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ORIGINAL PAPER

Overexpression of protein disulfide isomerase DsbA enhanced detoxification of organophosphates and enhanced detectability in the environment following degradation of pesticide residues

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Abstract In many cases the expressed protein is insoluble and accumulates in so-called inclusion bodies. Several strategies have been developed to improve the solubility of the expressed protein. Disulfide isomerase DsbA could effectively assist proteins folding,both in vivo coexpressed with the target protein,and in vitro replenished as foldases.Moreover,DsbA also has the chaperone-like activity in the assistant refolding of genetically engineered inclusion bodies.Coexpression of DsbA with the target protein could lead to higher levels of soluble protein. In this work, a significantly improved, recombinant *Escherichia coli* has been developed to degrade the toxic organophosphorus compound, methyl parathion, to non-toxic materials by coexpression of disulfide isomerase DsbA and Methyl Parathion Hydrolase (MPH)-GFP fusion protein. Whole cells expressing DsbA had significant enhancement of MPH activity compared to DsbA-free system and could be easily detected.

Keywords Biodegradation · Fusion expression · Disulfide isomerase · Methyl parathion hydrolase

Introduction

Organophosphorus pesticides such as parathion and methyl parathion (MP) have been used extensively in agriculture and household as pesticides, fungicides and herbicides. They are acute neurotoxins by virtue of their potent inhibition of acetylcholinesterase (Singh and Walker 2006). These compounds cause enormous damage to non-target organisms because the acetylcholinesterase is present in all vertebrates (Zhang et al. 2005).

The microbial degradation has become the focus of many studies, because it is economical and effective. Methyl parathion-degrading (*mpd*) gene has been intensively researched (Cui et al. 2001; Fu et al. 2004; Rani and Lalithakumari 1994; Yang et al. 2006; Zhang et al. 2006). We previously cloned *mpd* gene from chlorpyrifos-degrading *Stenotrophomonas* sp. and the gene was expressed in *Escherichia coli* (Yang et al. 2006). However, their use is still limited in the field because recombinant *E. coli* have very low production yield of MPH due to its low solubility (Yang et al. 2008b). Meanwhile, recombinant *E. coli* may cause new environmental contamination such as interference with the distribution and growth of the indigenous microbial populations. Therefore, a system that can enhance MPH production yield and can readily detect the released organisms in the environment is needed.

Efficient production of heterologous proteins with prokaryotic and eukaryotic hosts is often hampered by inefficient formation of the product. Limitation has been attributed to a low folding rate, and a rational solution is the overexpression of proteins supporting folding, like protein disulfide isomerase (Inan et al. 2006; Takegawa et al. 2010; Davis et al. 2000; Robinson et al. 2004; Sharma et al. 2009), or the unfolded protein response transcription factor (Mattanovich et al. 2007). DsbA, a monomeric protein of 21.1 kDa, is the catalyst of disulfide formation (Martin et al. 1993) in the periplasm of *E. coli*. DsbA could effectively assist proteins folding (Narayanan and Chou 2009; Schlapschy et al. 2006),both in vivo coexpressed

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with the target protein and in vitro replenished as foldases.Moreover,DsbA also has the chaperone-like activity in the assistant refolding of genetically engineered inclusion bodies (Xu et al. 2008a, b).In the present work, we have constructed a autofluorescent recombinant *E. coli* containing DsbA coexpression plasmids, we analyzed the effects of DsbA overexpression on whole cell MPH activity when produced in *E. coli*. To the best of our knowledge, this is the first published report to demonstrate that bacterial DsbA could be successfully used to improve bioconversion efficiency for organophosphates in a recombinant fluorescent *E. coli* whole cell system.

Materials and methods

Strains and plasmids

The strains and plasmids used are listed in Table 1.

Media and culture conditions

E. coli bearing plasmids was grown at 37°C in 50 ml LB growth medium (5 g yeast extract, 10 g tryptone, and10 g NaCl in1 l distilled water) containing kanamycin 20 μg ml^{-1} using a 250 ml flask. After the cells were cultivated at 37°C for 2 h (OD600 = 0.3), 0.1 mM IPTG was added and the cells were shifted to a lower temperature (25°C) for induction. At predetermined time intervals, 1 ml of the culture was collected to measure cell density, MPH activity and GFP fluorescence (in RFU, relative fluorescent unit). GFP fluorescence was detected using a fluorescence spectrophotometer.

Construction of recombinant plasmids

Standard cloning procedures were performed according to Sambrook and Russell (2003). The *mpd-gfp* gene was polymerase chain reaction (PCR)-amplified from plasmid pMG33 using primers P1 and P2. The product was digested with *Nde*I-*Xho*I, and cloned into plasmid pET30a to generate control vector pEMG (Fig. 1a). The *mpd-gfp* fusion gene was PCR-amplified from pMG33 with *Bam*HI-*Xho*I (P3 and P2) and subcloned into pET30a, subsequently the DsbA gene without signal peptides was PCR-amplified (P4 and P5) from the *E. coli* K-12 chromosomal DNA and subcloned into pET30a with *Nde*I-*Bam*HI to generate pDsbAMG(Fig. 1b). Transformations of BL21 (DE3) with these constructs were performed according to the manufacturer's instructions.

Soluble expression analysis of MPH-GFP

For quantification of protein soluble expression, cells harboring pDsbAMG were induced with 0.1 mM IPTG and cultured at 25°C for 6 h, then 1 ml cells were collected by centrifugation at 3,500 × g for 10 min. Harvested cells were washed, and resuspended in PBS buffer, and then

Table 1 Strains and plasmids used in this work

Strain, plasmid	Description	Source or reference
Strains		
E. coli K-12	Source of *DsbA* gene	This lab
E. coli DH5α	*supE44* Δ*lacU169*(*φ80 lacZ*ΔM15) *recA1 endA1 hsdR17*(r_K^- m_K^+) *thi-1 gyrA relA1* F^- Δ(*lacZYA-argF*)	Tiangen
E. coli BL21 (DE3)	F^- *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (DE3)	Novagen
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17*(r_K^- m_K^+) *supE44 relA1 lac* (*F^- proAB lacIqZ M15Tn10 $Tetr^-$*)	Stratagene
Plasmids		
pMG33	Source of *mpd-gfp* gene	Yang et al. (2008a)
pET30a	Vector for construction of *DsbA-mpd-gfp* fusion gene	Novagen
pEMG	pET30a derivative, control plasmid for expressing MPH-GFP intracellularly	This study
pDsbAMG	pET30a derivative, plasmid for expressing DsbA-MPH –GFP intracellularly	This study
Primers		
P1	5′-CATATGGCCGCACCGCAGGTGCGCA-3′	This study
P2	5′-CTCGAGTTACTTGTACAGCTCGTCCA-3′	This study
P3	5′-GGATCCGCCGCACCGCAGGTGCGCA-3′	This study
P4	5′-CATATGTTCGCACCGCTGAAATCG-3′	This study
P5	5′-GGATCCTTTTTTCTCGGACAG-3′	This study

The restriction sites are underlined

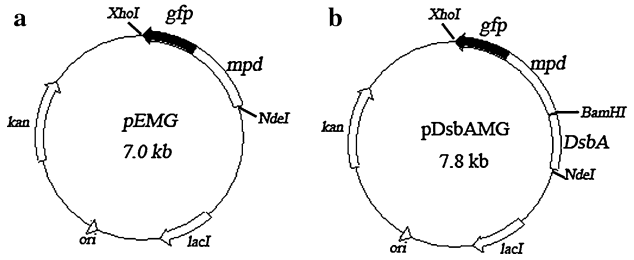

Fig. 1 Construction maps of the recombinant plasmid. The *mpd-gfp* gene was amplified by PCR from pMG33 and then inserted into pET-30a vector to construct pEMG plasmid (**a**). The *DsbA* gene was inserted into the upstream site of the *mpd-gfp* gene in the plasmid pET30a to form the pDsbAMG plasmid, in which a stop codon of the original *DsbA* gene was deleted (**b**)

treated with ultrasound sonication at 10 s × 6 cycles on ice. After a high spin (10,000 × g, 10 min), the supernatant and the deposition were examined by using SDS–PAGE with 10% (v/v) acrylamide. For Western blot analysis, the gel was transferred onto nitrocellulose membranes (Millipore) with a tank transfer system (Bio-Rad). Blotted membranes were placed in a blocking solution of 3% BSA in TTBS buffer (100 mM Tirs-Cl, 0.9% (w/v) NaCl, 0.1% (v/v) Tween 20) for 1 h. For immunodetection, primary GFP Polyclonal antibody (1:1,000) (Molecular Probes, Eugene, OR) was diluted in TTBS buffer. The second antibody was an alkaline phosphatase conjugated goat anti-rabbit IgG diluted 1:1,500 (Zhongshan Biotechnology, Beijing, China). The membrane was then stained with NBT/BCIP (Novagen, Darmstadt, Germany) for visualizing antigen–antibody conjugates.

Whole cell activity assay

Induced cells were suspended in a 100 mM phosphate buffer (pH 7.4) and diluted to an OD600 of 1.0. MPH activity assay mixtures (1 ml, 3% methanol) contained 50 μg ml^{-1} methyl parathion (added from a 10 mg ml^{-1} methanol stock solution), 960 μl of a 100 mM phosphate buffer (pH 7.4) and 10 μl of cells. Changes in absorbance (405 nm) were measured for 3 min at 30°C using a BeckmanDU800 spectrophotometer. Activities were expressed as units(1 μmol of p-nitrophenol formed per minute) per OD600 whole cells ($\varepsilon 405 = 17{,}700\ M^{-1}\ cm^{-1}$ for p-nitrophenol).

Stability study of resting cultures

Cells were grown in 50 ml of LB medium supplemented with 0.1 mM IPTG, 20 μg ml^{-1} kanamycin for 2 days, washed twice with 50 ml of 150 mM NaCl solution, resuspended in 5 ml of 100 mM phosphate buffer (pH 7.4) and incubated in a shaker at 25°C. Over a 2-week period, 0.1 ml of samples were removed each day. Samples were centrifuged and resuspended in 0.1 ml of 100 mM phosphate buffer (pH 7.4). MPH activity assays were conducted as described above.

Whole cell bioconversion kinetics

The values for kinetic parameters (*V*max and *Km*) were determined by analyzing Line weaver–Burk plots (Shuler & Kargi 1992) over the range 0.6–1.0 mM Methyl Parathion in 100 mM phosphate buffer, pH 7.4.

Measurement of Whole-Cell Fluorescence

Induced cells were suspended in a PBS buffer (pH 7.4) and diluted to an OD600 of 1.0, and the similarly diluted cells harboring pET30a were used as background references. The GFP fluorescence intensity was determined using a fluorescence spectrophotometer (F-4,500, HITACHI, Japan) with a bandwidth of 5 nm, an excitation wavelength of 488 nm and an emission wavelength of 510 nm.

Results and discussion

Coexpression of DsbA and MPH-GFP fusion protein

To evaluate the effect of DsbA coexpression on cells, cell density (OD600), MPH activity and fluorescence were measured during cell cultivation in LB media (Fig. 2). As shown in Fig. 2, We observed no notable changes on cell growth from the *DsbA* coexpressing (*DsbA*+) BL21 compared to the *DsbA* non-expressing (*DsbA*−) strain (see Fig. 2a). While the effect on cell growth was negligible, both the whole cell MPH activity and GFP fluorescence intensity in the (*DsbA*+) BL21 were much higher than those in (DsbA−) BL21. This can also be seen that MPH activity and GFP fluorescent intensity of the (DsbA−) BL21 strain (see Fig. 2b) began to decrease after about 12 h. However, (DsbA+) BL21 show the same trend ever after 18 h (Fig. 2b). To monitor the stability of suspended cultures, whole-cell activity was determined periodically over a 2-week period. As shown in Fig. 3, whole-cell activity remained at essentially the original level over the 2-week period.

To verify the synthesis of DsbA-MPH-GFP fusion, Western blot was performed with the supermatant of BL21(DE3) carrying pDsbAMG after induction with 0.1 mM IPTG. DsbA-MPH-GFP fusion was observed from cells carrying pDsbAMG at the position of ca. 80 kDa, which matched well with the molecular mass estimated from the deduced amino acid sequence of the fusion (Fig. 4). However, no such protein was detected with the control cells without IPTG induction. To assess the distribution of the fusion between

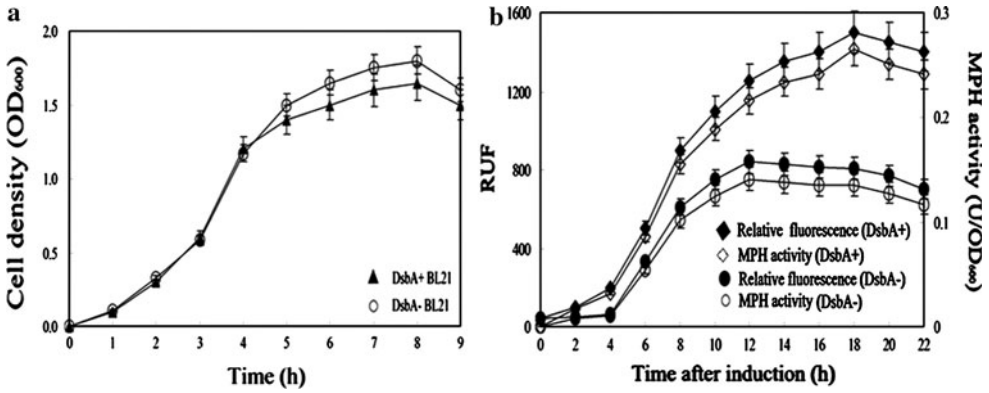

Fig. 2 Time-course profiles of cell growth (**a**), methyl parathion hydrolase (MPH) activity and fluorescence intensity (**b**) in DsbA− and DsbA + *E. coli* BL21. All data are plotted as a mean with standard deviation ($n = 3$)

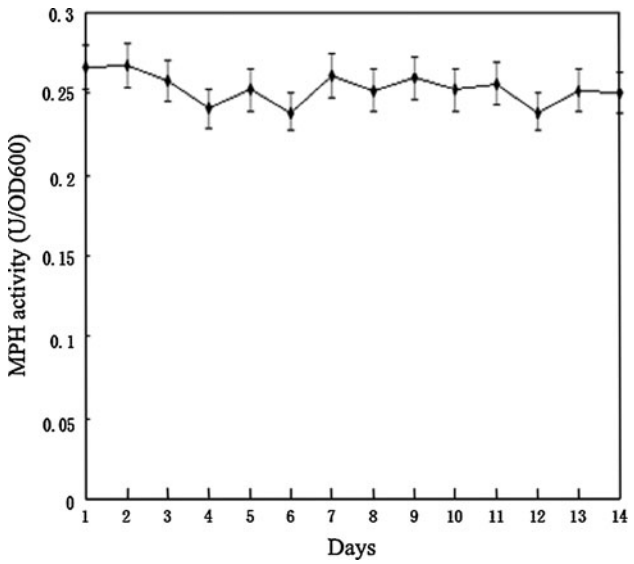

Fig. 3 Whole-cell MPH activity in suspended *E. coli* cultures expressing the fusion protein. Data are mean values with standard deviations from three replicates

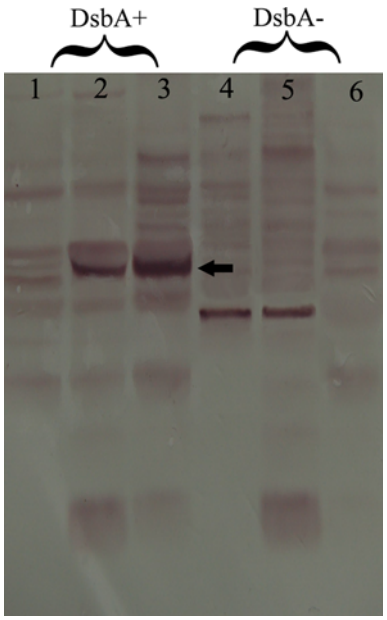

Fig. 4 Western blotting analysis of DsbA coexpression. Both DsbA + and DsbA− strains are shown. Lanes 1 and 6, without induction; lanes 2 and 5, insoluble fraction; lane 3 and 4, soluble fraction. The arrow indicates the fusion protein DsbA-MPD-GFP (80 kDa)

the insoluble and soluble fractions, deposition and soluble fractions were probed with anti-GFP serum. As shown in Fig. 4, more than 70% of the fusion was associated with the soluble fraction as judged by the intensity of the protein band. To investigate the effect of disulfide isomerase DsbA on the efficiency of soluble expression, Western blot was also performed with E. coli harboring pEMG. However, only 50% of the fusion was associated with the soluble fraction of *E. coli* (Fig. 4).

Effect of DsbA on whole cell MPH activity

The presence of DsbA had a significant impact on MPH activity. As shown in Fig. 2b, whole cell MPH activity of the *DsbA* + *E. coli* was higher than that of the *DsbA*–strain from an initial induction point. Difference of whole cell MPH activity between two strains increased with culture time. Finally, the *DsbA* + strain showed 75% enhanced whole cell MPH activity compared to the *DsbA*−strain. We obtained a similar result of MPH activity enhancement by DsbA coexpression in the other *E. coli* X-blue. From these results, we found that DsbA coexpression successfully worked to enhance MPH production yield and could be applied to all types of *E. coli* cells.

We performed whole cell bioconversion reaction with various substrate (methyl parathion) concentrations. Whole cell reaction rates (v) were plotted against substrate concentrations (S) as depicted in Fig. 5a. All data shown here were based on unit whole cell concentration (1 OD_{600} cells ml^{-1}). Both reactions showed Michaelis–Menten kinetic patterns and the *DsbA* + strain exhibited higher bioconversion rates in all ranges of methyl parathion concentrations. It is found that the biodegradation rate in the

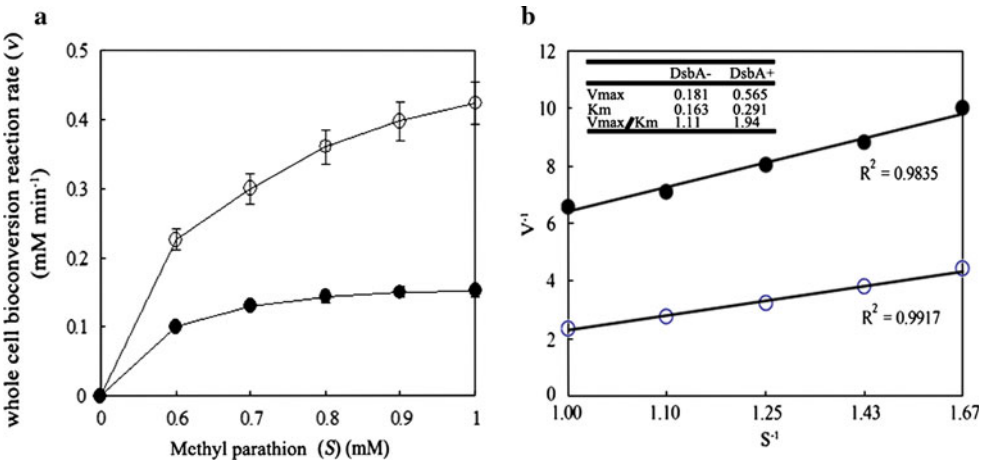

Fig. 5 Whole cell bioconversion reactions (**a**) and Lineweaver–Burk plot analysis (**b**) with DsbA− (*filled circle*) and DsbA + (*open circle*) *E. coli* BL21. Bioconversion reactions were performed in 'resting cell' condition. All data were based on unit cell concentration (1 OD_{600} cell ml^{-1}). Data of Lineweaver–Burk plot analysis are plotted as a mean ($n = 3$)

DsbA + strain was much increased with substrate concentrations compared to that in the *DsbA*−strain and showed a 2.8-fold higher conversion rate with 1.0 mM methyl parathion. These results demonstrated that the developed whole cell bioconversion system could more efficiently degrade organophosphates, especially high concentration of organophophate.

From the linear Line weaver–Burk analysis (see Fig. 5b), kinetic parameters *V*max and *Km* were determined for both the strains. The *DsbA* + whole cells showed 3.1-fold higher bioconversion rate, *V*max (0.565 mM min^{-1} per unit cell concentration) compared to *DsbA*−strain (0.181 mM min^{-1}). However, the *DsbA* + strain had a higher Michaelis–Menten constant, implying that substrate affinity of the *DsbA* + whole cell biocatalyst was lower. When we calculated overall reaction efficiency, *V*max/*Km*, the *DsbA* + strain exhibited highly enhanced bioconversion efficiency by 75%. Therefore from these results we confirmed that DsbA co-expression could be successfully employed to develop a whole cell biocatalysis system with notable enhanced bioconversion efficiency and capability for organophosphate, methyl parathion degradation.

Effect of DsbA on detection of whole cell

At the optimum temperature for its growth (37°C), *E. coli* tends to accumulate heterologous proteins in insoluble form. In this work, fusion DsbA confers higher level of solubility to heterologous proteins GFP in a reproducible way, even when *E. coli* is grown at 37°C. The whole-cell fluorescence of *E. coli* BL21/pDsbAMG was ∼2-fold higher than that of BL21/pMG (Fig. 2b) at 25°C. The fluorescence increased gradually after induction with 0.1 mM IPTG and reached maximum at 18 h (Fig. 2b).

Coexpression of disulfide isomerase DsbA with the target protein has enormous potential in the synthesis of a wide variety of valuable products (Joly et al. 1998; Locher and Griffiths 1999). Up to now, there is no report that bacterial DsbA could be successfully used to improve bioconversion efficiency for organophosphates in a recombinant *E. coli* whole cell system. In the previous work, we used an INP-based display system to functionally express GFP-MPH on the cell surface (Yang et al. 2008a). However, the external environment affects the stability of the protein and protein activity, including acidic pH environment and a number of protein inhibitory compounds. Therefore, the effective intracellular expression is still one of the choices for whole-cell catalyst. In this study, MPH soluble expression in *E. coli* was achieved by coexpression of DsbA. The correct disulfide pairing is a prerequisite for biological function, while the DsbA can enhance the expression of the matching and isomerization of disulfide bonds (Stewart et al. 1998). However, we noted that the enzyme activity was lower than expected even in the coexpression of DsbA, suggesting that a part of soluble expression products is no biological activity because of incorrect folding. This may be related to the intracellular reduction environment of *E. coli*. In the intracellular reduction environment, the formation of disulfide bonds are extremely limited and ultimately lead to the incorrect expression of disulfide bonds. Generally, it is believed that intracellular reduction is maintained by thioredoxin reductase (*TrxB*) and glutathione in E. coli (Locher and Griffiths 1999; Aslund and Beckwith 1999). Mutation of *trxB* genes and/or the glutathione redox enzyme gene (*gor*) can change the intracellular reduction environment in *E. coli* and promote the formation of intracellular disulfide bonds (Stewart et al. 1998). Thus, strains of *E. coli* deleted *trxB* or *gor* gene were used to further overexpress functional heterologous protein.

The recombinant *E. coli* is very useful not only for the detoxification of OPs but also for the rapid detection of OPs. Although, it has been reported that surface-expressed GFP exhibits a stronger fluorescence compared to cytosol

expressed GFP (Shi and Su 2001), our results showed that the fluorescence of whole cells displaying MPH-GFP fusion was very sensitive to extracellular pH changes (Yang et al. 2008a). Therefore, it does not correctly reflect the precise relationship between the fluorescence and the enzyme activity. In this study, the enhanced fluorescence intensity addressed the shortcomings of low fluorescence intensity of intracellular expression.

In conclusion, we obtained much improved bioconversion capability and efficiency for Methyl parathion as a model organophosphorus compound using the recombinant *E. coli* coexpressing MPH and DsbA as a whole cell biocatalyst. The strain expressing DsbA also showed highly increased biodegradation rate with increase of substrate concentrations compared to the control strain expressing MPH only, demonstrating that the DsbA coexpressed whole cell bioconversion system can more efficiently detoxify high concentration of organophosphates. Since the hydrolysis of OPs by MPH generates protons, it is possible to develop whole-cell biosensors for OP detection on the basis of the changes in pH by utilizing the whole cells coexprssing DsbA.

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