Over-expression and Purification of Recombinant Methylparathion Degrading Enzyme by Lactose Induction

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Abstract—Organophosphorus (OP) degrading enzymes were widely used in economical and safe detoxification of organophosphorus pesticides from soil, water, food, and aquatic product. In this study, a novel methylparathion degrading enzyme (MPD) was over-expressed in *Escherichia coli* BL21 (DE3) as a His-tagged fusion protein by the use of lactose as inducer instead of IPTG. SDS-PAGE combined with enzyme activity analysis indicated that lactose-induced expression yield of active MPD was increased nearly 2-fold compared with IPTG as inducer. The optimum temperature and concentration for lactose induction was 37 °C and 0.5 % (w/v), respectively. The expressed fusion proteins induced by the two inducers were both purified by Ni-metal-affinity chromatography. The specific activity of the purified recombinant MPD induced by lactose, reaching at 140 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$, was nearly 0.5-fold higher than that of the purified enzyme induced by IPTG. Thus, lactose was a well alternative inducer to produce active MPD and gave an advantage over enzyme purification. This study suggested a more effective method to produce purified MPD, an ideal enzyme to detoxify OP pesticides for ecosystem restoration.

Keywords—ethylparathion degrading enzyme; pollutant detoxification; Lactose inducer; Enzyme purification.

I . INTRODUCTION

Organophosphorus pesticides (OPs), widely used as a large group of pesticides in agriculture, are regarded as the

main pollution sources due to their severe toxicity to environment as well as to human beings [1]. The microbial degradation of OPs offers a promising strategy to solve the problems by screening organophosphorous degrading microorganisms as well as using bacterial enzymes. Methylparathion-degrading gene (*mpd*) has been intensively investigated and widely used in ecosystem restoration [2-5]. We previously cloned *mpd* gene from a novel organophosphorous-degrading bacterium naming HS-D36 and the intact *mpd* gene was expressed in *E.coli* by using pET expression system [6].

IPTG, the typical inducer for heterologous gene expression in *E.coli* expression systems, has main drawback of its high expense and toxicity to organisms. Lactose, the natural inducer for the *lac* operon, is therefore exploited to instead IPTG, especially in large scale fermentations [7-8].

In our previous research, the recombinant MPD induced by IPTG predominantly expressed as insoluble inclusion bodies. It significantly reduced the yield of active MPD as well as the purified MPD. In order to improve the efficiency of active MPD expression and purification for environmental pollutant detoxification, in this paper, we choose lactose as an alternative inducer to produce recombinant MPD in *E.coli* cells and purified the lactose induced proteins by Ni-metal affinity chromatography.

II . MATERIALS AND METHODS

A. Strains, plasmids, and chemicals

The bacterial strain HS-D36 containing the *mpd* gene, *E.coli* BL21 (DE3) used as heterologous expression hosts and plasmid pET-28a used for over-expression of recombinant MPD were stored in our laboratory. Enzymes for molecular cloning were purchased from TaKaRa. Other reagents including IPTG and lactose were purchased from Wuhan Yixin Biotech. Co. Plasmid pMD-mpd, the source of the *mpd* gene, and the expression recombinant plasmid pET-mpd were constructed as described previously [6].

B. Expression of recombinant MPD by induction of IPTG or lactose

BL21 (DE3) strains harboring pET-mpd were activated overnight followed by transferring and growing in LB medium plus 50 $\mu\text{g}\cdot\text{ml}^{-1}$ kanamycin at 37 °C to the optimum density ($\text{OD}_{600} = 0.6$). IPTG was added at a final concentration of 1.0 $\text{mmol}\cdot\text{L}^{-1}$ as inducer to produce the recombinant MPD. Meanwhile, lactose, an alternative inducer, was added from its stock solution to give the desired concentrations (w/v) of 0.05%, 0.1%, 0.2%, 0.5%, and 1.0%. Recombinant *E.coli* cells were harvested after 2- 16 h of induction. For lactose experiments, the temperature was reduced to 28 or 20 °C at the time point of induction. All the samples were harvested by centrifuging at 10,000 g for 10 min and the expressed proteins were analyzed by SDS-PAGE.

C. Purification of His₆-tagged MPD

The over-expressed His₆-tagged MPD by 8 h induction of 1 mM IPTG or 0.1% (w/v) lactose was determined by analysis of SDS-PAGE before purification by affinity chromatography. The HisTrap affinity column (1-ml) purchased from GE Healthcare was used for one-step purification of the recombinant MPD by using AKTA purifier system (GE Healthcare).

For the preparation of the crude enzyme, 1) the induced *E.coli* BL21 (DE3) cells harboring pET-mpd were harvested from 1.0 L cultures by centrifugation (10,000 g, 4 °C, 10 min); 2) the cell pellets were resuspended in

binding buffer (20 $\text{mmol}\cdot\text{L}^{-1}$ Tris-HCl, 500 $\text{mmol}\cdot\text{L}^{-1}$ NaCl, and 5 $\text{mmol}\cdot\text{L}^{-1}$ imidazole, pH 8.3) at 10 $\text{ml}\cdot\text{g}^{-1}$ of wet cells; 3) this cell suspension with the adding of 0.1 $\text{mmol}\cdot\text{L}^{-1}$ (final concentration) PMSF (protease inhibitor) was sonicated for about 5 min until the mixture was no longer viscous; and 4) the lysate was centrifuged (12,000 g, 4 °C, 15 min) to remove the debris.

The supernatant was subjected to a 1-ml Ni-metal-affinity column which had been equilibrated with the binding buffer. After washing with 20 ml washing buffer (20 $\text{mmol}\cdot\text{L}^{-1}$ Tris-HCl, 500 $\text{mmol}\cdot\text{L}^{-1}$ NaCl, and 5 $\text{mmol}\cdot\text{L}^{-1}$ imidazole, pH 8.3), His₆-tagged MPD was eluted from the column with elution buffer (20 $\text{mmol}\cdot\text{L}^{-1}$ Tris-HCl, 500 $\text{mmol}\cdot\text{L}^{-1}$ NaCl, and 90 $\text{mmol}\cdot\text{L}^{-1}$ imidazole, pH 8.3). The purified enzyme was subjected to specific activity determination for access of purification efficiency.

D. Assay of MPD activity

1 ml of MPD activity assay mixture was consisted of 50 μl methyl parathion (MP) (2 $\text{mg}\cdot\text{ml}^{-1}$), 850 μl assay buffer (50 $\text{mmol}\cdot\text{L}^{-1}$ Tris-HCl, pH 8.9), and 100 μl of crude enzyme MPD. A solution without the crude enzyme was set as a negative control. The rate of p-nitrophenol (PNP) release at room temperature in the dark was spectrophotometrically measured at 405 nm to determine MPD activity. One unit (U) of activity is defined as the formations of 1 μmol PNP per minute.

E. Assay of soluble protein content

The concentration of total soluble proteins was determined according to the method described by Bradford [9] using BSA as the standard.

III. RESULTS AND DISCUSSION

A. Over-expression of recombinant MPD by lactose induction

In *E.coli* expression system, the natural inducer of *lac* operon is lactose which has been used in large-scale fermentations [7]. In this study, we tested whether recombinant MPD could be expressed at high level in

E.coli BL21 (DE3) cells with lactose inducer.

As shown in Fig.1, 8-16 h induction of IPTG and lactose could both make recombinant *E.coli* BL21 (pET-mpd) produce an over-expressed protein with the molecular weight of 35 kD which was identical to the calculated mass of MPD. Thus, lactose could be used to instead IPTG for inducing recombinant MPD in BL21 (DE3). Interestingly, as observed in SDS-PAGE, another protein (about 37 kD) which band located a little upper to the 35 kD protein was also over-expressed with the lactose induction. Here, whether the two over-expressed protein bands around 35 kD were two different forms of *mpd* gene product needed further identification, such as western blotting analysis.

Figure 1. SDS-PAGE analysis of recombinant MPD expressed in BL21 (DE3) by IPTG or lactose induction. M: standard protein molecular weight (KD); the final concentrations of IPTG and lactose used in induction media were 1.0 mM and 0.1% (w/v), respectively; 0, 8, and 16 h were expressed as induction time points.

Nevertheless, in this study, the use of different dose of lactose (w/v) from 0.05% to 1.0% as the inducer at 37 °C all obtained higher enzyme activity compared to that of IPTG induction (data not shown). Under the optimum conditions for lactose induction (Fig.2), the activity of recombinant MPD reached to 83.9 U/ml, which was nearly 60% higher than that in IPTG induction (52.5 U/ml; see reference [6]). Therefore, lactose induction favored the yield of active MPD. As reported previously [10], recombinant protein expression in *E.coli* by IPTG induction often resulted in the accumulation of large amounts of unfolded proteins in the form of insoluble inclusion body. This would be more or less reduced in lactose induction, as described in our present experiments.

The optimum conditions for lactose induction were investigated in this paper. As shown in Fig.2, the optimum

temperature and induction time were 37 °C and 8 h,

Figure 2. Effect of temperature (A) and induction time (B) on the activity of recombinant MPD expressed in BL21 (DE3) by lactose induction. The final concentration of lactose in induction systems were 0.5% (w/v).

respectively, with 0.5% (w/v) of lactose. It yielded the maximum MPD activity of 83.9 U/ml at 37 °C for 8 h. However, the yield of active MPD for the same induction time was deduced by 32% at 28 °C, and by 51% at 20 °C. At the lower temperatures, the recombinant *E.coli* cell density decreased in accordance (data not shown). The optimum lactose concentration for the induction of MPD expression in our study was 0.5% (w/v) or below. Higher concentrations of lactose (above 1.0%) inhibited protein expression, which was also observed in MPH expression induced by lactose [11]. This decrease in enzyme expression was assumed to a result of lactose metabolism, but not due to cell death or *E.coli* growth restriction, for there was no negative change in cell density.

B. Purification of His₆-tagged MPD

The expressed His₆-tagged MPD induced by IPTG as well as lactose were both subjected to Ni-affinity chromatography for the enzyme purification. The crude extracts after sonication were applied to the Ni-NTA column under non-denaturing conditions. As shown in Fig.3, undesired proteins were washed away with buffer containing 5 mmol·L⁻¹ imidazole, and His₆-tagged MPD

was eluted with buffer containing 90 mmol·L⁻¹ imidazole.

Figure 3. SDS-PAGE analysis of recombinant MPD purification by Ni²⁺-affinity chromatography. M: standard protein molecular weight (KD); Lane 1: enzymatic supernatant extracted from BL21 (DE3) cells harboring pET-mpd with lactose induction; Lane 2: proteins not binding to Ni²⁺-affinity column; Lane 3: undesired proteins washed away with washing buffer; Lane 4: proteins eluted by buffer containing 30 mmol·L⁻¹ imidazole; Lane 5: proteins eluted by buffer containing 90 mmol·L⁻¹ imidazole.

Further, the specific activity of purified MPD using lactose as inducer was increased by 1.5-fold compared to that using IPTG as inducer (Table 1). It was assumed to be resulted from more yield of active MPD in lactose induction. Several organophosphorus hydrolases have been reported to be purified with specific activities of not more than 100 U/mg [3, 12]. In our study, the specific activity of MPD induced by lactose reached at 150.7 U/mg. It suggested that lactose induction system could be a well alternative to produce recombinant MPD for enzyme purification with higher efficiency.

Table 1. Purification of recombinant MPD by Ni-metal-affinity chromatography

Enzyme	Specific activity (U/mg protein)		Fold
	Before	After	
	purification	purification	
MPD induced by IPTG	30.8	72.4	2.4
MPD induced by lactose	41.2	150.7	3.7

IV. CONCLUSION

The results presented in this paper demonstrated the over-expression of the recombinant MPD by lactose induction. The optimum temperature and inducer concentration were 37 °C and 0.5% (w/v), respectively.

The lactose induction could produce more active MPD protein and yield better purified recombinant enzymes by using Ni-metal affinity chromatography. Here, we reported a more efficient method to express and purify MPD for OP pesticide detoxification in practice.

ACKNOWLEDGEMENTS

This work was partially supported by National Natural Science Foundation of China (30771429); The National High Technology Research and Development Program of China (2007AA05Z417); Specialized Research Fund for the Doctoral Program of Higher Education (20060511002) and by the Construction Fund for “211” Project of the Ministry of Education of China.

REFERENCES

- [1] Singh BK, Walker A. Microbial degradation of organophosphorus compounds. *FEMS Microbiol Rev*, 2006, 30: 428–471
- [2] Cui ZL, Li SP, Fu GP. Isolation of methyl parathion degrading strain M6 and cloning of the methyl parathion hydrolase gene. *Appl Environ Microbiol*, 2001, 67: 4922–4925
- [3] Fu GP, Cui ZL, Huang T, Li SP. Expression, purification, and characterization of a novel methyl parathion hydrolase. *Protein Expr Purif*, 2004, 36: 170–176
- [4] Yang C, Liu N, Guo X, Qiao C. Cloning of mpd gene from a chlorpyrifos-degrading bacterium and use of this strain in bioremediation of contaminated soil. *FEMS Microbiol Lett*, 2006, 265: 118–125
- [5] Zhang R, Cui ZL, Zhang X, Jiang J, Gu J, Li SP. Cloning of the organophosphorus pesticide hydrolase gene clusters of seven degradative bacteria isolated from a methyl parathion contaminated site and evidence of their horizontal gene transfer. *Biodegradation*, 2006, 17: 465–472
- [6] Wang BB, Xiong L, Zheng YL, Zhang ZX, Tong WY, Liu S, Chen YH, Xiao WJ, Liu DL. Cloning and expression of the mpd gene from a newly isolated methylparathion-degrading strain of bacteria. *Acta Scientiae Circumstantiae*, 2008, 28: 1969–1975
- [7] Neubauer P, Hofmann K. Efficient use of lactose for the lac promoter-controlled overexpression of the main anti-genic protein of the foot and mouth disease virus in *E. coli* under fed-batch fermentation conditions. *FEMS Microbiol Rev*, 1994, 14: 99–102
- [8] Monteiro R, Souza EM, Yates MG, Pedrosa FO, Chubatsu LS. Use of lactose to induce expression of soluble NifA protein domains of *Herbaspirillum seropedicae* in *E. coli*. *Can J Microbiol*, 2000, 46: 1087–1090
- [9] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, 1976, 72: 248–254
- [10] Schein CH, Noteborn MHM. Formation of soluble recombinant proteins in *E. coli* is favored by lower growth temperature. *Biotechnology*, 1988, 6: 291–294
- [11] Yang JJ, Yang C, Jiang H, Qiao CL. Overexpression of methyl parathion hydrolase and its application in detoxification of organophosphates. *Biodegradation*, 2008, 19: 831–839
- [12] Chu XY, Wu NF, Deng MJ, Tian J, Yao B, Fan YL. Expression of organophosphorus hydrolase OPHC2 in *Pichia pastoris*: purification and characterization. *Protein Expr Purif*, 2006, 49: 9–14