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ORIGINAL ARTICLE

Biodegradation of Tolclofos-methyl by Extracellular Secreted Organophosphorus Hydrolase in Recombinant *Escherichia coli*

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Abstract Fungicide tolclofos-methyl [(O,O-dimethyl O-(2,6-dichloro-4-methylphenyl) phosphorothioate)], belonging to the group of organophosphorus compounds, has been usually applied for the effective control of soil born diseases by *Rhizoctonia solani*. However, its excessive usages have leaded to the accumulation of this compound on soil and ginseng itself. Therefore, we practiced the actual degradation of tolclofos-methyl using organophosphorus hydrolase (OPH). A gene encoding OPH from *Flavobacterium* sp. strain ATCC 27551 was cloned and expressed in *Escherichia coli* with or without signal peptide-encoding sequences under control of a T7 promoter. High-level expression of recombinant OPH was verified by specific OPH activity assay. In addition, although the extracellular secretion of OPH in *E. coli* has never been reported till now, secretion of the recombinant OPH was observed when a signal peptide of the gene was truncated. Recombinant *E. coli* strain removed a maximum of 80% of the organophosphorus compound tolclofos-methyl, as determined by an *in vitro* assay. The present study reports for the first time on the secretion of recombinant OPH not affected by signal peptide sequence in *E. coli* and biodegradation of tolclofos-methyl by extracellular secreted OPH from recombinant *E. coli.*

Keywords biodegradation · organophosphorus hydrolase · secretion · tolclofos-methyl

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Introduction

A soil-borne plant pathogen (*Rhizoctonia solani*) causing damping-off diseases in vegetable seedlings and tuber crops has been controlled by the fungicide tolclofos-methyl [(O,O-dimethyl O-(2,6-dichloro-4-methylphenyl) phosphorothioate], a member of the organophosphorus fungicides, by inhibiting spore germination or fungal mycelium growth (Tomlin, 1997; Yobo et al., 2010). Tolclofos-methyl has been widely used to control the root rot damping-off disease which occurs in ginseng growing fields in Korea. However, the accumulation of tolclofos-methyl in soil as well as inside the ginseng has raised the necessity of cleaning up the compound due to concerns regarding environmental pollution and the market value of ginseng (MAF and NACF, 2006).

Bioremediation describes environmental remediation techniques which employ the microbial capabilities to remove various pollutants, including man-made toxic chemicals, from nature. These methods have various advantages such as cost-effectiveness and environmentally friendly, compared to the removal methods that employ physical- and chemical treatments. Biodegradation of organophosphorus pesticides by organophosphorus hydrolase from soil bacteria could be a typical example of a bioremediation strategy for practical detoxification (Singh and Walker, 2006; Singh et al., 2008).

Organophosphorus hydrolase (OPH, also referred to as phosphotriesterase or parathion hydrolase; EC 3.1.8.1), is known to effectively hydrolyze organophosphorus compounds as well as various phosphorus-containing bonds such as P-O, P-CN, P-F, and P-S bonds. In *Flavobacterium* sp. strain ATCC 27551 and *Brevundimonas diminuta* (formerly *Pseudomonas diminuta* MG), the native OPH has been identified as a membrane-bound homodimeric metalloenzyme containing zinc ions and a highly conserved organophosphorus degradation (*opd*) gene in each indigenous plasmid (McDaniel et al., 1988; Mulbry and Karns,

1989; Gorla et al., 2009). Because most microorganisms producing OPH have been reported as Gram-negative bacteria, which release OPH into their cytoplasm (Singh and Walker, 2006), inefficient degradation rates of targeted organophosphorus xenobiotics were observed when the bacteria were directly applied for the bioremediation of organophosphorus compounds. This inefficiency was caused by cell membrane of the wild type strain, which could act as permeability barrier, thus limiting interaction between pesticide and OPH (Yang et al., 2010b).

Escherichia coli has been regarded as the most attractive host strain for the expression of recombinant proteins, because it grows rapidly and has easy applications based on the well-characterized genetic information (Miksch et al., 2005). However, despite many efforts to achieve the high yield production of OPH, *E. coli* has only been able to produce relatively low levels of recombinant OPH and was produced as inclusion bodies under the strong inducible promoters (Mulbry and Karns, 1989; Pandey et al., 2009). Moreover, although the expression of recombinant OPH was detected, the degradation by this enzyme was not efficient, because Gram-negative cell envelope of *E. coli* can act as another barrier to prevent contact between the expressed OPH and target pesticides (Chen and Mulchandani, 1998; Ni and Chen, 2004). To overcome these limitations of *E. coli* expression, various strategies, such as using a green fluorescent protein (GFP) fusion system (Cha et al., 2000; Wu et al., 2000; 2001), applying a cell surface display system using fusion with lipoprotein-outer membrane A (Lpp-OmpA), attaching an ice nucleation protein (INP) motif of *Pseudomonas syringae*, or using an auto-transporter secretion system (Richins et al., 1997; Shimazu et al., 2001; Kang et al., 2008; Li et al., 2008; Yang et al., 2008), have been applied for OPH expression. A periplasmic secretion method using recombinant OPH has also been reported. The attachments using a *pelB* signal sequence representing the general secretory (Sec) pathway and a signal sequence of *E. coli* trimethylamine N-oxide reductase (TorA) representing the twin arginine translocation pathway, led to a successful periplasmic expression of OPH (Kang et al., 2005; 2006).

In the present study, an *opd* gene from *Flavobacterium* sp. strain ATCC 27551 was cloned and expressed in *E. coli*. We report the secretion of recombinant OPH from an *E. coli* strain harboring only a structural *opd* gene without any tagging system. Biodegradation of tolclofos-methyl using these recombinant *E. coli* strain is also reported.

Materials and Methods

Bacterial strains, plasmids, and culture conditions. Strain *Flavobacterium* sp. ATCC 27551 was obtained from the American Type Culture Collection (ATCC; USA) and cultured in tryptic soy broth (TSB; Difco Laboratories Inc., USA) at 30°C with shaking at 220 rpm. Luria-Bertani (LB; 1.0% tryptone, 0.5% yeast extract, and 0.5% NaCl) medium was used as the basal medium for *E. coli* growth. For supplementary antibiotics, 100 μg/mL of ampicillin (Sigma Chemical Co., USA) or 10 μg/mL of tetracycline (Sigma Chemical Co.) was added when necessary. A plasmid vector pET21a (+) (Novagen Inc., USA) was used for the expression of recombinant OPH in *E. coli.* Strain DH5α was used as a host strain for manipulating plasmids. Strains used for the recombinant OPH expression were as follows: *E. coli* BL21(DE3) (F^-, *ompT*, $hsdS_B(r_B^- m_B^-)$, *gal*, *dcm*), *E. coli* BLR(DE3) [F^-, *ompT*, $hsdS_B(r_B^- m_B^-)$, *gal*, *dcm* (DE3) Δ (*srl-recA*)306::Tn*10* (Tet^R)], and *E. coli* HMS174(DE3) [F^-, *recA1*, $hsdR(r_{k12}^- m_{k12}^+)$ (DE3)] (Novagen Inc.). For the expression of recombinant protein, sub-cultures of each recombinant *E. coli* strain were grown overnight and inoculated into the fresh LB media at a final concentration of 2% (v/v). After incubation for 4 h at 30°C with shaking at 220 rpm, isopropyl β-D-thiogalactopyranoside (IPTG; Sigma Chemical Co.) was added at a final concentration of 1 mM, and the cells were cultured overnight at 30°C with shaking at 220 rpm.

Construction of OPH expression plasmids. General polymerase chain reaction (PCR) reaction was performed as follows: an initial cycle at 97°C for 1 min, 30 cycles at 97°C for 1 min, 56°C for 30 s, and 72°C for 2 min using *Pfu* DNA polymerase (Stratagene Co., USA). Primer OH-S-UP (5'-ctgctagcatgcaaacgagaagggtt-3', *Nhe*I site underlined) and primer C-His-TEV (5'-cgaattcatta gtggtggtggtggtggtg***gccctgaaagtacaggttctc***-3', *Eco*RI site underlined and TEV protease cleavage site in bold-italic font) were used for PCR amplification of the *opd* structural gene from chromosomal DNA of *Flavobacterium* sp. strain ATCC 27551 as a template. Primer OH-UP (5'-ctgctagctcgatcggcacaggcgat-3', *Nhe*I site underlined) and primer C-His-TEV were used for amplification of the *opd* gene without a Tag signal sequence. Primer XB-S-UP (5'-ct gctagcatgaacacgctcgtccat-3', *Nhe* site underlined) and primer XB-OH-DOWN (5'-gcctgtgccgatcga***cgcccgggcggtgcc***-3'), sequence corresponding to the 5' end of the structural *opd* gene which was a truncated Tat signal sequence is underlined; sequences corresponding to the 3' end of the XynB signal sequence in bold were used for amplification of the signal peptide encoding the sequence of XynB from *Streptomyces thermocyaneoviolaceus* (Shin et al., 2009). A DNA fragment of the XynB signal sequence was mixed with a DNA fragment of the *opd* gene without a Tag signal sequence, and a second round of PCR was conducted using primers XB-S-UP and C-His-TEV to generate the *opd* gene with a XynB signal sequence. All inserted DNA sequences of recombinant plasmids were confirmed by DNA sequencing analysis.

Cell fractionation. Cell fractionation of recombinant *E. coli* samples was performed with the culture broth of recombinant *E. coli* strain BLR(DE3) harboring plasmid pOPH (20 mL) which had been incubated for 24 h at 30°C and 220 rpm shaking with IPTG induction. To produce the total cell lysate fraction, culture broth was centrifuged, and the pellet was resuspended in 50 mM Tris-HCl (pH 8.0). The suspension was sonicated (10 s pulse on/off for 1 min with 50% amplitude; Vibra-cell VCX-130; Sonics

and Materials Inc., USA) and used as a total cell lysate fraction. The supernatant fraction was obtained after centrifugation of the culture broth at 17,700×g for 10 min. The cell pellet was further fractionated based on a method described in the pET system manual (Novagen Inc.). The cell pellet was first washed with 50 mM Tris-HCl (pH 8.0) and then centrifuged at 17,700×g for 3 min. The collected cell pellets were resuspended in 50 mM Tris-HCl (pH 8.0) containing 20% sucrose and 1 mM EDTA, and incubated for 10 min at 25°C. After centrifugation at 10,000×g for 10 min at 4°C, the harvested cells were resuspended in 2 mL of ice-cold 5 mM $MgSO_4$. The suspension was incubated in ice for 10 min and centrifuged at 10,000×g for 10 min at 4°C. The obtained supernatant was used as a periplasmic fraction. The pellet was resuspended in 50 mM Tris-HCl (pH 8.0), sonicated as described above, and centrifuged. The obtained supernatant was used as a cytoplasmic fraction. The purities of the cell fractions were verified using an assay for alkaline phosphatase activity with *p*-nitrophenyl phosphate (PNP; Sigma Chemical Co.) as a substrate (Jung and Tae, 2000).

Analytical assays. The activity of recombinant organophosphorus hydrolase was assayed using parathion (*O*,*O*-diethyl-*O*-4-nitrophenyl phosphorothioate; Sigma Chemical Co.) as a substrate. The enzyme assay mixture consisted of 100 μL enzyme sample [100 μL of enzyme solution or 100 μL of whole cell suspension in 50 mM Tris-HCl (pH 8.0)] and 50 mM Tris-HCl (pH 8.0) containing 3% methanol. Subsequently, 172 μM parathion (used as stock solution, 10 mg/mL in methanol; Sigma Chemical Co.) was added, and the increase of absorbance was monitored at 412 nm for 10 min at 25°C using a UV-Vis spectrophotometer (UV-1800; Shimadzu Co., Japan). One unit of OPH activity was defined as the amount of enzyme needed to release 1 nmol of *p*-nitrophenol per min (ε_{412} =16,500 M^{-1} cm^{-1} for *p*-nitrophenol). The cell density of recombinant *E. coli* strains was determined by measuring the absorbance at 600 nm (OD_{600}) with a UV/Vis spectrophotometer (UV-1800; Shimadzu Co.). Protein concentration was determined using the Bradford method with Bovine Serum Albumin (BSA; Sigma Chemical Co.) as the protein standard (Bradford, 1976).

Zymogram assay. For the preparation of protein solution containing secreted recombinant OPH, the recombinant *E. coli* BLR(DE3) strain having plasmid pOPH was cultured in LB medium supplemented with 100 μg/mL of ampicillin and 10 μg/mL of tetracycline for 24 h at 30°C with shaking at 220 rpm. The culture solution was centrifuged at 17,700×g for 5 min, and the obtained cell-free culture supernatant was concentrated by addition of ammonium sulfate to a final concentration of 50%. After centrifugation at 17,700×g for 10 min, the precipitated protein was suspended in 50 mM Tris-HCl (pH 8.0). This protein solution was dialyzed against 50 mM Tris-HCl (pH 8.0), and used as the protein fraction for the zymogram assay. A solution from recombinant *E. coli* BLR(DE3) strain containing plasmid vector pET21a(+) was used as the control protein fraction. The zymogram assay for secreted recombinant OPH was conducted using non-denaturing gel electrophoresis. The prepared protein solution was electrophoresed on an 8% polyacrylamide gel at 4°C for 4 h with a constant current of 110 V (Bio-Rad Laboratories Inc., USA). The gel was washed using 50 mM Tris-HCl (pH 8.0), and attached to a gel containing 40 ppm of parathion (stock solution, 10 mg/mL in methanol; Sigma Chemical Co.) as a substrate. The OPH activity was determined by development of color on the upper surface of the gel.

Degradation of tolclofos-methyl. Recombinant *E. coli* strains bearing OPH expressing recombinant plasmids were cultured in 1/10 strength LB medium (0.1% tryptone, 0.05% yeast extract, and 0.05% NaCl) supplemented with 100 μg/mL of ampicillin and 10 μg/mL of tetracycline at 30°C with shaking at 220 rpm for 48 h. Twenty milligrams per liter of tolclofos-methyl (stock solution, 2×10^4 mg/L in acetone; Dongbang Agro Co., Korea) and 0.5 mM Co^{2+} ion were co-added, when 1 mM IPTG was added for OPH induction. After incubation for 5 days, ethyl-acetate (Burdick & Jackson, USA) was added as the same volume of culture broth, and thoroughly mixed by vortexing. Subsequently, a 1/3 portion of the upper fluid phase was removed, concentrated using a vacuum evaporator (Thermo Fisher Scientific Inc., USA), and resuspended in the same volume of acetonitrile (Burdick & Jackson, USA). The extract was filtered through a 0.45-μm PVDF syringe filter (Millipore Co., USA) and analyzed by an high-performance liquid chromatography (HPLC) (Waters Co., USA) equipped with a UV-8010 detector (200 nm; TOSOH Co., Japan) and a ZORBAX Eclipse XRD-C18 column (4.6×150 mm; Agilent Technologies Inc., USA). A solution of acetonitrile-water (70:30, v/v) was used as the mobile phase with a flow rate of 2 mL/min. Tolclofos-methyl was detected at elution times of ~1.45 min, at 200 nm which was determined using maximum absorption wavelength (λ_{max}) scanning (from 200 to 400 nm) of tolclofos-methyl. Under this analytical condition, recovery rate of tolclofos-methyl was 86.8% ±17.7.

Prediction of secondary structure of mRNA. A DNA sequence region ranging from a transcriptional initiation site of the T7 promoter sequence to a downstream site of the *opd* structural gene of each recombinant plasmid with or without the signal peptide sequence was selected for the prediction of *opd* mRNA secondary structure and 5' mRNA local folding energy (ΔG value, kcal/mol). Potential ΔG values of these sequences were obtained from analysis using the RNA folding form at the mFold Web Server (http://mfold.rna.albany.edu) with conditions set at 30°C and 1 M NaCl (Markham and Zuker, 2008).

Statistical analysis. Statistical significance was evaluated with one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (p <0.05), using SPSS 19.0 (SPSS Inc., USA).

Results and Discussion

Construction of recombinant OPH expression plasmids. A PCR amplified *opd* gene encoding for organophosphorus hydrolase

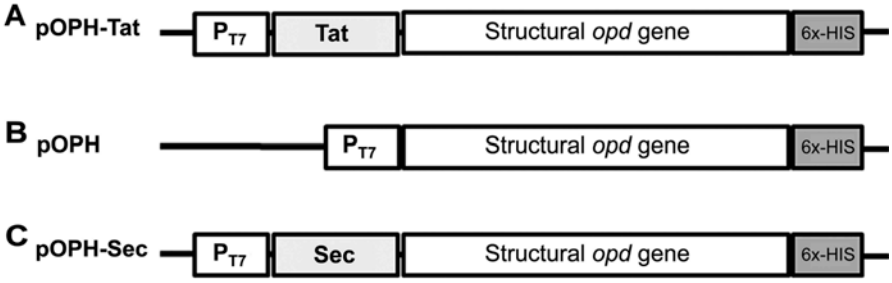

Fig. 1 Schematic diagram for the construction of recombinant OPH expression plasmids. All recombinant plasmids were constructed based on plasmid vector pET21a(+). Abbreviations: pOPH-Tat, recombinant plasmid containing *opd* gene with a Tat-type signal peptide sequence; pOPH, recombinant plasmid containing only structural *opd* gene; pOPH-Sec, recombinant plasmid containing *opd* gene with a Sec-type signal peptide sequence; P_{T7}, T7 promoter; Tat, Tat-type signal peptide sequence; Sec, Sec-type signal peptide sequence; 6 × -HIS, 6 × histidine taq sequence (HHHHHH).

from *Flavobacterium* sp. ATCC 27551 was cloned into *E. coli* plasmid vector pET21a (+), and designated as pOPH-Tat (Fig. 1A). The original *opd* gene has a twin arginine translocation signal sequence designated as the Tat signal sequence. Mulbry and Karns (1989) reported that removing the Tat signal sequence from the gene produced a 2-fold increase of OPH production in an *E. coli* recombinant expression system under the control of *lac* promoter. Pandey et al. (2009) reported that deletion of the Tat signal sequence could lead to a ~3.28-fold increase of OPH expression when using the T7 promoter in an *E. coli* strain, although this expression was mostly manifested by formation of inclusion bodies. Therefore, an OPH expression plasmid was constructed without the Tat signal sequence. The amplified PCR fragment containing only the structural *opd* gene without the native Tat signal sequence was cloned into the same vector, and it was designated as pOPH (Fig. 1B).

To determine whether the *opd* gene had secretory signal sequences other than the original Tat, an OPH expression plasmid was constructed with another signal sequence. Xylanase (XynB) of *S. thermocyaneoviolaceus* is known to contain ~40 amino acids, which form a general secretory (Sec) signal sequence. Furthermore, XynB carrying this signal sequence has already been shown to express under the T7 promoter in *E. coli* BLR(DE3) strain, and the produced recombinant XynB could be secreted into the culture medium (Shin et al., 2009). Based on these reports, the XynB signal sequence was used for the construction of the recombinant plasmid, which could be expected to show easy secretion of overproduced recombinant OPH. For construction of an *opd* gene with the Sec signal peptide sequence, secondary PCR was performed with each gene fragment as described in Materials and Methods. The plasmid containing the *opd* gene with the Sec signal peptide sequence was designated as pOPH-Sec (Fig. 1C).

Expression of recombinant OPH in *E. coli*. The expression plasmids were transformed into the three different *E. coli* hosts (BL21(DE3), BLR(DE3), and HMS174(DE3)), and the recombinant strains were grown in LB medium; the recombinant proteins were induced as described in Materials and Methods. The induced and non-induced cells were harvested, and total protein was analyzed by SDS-PAGE. However, specific bands were not detected in any host-plasmid combination (data not shown). Recombinant OPH production was confirmed by a test for OHP activity. The whole cells of each culture broth were directly used as enzyme fractions for the OPH activity assay. Recombinant *E. coli* BLR(DE3) strain harboring plasmid vector pET21a(+) was used as a negative control. The levels of recombinant OPH expression were significantly different between the *opd* gene with the signal peptide sequence and the *opd* gene without the signal peptide sequence (Fig. 2A).

The highest OPH activity was obtained from the *E. coli* BLR(DE3) strain bearing plasmid pOPH, which has only the structural *opd* gene without a signal peptide sequence. On the other hand, the *E. coli* BLR(DE3) strain bearing plasmid pOPH-Tat having Tat signal peptide sequence in front of the structural gene was found to show a low level of OPH activity compared to that of pOPH.

The *E. coli* with pOPH-Sec, which had a Sec signal peptide, also showed low levels of OPH activity. *E. coli* transformant of signal peptide harboring plasmids showed meaningful OPH activity compared to the control strain; however, the activity levels did not reach that of the *opd* gene transformant without a signal peptide sequence. Strains with pOPH-Tat and pOPH-Sec showed only 10 and 2% OPH activities, respectively, compared to the activity of the pOPH transformant.

Whether the increased OPH activity produced by deletion of the Tat signal sequence could be influenced by various characteristics of the *E. coli* expression host strain itself is yet doubtful. Therefore, pOPH was transformed to other strains such as *E. coli* BLR(DE3), which is a *recA*-mutated expression host strain derived from *E. coli* BL21(DE3) and *E. coli* HMS174(DE3) strain derived from *E. coli* K-12 strain. All recombinant *E. coli* strains were inoculated into LB medium supplemented with ampicillin and incubated for 4 h at 30°C with shaking at 220 rpm. After induction with 1 mM IPTG, the cells were incubated for an additional 21 h, and whole cells of the culture broth from each recombinant *E. coli* strain were used to assay the specific activity of OPH (Fig. 2B).

Secretion and localization of recombinant OPH in *E. coli*. The

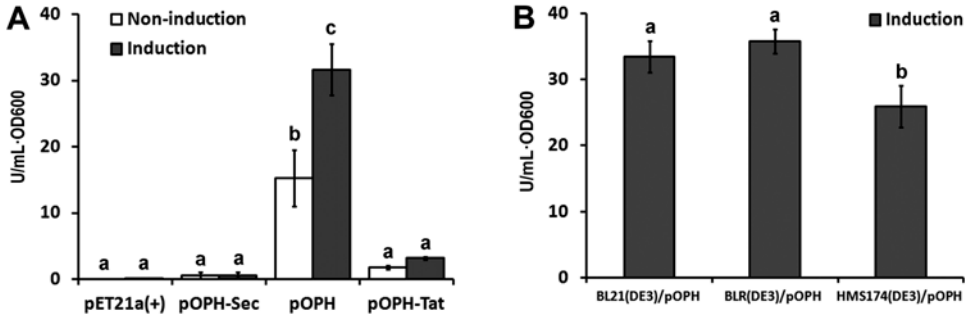

Fig. 2 Recombinant OPH produced from *E. coli*. All recombinant *E. coli* strains were incubated in LB media at 30°C with 220 rpm shaking for 21 h after induction with 1 mM IPTG. Whole cells of culture broth from each recombinant *E. coli* strain were used for the assay of OPH-specific activity as described in Materials and Methods. Data are means of three determinations, and error bars represent the standard deviation. Lower case letters indicate significant differences among the groups (p <0.05, one-way ANOVA followed by Duncan's multiple range test). (A) Effects of signal sequence on the production of recombinant OPH in *E. coli*. Abbreviations: pOPH-Sec, *E. coli* BLR(DE3) strain harboring plasmid pOPH-Sec (containing the XynB signal sequence of *S. thermocyaneoviolaceus*); pOPH, *E. coli* BLR(DE3) strain harboring plasmid pOPH (containing the structural *opd* gene without Tat signal sequence); pOPH-Tat, *E. coli* BLR(DE3) strain harboring plasmid pOPH-Tat (containing the structural *opd* gene with a Tat signal sequence). (B) Comparison of specific OPH activity among various recombinant *E. coli* host strains.

recombinant OPH activity with signal peptides was significantly lower than that without signal peptides. Therefore, the localization of the recombinant protein was investigated before applying the recombinant strain for bioremediation. An IPTG-induced culture of *E. coli* BLR(DE3) harboring pOPH was fractionated as described in Materials and Methods. The successful fractionation was confirmed when the alkaline phosphatase activity was found only in the periplasmic fraction. The proportion of localized recombinant OPH was represented as the relative percentage value of specific activity.

OPH activity was found in the cytoplasmic fraction (52%; ~125.99 U/mL of culture), whereas only 4% of the activity was found in the periplasmic fraction (~10.31 U/mL of culture) (Fig. 3A). In addition, 44% of the activity existed in the cell-free culture supernatant (~105.6 U/mL of culture).

In a previous study, very limited secretion of recombinant OPH was observed from an *E. coli* strain bearing the *opd* gene. Kang et al. (2005) showed that only 1.7% of the OPH activity was found in the periplasmic fraction, and no activity was observed in the supernatant, even though they used the TorA signal sequence for better secretion. Surprisingly, significant OPH activity was recovered from the supernatant fraction as well as the cytoplasmic fraction from recombinant *E. coli*, even without any signal sequence. This is the first report describing meaningful secretion of recombinant OPH from an *E. coli* system. The extracellular secretion of the recombinant OPH was reconfirmed using the zymogram assay. Cell-free culture supernatant from recombinant *E. coli* BLR(DE3) strain harboring plasmid pOPH was prepared as described in Materials and Methods, and the activity of secreted recombinant OPH was observed through the zymogram assay. Furthermore, yellow color development was generated on the gel from the supernatant of *E. coli* BLR(DE3)/pOPH (Fig. 3B). Pandey et al. (2009) reported that the recombinant OPH without a signal sequence was formed as inclusion bodies in *E. coli*.

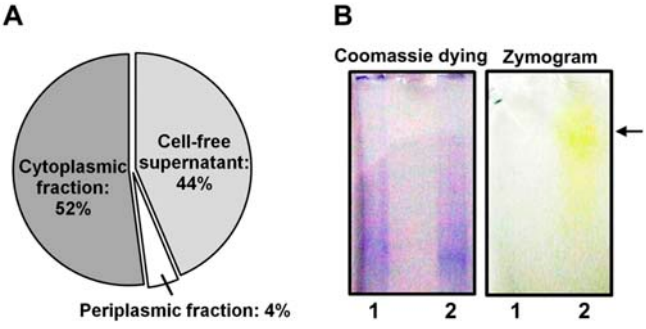

Fig. 3 Secretion of recombinant OPH in *E. coli*. (A) Confirmation of localization of recombinant OPH produced from *E. coli*. Recombinant *E. coli* BLR(DE3) strain harboring plasmid pOPH was grown in LB medium at 30°C with 220 rpm shaking for 24 h after induction with 1 mM IPTG. Preparation of each cell fraction and OPH-specific activity assay was conducted as described in Materials and Methods. Proportion of localized recombinant OPH is presented as the relative percentage values of OPH-specific activity per 1 mL culture broth of *E. coli* BLR(DE3)/pOPH. (B) Zymogram assay using the cell-free supernatant solution. Cell-free culture supernatant solution from recombinant *E. coli* strain containing plasmid pOPH was concentrated by ammonium sulfate precipitation to a final concentration of 50%. After dialyzing against 50 mM Tris-HCl buffer (pH 8.0), the obtained protein solution was used for zymogram assay. Protein solutions from the recombinant *E. coli* strain containing plasmid vector pET21a(+) were used as controls. Arrow indicates the region showing yellow color development on the surface of gel resulting from the production of *p*-nitrophenol. Lanes: 1, Protein solution from the recombinant *E. coli* BLR(DE3) strain containing plasmid vector pET21a(+); 2, Protein solution from the recombinant *E. coli* BLR(DE3) strain containing plasmid pOPH (containing the structural *opd* gene without a Tat signal sequence).

However, no significant level of inclusion body was found in *E. coli* BLR(DE3)/pOPH under both induced and non-induced conditions. Extracellular secreted OPH may have a greater capability to remove toxic organophosphate insecticides.

In *E. coli*, a recombinant protein containing a signal peptide

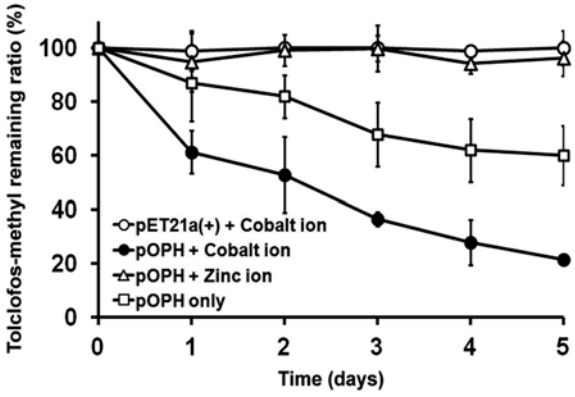

Fig. 4 Biodegradation of tolclofos-methyl by *E. coli* BLR(DE3)/pOPH producing recombinant OPH. Cells were incubated in 1/10 strength LB media at 30ºC with 220 rpm shaking for 48 h followed by addition of 0.5 mM Co^{2+} ion, 1 mM IPTG, and 20 mg/L tolclofos-methyl, and further incubated for 5 days at 30ºC with shaking at 220 rpm. The ratio of tolclofos-methyl remaining in culture broth was analyzed by HPLC. Recombinant *E. coli* BLR(DE3) strain harboring plasmid vector pET21a(+) was used as a control. Data are means of three independent experiments with at least three replicates per each test. Error bars represent the standard deviation. Abbreviations: ○, Recombinant *E. coli* BLR(DE3) strain harboring plasmid vector pET21a(+) cultured with Co^{2+} ion; ●, Recombinant *E. coli* BLR(DE3) strain harboring plasmid pOPH (containing the structural *opd* gene without a Tat signal sequence) cultured with Co^{2+} ion; △, Recombinant *E. coli* BLR(DE3) strain harboring plasmid pOPH cultured with Zn^{2+} ion; □, Recombinant *E. coli* BLR(DE3) strain harboring plasmid pOPH cultured without metal ion.

could be targeted for a secretion system functioning through the inner membrane into the periplasmic space, and could normally be retained within this extra-cytoplasmic compartment (Rinas and Hoffmann, 2004). In addition, *E. coli* can not secrete Tat substances into a growth culture medium, because the Tat secretion transport system only crosses the cytoplasmic membrane (Voulhoux et al., 2001; Yang et al., 2010a). Several possibilities, such as the protein leakage (Tong et al., 2000) or involvement of a type II secretion system (Voulhoux et al., 2001) could be proposed to explain the secretion of recombinant OPH observed in the present study.

Biodegradation of tolclofos-methyl using recombinant OPH. Recombinant *E. coli* BLR(DE3)/pOPH was tested for its ability to degrade the organophosphorus fungicide, tolclofos-methyl. The strain was cultured in 1/10 strength LB medium with 20 mg/L of tolclofos-methyl, with or without 0.5 mM of Co^{2+} ion or Zn^{2+} ion for 5 days at 30ºC with shaking at 220 rpm. The remaining ratio of tolclofos-methyl in the culture broth was analyzed by HPLC, and biodegradation efficacy was calculated. The recombinant *E. coli* strain with Co^{2+} ion successfully degraded tolclofos-methyl to around 80%; however, only around 40% of degradation was observed without Co^{2+} ion. Surprisingly, no activity was found with Zn^{2+} ion (Fig. 4).

The OPH, homodimeric organophosphotriesterase, requires a metal ion as a cofactor. Zinc ion and several other divalent metal ions such as Co^{2+}, Cd^{2+}, and Ni^{2+} have been proven to be effective cofactors. In particular, Co^{2+} ion has been reported to produce higher OPH activity levels than those produced by other native metal ions (Omburo et al., 1992; Theriot and Grunden, 2011). However, different from the results of previous studies, no promoting effect was observed when Zn^{2+} ion was added. In addition, significant differences were found in activity with or without Co^{2+} ion, indicating the influence of metal ions on the improved folding of expressed protein (Manavathi et al., 2005).

Bioremediation strategies by microorganisms having degrading capacity against toxic pollutants generally requires proper resolutions related with the potential limitations such as poor bioavailability of chemicals, inadequate supply of nutrients, as well as insufficient biodegradation *in situ* (Dua et al., 2002).

Due to these concerns, extracellular secretion of OPH from recombinant *E. coli* BLR(DE3)/pOPH could have advantage for *in situ* environmental applications.

Effect of the signal peptide sequence on expression. In recent years, for the high yield expression of a recombinant protein in *E. coli*, many studies have suggested a correlation between the efficiency of translation initiation and the stability of mRNA secondary structure, especially at the region near the ribosomal binding site (Shine-Dalgarno sequence). Kudla et al. (2009) suggested a correlation between the translation efficiency of a recombinant protein and the stability of mRNA structure near the translation start site, by using the synthesized GFP library in *E. coli*. In their study, protein expression level was reduced if mRNA had a more stable structure near the translation start region. In addition, Gu et al. (2010) surveyed the reduction in stability of mRNA secondary structure near the translation start codon by using the genomes of 340 species. In addition, Pandey et al. (2009) noticed that recombinant OPH could be increased ~3.3 fold in *E. coli* BL21(DE3) strain when OPH was expressed without the signal peptide sequences. In their study, this increasing tendency was explained by examining the changes of ΔG values (kcal/mole) resulting from mutation only in the 87 bp region of the signal peptide.

Based upon these reports, it could be suggested that the putative involvement between the local mRNA secondary structures originated from the *opd* transcript and the expression level of recombinant OPH in *E. coli*. The expression level of recombinant OPH could be affected by a stronger mRNA secondary structure. The folding energy (ΔG, kcal/mole) of the 5' end of the coding region, which has been suggested to influence the stability of mRNA secondary structure, was analyzed using three kinds of recombinant plasmids, such as plasmid pOPH (carrying only structural *opd* without a Tat signal sequence), plasmid pOPH-Tat (carrying structural *opd* with a Tat signal sequence), and plasmid pOPH-Sec (carrying structural *opd* with a Sec signal sequence). With consideration of the report by Kudla et al. (2009), the region covering a transcriptional start site of the T7 promoter sequence to a downstream sequence site of the structural *opd* gene was selected for the analysis of ΔG and analyzed using the RNA folding form of the mFold Web Server with setting conditions of 30ºC and 1 M NaCl (Gu et al., 2010).

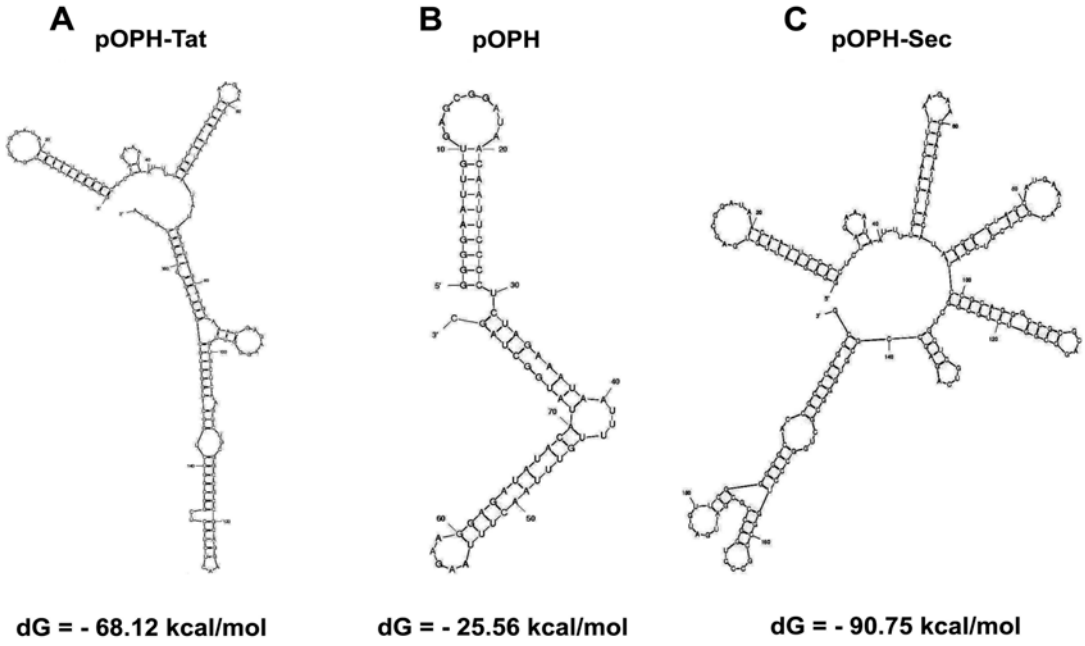

Fig. 5 Stability prediction of *opd* mRNA secondary structure. A DNA sequence covering the region from a transcriptional start site of the T7 promoter sequence to downstream site of the structural *opd* gene of each recombinant plasmid vector was analyzed for both the prediction of mRNA secondary structure and potential ΔG values, using the RNA folding form at the mFold Web Server (http://mfold.rna.albany.edu) set at conditions of 30°C and 1 M NaCl. Abbreviations: pOPH-Tat, *E. coli* BLR(DE3) strain harboring plasmid pOPH-Tat (containing the structural *opd* gene with a Tat signal sequence), pOPH, *E. coli* BLR(DE3) strain harboring plasmid pOPH (containing the structural *opd* gene without a Tat signal sequence); pOPH-Sec, *E. coli* BLR(DE3) strain harboring plasmid pOPH-Sec (containing the XynB signal sequence of *S. thermocyaneoviolaceus*)

Potential ΔG values of the 5' end of the *opd* mRNA-coding region generated from the recombinant plasmids pOPH-Tat and pOPH-Sec were -68.12 and -90.75 kcal/mol, respectively, whereas the ΔG value of pOPH was -25.56 kcal/mol (Fig. 5). As mentioned in previous reports, a higher negative potential ΔG value could be interpreted as the existing mRNA state having a more stable secondary structure, which has been considered not to lead to a more efficient expression of target protein having these kinds of feature of mRNA state. Compared to the predicted ΔG values, the more positive and higher ΔG value was assumed in the case of recombinant plasmid pOPH, which already showed a higher OPH activity than those of recombinant plasmids pOPH-Tat and pOPH-Sec. The actual activity matched well with the predicted activity. The recombinant *E. coli* BLR(DE3)/pOPH showed significantly higher activity than those of plasmids encoding lower ΔG values at the 5' end of transcripts (Fig. 2A). Both strains harboring plasmids with a signal peptide showed meaningful OPH activity, and their expression was induced by IPTG.

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