-1,2-fucosyltransferase (FucT2) from *Helicobacter pylori* [31, 33]. More research is in progress to find $PLA_2$ from other bacteria with increased stability, of which specific activity is not affected significantly by the attachment of repeated aspartates.

In conclusion, this study demonstrated that an *E. coli*-based $PLA_2$ production system could be more efficient in terms of $PLA_2$ productivity, as compared to the *P. pastoris*-based system. Among the *E. coli* host strains harboring the authentic $PLA_2$ gene (P-$PLA_2$) or codon- optimized $PLA_2$ (P-Opt. $PLA_2$), the recombinant *E. coli* BL21 star (DE3) P-$PLA_2$ has exhibited the highest activities of $21.7 \pm 0.7$ U/ml in the culture broth and $17.0 \pm 1.3$ U/ml in the intracellular fraction. Moreover, the extracellular $PLA_2$s from the recombinant *E. coli* P-$PLA_2$ culture was able to hydrolyze 31.1 g/l of crude soybean lecithin to linoleic acid and lysolecithin at a conversion yield of at least 95%. Therefore, it was concluded that the recombinant *E. coli* P-$PLA_2$ system could be used as a microbial cell factory to produce a catalytically active $PLA_2$ for hydrolysis of the selective sn-2 position of plant lecithins.

## Acknowledgments

## Conflict of Interest

The authors have no financial conflicts of interest to declare.

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