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DOI: 10.2225/vol14-issue6-fulltext-2 *SHORT COMMUNICATION*

Expression and non-chromatographic purification of 1,3-propanediol oxidoreductase in *Escherichia coli*

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Abstract The gene *dha*T from *Klebsiella pneumoniae* encodes 1,3-propanediol oxidoreductase (PDOR). Thermally responsive elastin-like polypeptides (ELPs) was used as a fusion tag to purify the proteins (PDOR). The ELP gene was attached to *dha*T and ligated into the pET-22b vector. Different NaCl concentrations were employed to decrease the transition temperature (T_t) which was diminished as salt concentration increased. The optimal final concentration of NaCl was 1 M and the corresponding T_t was 39.5ºC. Enzymatic assays were determined via every step for purification of fusion PDOR. PDOR showed good stability during the purification process, the specific activity in the first and second round of inverse transition cycling (ITC) was 276.1 ± 13.3 and 213.3 ± 10.8 U/mg, respectively. The ELPs fusion PDOR was superior to histidine tagged PDOR in both yield and activity after the purification.

Keywords: 1,3-propanediol oxidoreductase, elastin-like polypeptides, *Escherichia coli*, fusion protein, non-chromatographic purification

INTRODUCTION

1,3-propanediol (1,3-PD), a bulk chemical, can be formulated into a variety of industrial products especially in the synthesis of polytrimethylene terephthalate and other polyester fibers (Zheng et al. 2004). In the model organism, *Klebsiella pneumonia*, the glycerol metabolism primarily involves two branch pathways: the reductive branch and the oxidative branch. 1,3-propanediol oxidoreductase (PDOR, E.C. 1.1.1.202) encoded by *dha*T gene is the key enzyme in oxidative procedure, which catalyzes 3-hydroxypropionaldehyde (3-HPA), production of reductive branch into 1,3-PD under the consumption of reducing power $NADH_2$ (Fenghuan et al. 2005). The crystallographic structure of PDOR, a type III Fe-NAD-dependent alcohol dehydrogenase, has been determined to be a decameric structure with exactly the same monomer (Marçal et al. 2009).

Large-scale protein separation and purification are key problems in the current biological engineering. Purification procedures account for about 60% to 70% of the total costs (Lim et al. 2007). A variety of affinity based chromatographic purification schemes have been developed to simplify protein purification in the laboratory scale, for example, the fusion tag technique which greatly simplifies the purification of target proteins (Nilsson et al. 1997). Through the integration of a specific polypeptide (*e.g.*, histidine tag), targeted proteins can be purified by high affinity or high specificity. However, the high cost of chromatography requires not only the special equipment but also the customized solid phase extraction. It is difficult to apply in the industrial scale owing to its low efficiency (Lim et al. 2007). Elastin-like polypeptides (ELPs), derived from the elastin hydrophobic region, are oligomeric repeats of

the pentapeptide Val-Pro-Gly-Xaa-Gly (where the "guest residue" Xaa is any amino acid except proline) that undergo a reversible inverse transition. They are highly soluble in water below the inverse transition temperature (T_t), but undergo a sharp (2-3ºC range) phase transition when the temperature is raised above T_t, leading to desolvation and aggregation of the polypeptide (Urry, 1997; Urry et al. 2010). As the result of this special character, ELPs have been widely used in protein purification and plasmid DNA separation (Kostal et al. 2004; Kim, 2005a; Kim, 2005b; Gao et al. 2006).

Previously, we designed a new type of ELP (*i.e.* ELP [KV8F-20]), which designated the guest residues to three amino acids, Lys, Val, Phe, in the ratio of 1:8:1. It had 20 pentapeptide repeats. The *de novo* designed gene was synthesized and successfully expressed in the host bacterium (Huang et al. 2011). In this study, we had PDOR gene *dha*T fused with ELPs and expressed in two different *E. coli* strains. Histidine tag attached to PDOR was inefficient and complex during chromatography purification. We now report the application of ELP tag to separate PDOR from cell-free extract with satisfying results.

MATERIALS AND METHODS

Materials and microorganisms

E. coli BL21 (DE3) and BLR (DE3) were conserved in our laboratory. Plasmid pUC19-ELP (Huang et al. 2011) and pUC57-*dha*T (Li et al. 2011) were previously constructed. Plasmid pET-22b(+) were purchased from Novagen (Darmstadt, Germany). NAD was bought from Roche (Shanghai, China). Restriction enzymes were bought from TaKaRa (Dalian, China). All other chemicals used in this study were of analytical grade commercially available.

Construction of expression vector for ELP fusion PDOR

In Figure 1, pUC19-ELP vectors were extracted and double digested by *Nde*I and *Hin*dIII restriction enzymes. DNA fragments were purified and ligated into pET-22b(+), then the plasmid pET-22b-ELP was transformed into *E. coli* DH5α. The transformants were screened and tested by double digestion. The positive strains were sent to Genscript Co., Ltd. for sequencing.

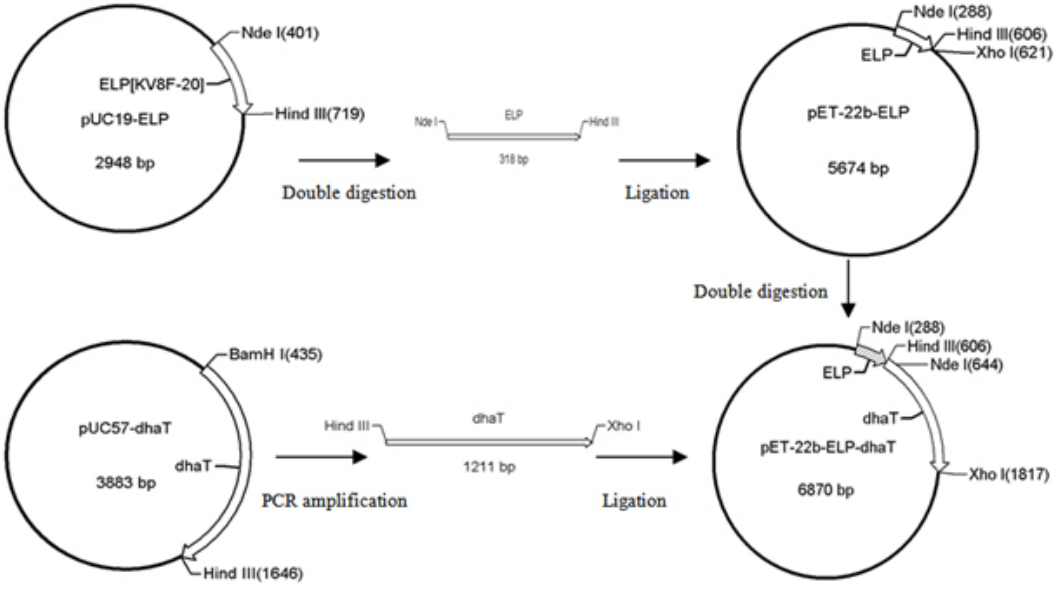

Fig. 1 The procedure of pET-22b-ELP-*dha*T construction.

We utilized PCR to amplify *dha*T gene in the vector pUC57-*dha*T and replace *Bam*HI by *Xho*I. Upstream primer (5'-ACTTAAGCTTGCATGCAGGCCTCTGCAGT-3') was in strict accordance with the sequence, but we added a palindromic sequence which recognized by *Xho*I in the downstream primer (5'-CAGCTCGAGTTAGAAGGCCTGACGGAAAATC-3'). Meanwhile, as the *dha*T gene was inserted to pUC57 in reverse order owing to single restriction enzyme *Sma*I which would cause random directions, the correct forward direction was from *Hin*dIII to *Xho*I after PCR.

The purified gene with a length of 1211 bp was ligated into pET-22b-ELP, which had already been double digested by *Hin*dIII and *Xho*I. The recombinant plasmid pET-22b-ELP-*dha*T was constructed and testified by single and double digestion, positive clones were sent to sequence. Between the ELP and PDOR genes, there was a transition interval, the exact position was from *Hin*dIII(606) to *Nde*I(644).

PDOR expression and activity assay

The transformed single colony was inoculated to 5 mL LB medium (10 g/L NaCl, 10 g/L tryptone, 5 g/L yeast extract) and cultured for 16 hrs at 37ºC. Then cell culture was inoculated to 50 mL fermentation medium (12 g/L tryptone, 24 g/L yeast extract, 3 g/L NH_4Cl, 4.5 mL/L glycerol, 1.7 mM KH_2PO_4, 7.2 mM K_2HPO_4) for 2 hrs at 30ºC. The inducer, isopropyl β-D-thiogalactoside (IPTG) was added to the medium at the final concentration of 1 mM. Induced *E. coli* were collected by centrifugation and broken up by sonication at 300 W for 2 sec operation and 2 sec interval with 60 cycles in ice bath, then centrifuged at 15,000 g for 15 min to collect supernatant. The Ni-IDA resin was previously used to purify PDOR by affinity chromatography (Li et al. 2011). Proteins in the cell-free extract and purified ones were detected by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Brilliant Blue R-250.

The activity of PDOR was determined through initial velocity method as described by Ahrens (Ahrens et al. 1998). Both cell-free extract and purified protein were analyzed by dynamic absorbance at 340 nm in potassium carbonate buffer. Enzymatic properties were studied by purified PDOR.

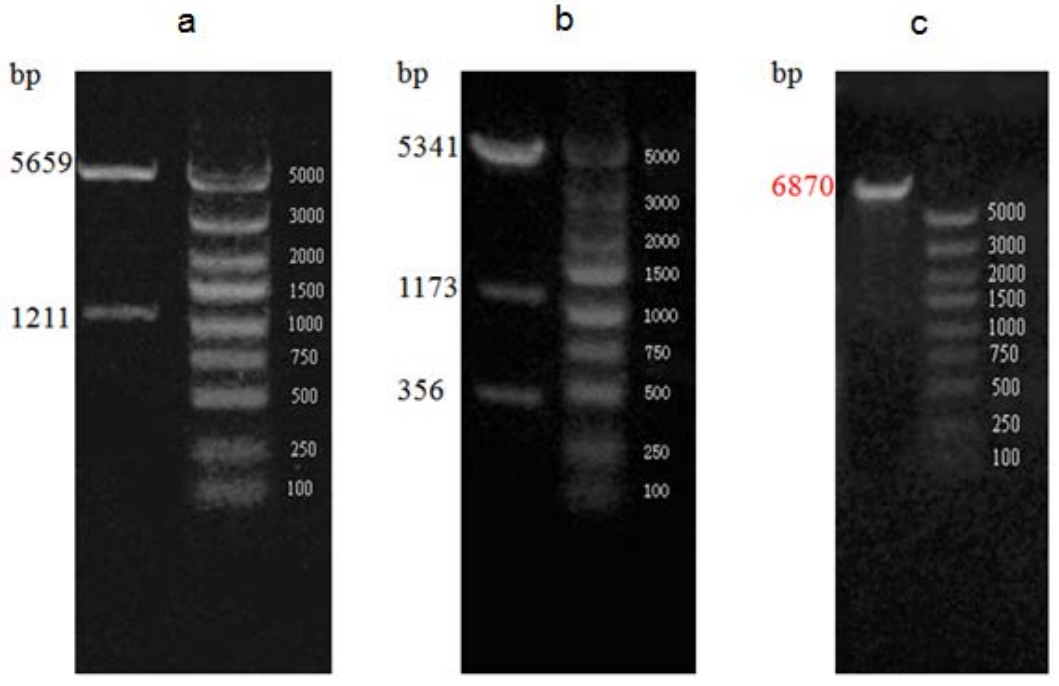

Fig. 2 Single and double digestion of pET-22b-ELP-*dha*T. (a) The vector was digested by *Hin*dIII and *Xho*I, the lower band was 1211 bp. (b) The vector was digested by *Nde*I and *Xho*I, the length of two lower bands was 356 and 1173 bp. (c) Single digestion of pET-22b-ELP-*dha*T by *Hin*dIII, the total length was 6870 bp.

Inverse transition cycling purification and detection

Through the purification by inverse transition cycling (ITC) (Meyer et al. 2001), ELP fusion proteins were aggregated by increasing the temperature of the cell lysate to ≤ 45ºC and/or by adding NaCl to a concentration ≤ 2 M. The aggregated fusion proteins were separated from the solution by centrifugation at 45ºC at 15,000 g for 15 min. The supernatant was decanted and discarded, and the pellet containing the fusion protein was resolubilized by agitation in the cold phosphate-buffered saline (PBS) buffer. The resolubilized pellet was then centrifuged at 4ºC to remove any remaining insoluble matter. Two rounds of ITC were carried out. Purified fusion proteins were then determined by SDS-PAGE.

Optical absorbance at 350 nm of ELP fusion solutions in the 10-60ºC range was monitored on an ultraviolet-visible spectrophotometer equipped with a temperature control device. The T_t was determined from the midpoint of the transition-induced change at a heating rate of 2ºC/min. The Weibull model was applied to fit the transition curve, quadratic equation was used to regress transition temperature versus the salt concentration curve. Both curves were fitted by marquardt iterative and evaluated by correlation coefficient and F value. The calculations were performed in DPS 7.05 software.

Table 1. Enzyme activity assay of PDOR fusion protein in the cell-free extract.

Strain	Enzyme activity (U/mL)	Protein concentration (mg/mL)	Specific activity (U/mg)
BL21 (DE3)	245.8 ± 4.3	9.9	24.8 ± 0.4
BLR (DE3)	356.3 ± 5.8	13.0	27.4 ± 0.5

RESULTS AND DISCUSSION

Construction of expression vector pET-22b-ELP-*dha*T

Two fragments were ligated exactly as designed and inserted correctly into the expression vector after sequential double subcloning operations. Single and double digestions were employed to verify the expression vector pET-22b-ELP-*dha*T (Figure 2) and followed by sequencing for further confirmation.

The validated vectors were transformed into competent cell *E. coli* BL21 (DE3) and BLR (DE3). These transformants were cultured overnight in LB medium with 100 μg/mL ampicillin added and inoculated to fermentation medium to express PDOR under the induction of IPTG. Enzyme activities and the SDS-PAGE of cell-free extract were shown in the Table 1 and Figure 3, respectively.

Through densitometry analysis to target bands by Tanon GIS (Shanghai, China) from Figure 3, BLR (DE3) strain was better than BL21 (DE3) in the level of protein expression and with higher enzymatic activity. So, we used cell-free extract from BLR (DE3) in the following purification procedures. BLR (DE3) strain was especially suitable for expression tandem repeat sequences like ELP gene (Schmidt et al. 2007). From the analysis of the cell-free extract, our target protein lay between marker 50 and 71 kDa. The pure PDOR was 42 kDa and ELP tag was 10 kDa, so the fusion protein was approximately 52 kDa. However, due to the special structure of ELP, which caused the lagged band (20% greater than the molecular weight) in SDS-PAGE (Meyer and Chilkoti, 2002; Cho et al. 2008), the fusion protein was obviously higher than 50 kDa marker.

ITC purification and fusion PDOR stability research

In each round of ITC purification, fusion protein was resolubilized in PBS buffer to 1/30 of culture volume. The resolubilized fusion protein in each round was determined by SDS-PAGE. As shown in Figure 3, there was a sharp drop of protein impurities after first round ITC and hardly any protein impurities remained after the second round ITC was completed.

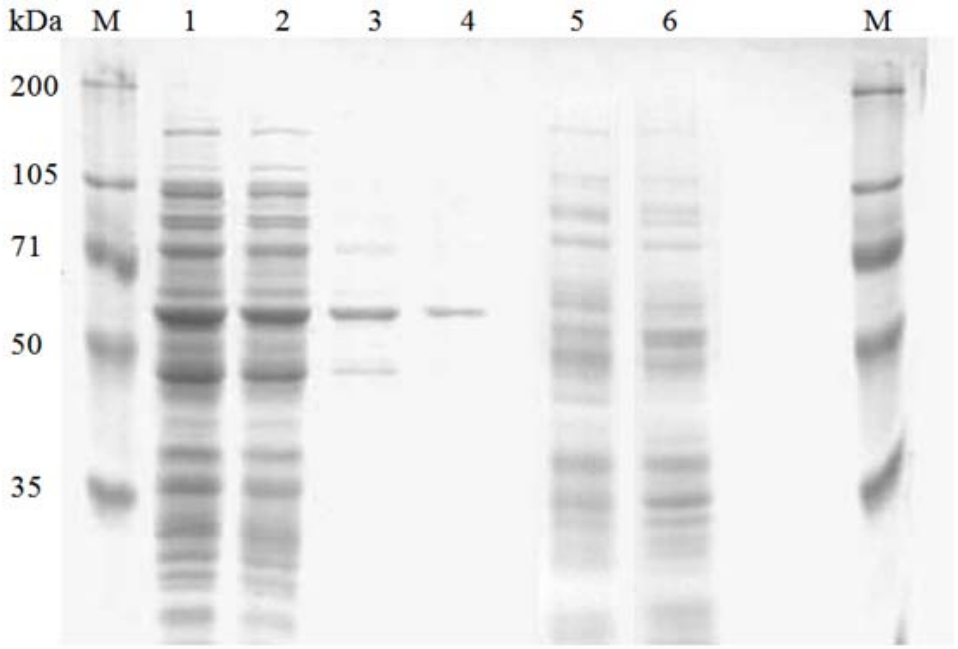

Fig. 3 SDS-PAGE of analysis of PDOR fusion protein. *Lane M* protein molecular marker; *lane 1* cell-free extract from BLR (DE3) strain induced by IPTG; *lane 2* cell-free extract from BL21 (DE3) strain induced by IPTG; *lane 3* purified fusion protein from BLR (DE3) strain of first ITC purification; *lane 4* purified fusion protein from BLR (DE3) strain of second ITC purification; *lane 5* cell-free extract from BLR (DE3) strain without induction; *lane 6* cell-free extract from BL21 (DE3) strain without induction; all samples were normalized by wet cell weight, dilution multiple was 50 for cell-free extract samples, loading amount from lane 1 to 6 were 7.8, 6.0, 1.1, 0.7, 3.8, 4.2 μg, respectively.

The relative turbidity was calculated by Meyer's method (Meyer and Chilkoti, 1999). At first we determined the change of relative turbidity without NaCl. A sharp increase in turbidity was occurred within the range from 54 to 58ºC because of the aggregation of ELP in Figure 4a. The Weibull model was applied to fit the calculated curve, the equation was presented as following. R was the correlation coefficient; F value was calculated to identify if the regression model fit the data well, great F value meant that the independent variables had significant effect on the dependent variable; x means temperature; y means relative turbidity. Weibull model was suitable to depict dramatic changes in the short term, so this model was able to predict the results for temperatures above 54ºC (Figure 4a) more appropriately than that below 54ºC. Their mean absolute errors were 0.019 and 0.036 respectively.

$$y = 1.02\left(1 - e^{-\left(\frac{x+215394.32}{215451.18}\right)^{101745.82}}\right) \quad \text{(R=0.99 F=866.43)}$$

[Equation 1]

T_t, defined to be the temperature at 50% maximal turbidity, is a convenient parameter to describe this process. The T_t for fusion protein was 56ºC when no salt was added and it was lower than that of free ELP (Huang et al. 2011), presumably because the much higher molecular weight of fusion protein contributed to the aggregation. Ionic strength effect was determined in Figure 4b: the transition temperature T_t decreases as the NaCl concentration increases, meanwhile, T_t and NaCl concentration show a significant quadratic relationship which x means the NaCl concentration and y means T_t value.

$$y = 55.53 - 21.31x + 4.86x^2 \quad \text{(R=0.99 F=182.43)}$$

[Equation 2]

However, the transition temperature of fusion protein decreased less obviously than that of free ELP (Huang et al. 2011), especially when salt concentration exceeded 1.5 M. On the one hand, low transition temperature ensured the mild operation temperature in purification and alleviated the enzyme activity loss caused by high temperature; on the other hand, salt should be added the less the better (Lim et al. 2007). After a comprehensive consideration, we chose 1.0 M as the optimal NaCl concentration during ITC purification.

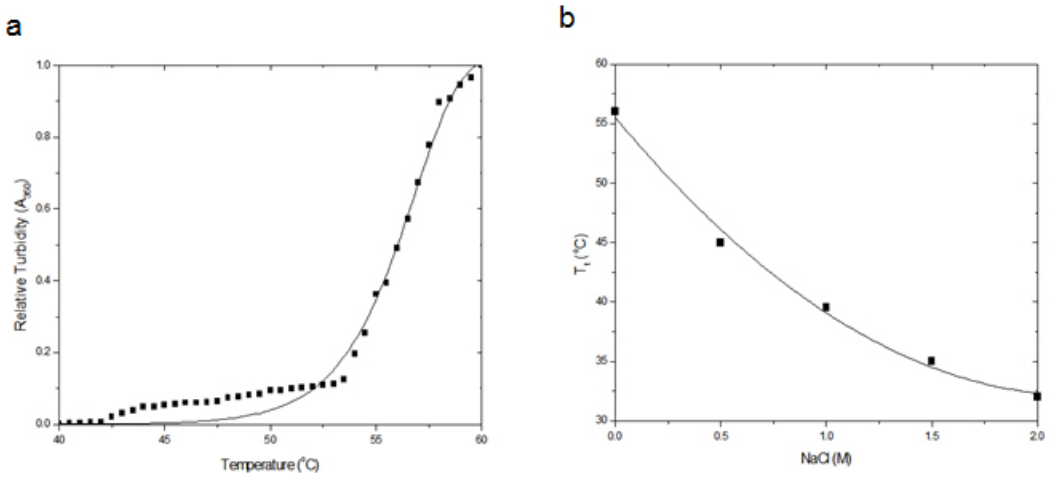

Fig. 4 Characterization of the inverse transition of ELP fusion protein in PBS buffer. (a) Theoretical curve calculated according to the Weibull model and (b) transition temperature *vs* salt concentration curve for ELP fusion protein.

NaCl was added at the concentration of 1.0 M to the cell-free extract to conduct two rounds ITC purification at 45ºC, the specific activity of PDOR fusion protein was determined at each round. As we can see in Table 2, PDOR activity was enhanced significantly from 27.4 ± 0.5 to 276.1 ± 13.3 U/mg after the first round of ITC. Because the first round removed a large amount of impure proteins in the cell-free extract. Moreover, the enzyme was previously tested to be stable at 45ºC (Li et al. 2011), so the quick and simple purification operation retain on the enzyme activity. After the second ITC round, the specific activity decreased to 213.3 ± 10.8 U/mg as the loss of fusion protein during purification and the reduction of enzyme activity itself at 45ºC. The protein concentration after first and second round of purification was 0.18 mg/mL and 0.12 mg/mL to the final volume of 1.3 mL. When harvesting the same quantity of wet cells with purification by histidine tag affinity chromatography, the purified protein of histidine tagged enzyme (48 kDa) was 0.075 mg/mL at the volume of 1.0 mL and the specific activity was 50.6 ± 3.9 U/mg (Figure 5).

Our ELPs fusion enzyme was quickly and simply purified by two rounds of ITC in this study. Meanwhile, the enzyme activity retained a lot after purification and highly surpassed (14-fold) that of histidine tagged enzyme, their recovery rates were 14.0% and 1.1%, respectively. The recovery rate of the second round ITC decreased to 7.2% because of the protein and activity losses during process of purification. Therefore, first round of ITC was considered to be more appropriate for enzyme acquisition and enzymatic reaction usage, although the purity was higher after two rounds of ITC.

Table 2. Purification comparison between ELP tag and histidine tag. Protein losses for each type of purification were compared with total protein amount of cell-free extract.

Purification type	Enzyme activity (U/mL)	Protein concentration (mg/mL)	Volume (mL)	Specific activity (U/mg)	Protein loss (mg)	Recovery rate (%)	Purification fold
Cell-free extract from *E. coli* BLR (DE3)	356.3 ± 5.8	13.0	1.3	27.4 ± 0.5	-	100	1
ELP tag (First round ITC)	49.7 ± 2.4	0.18	1.3	276.1 ± 13.3	16.67	14.0	10.1
ELP tag (Second round ITC)	25.6 ± 1.3	0.12	1.3	213.3 ± 10.8	16.74	7.2	7.8
Histidine tag (Affinity Chromatography)	3.8 ± 0.3	0.075	1.0	50.6 ± 3.9	16.83	1.1	1.8

Many kinds of fusion proteins like thioredoxin (Lim et al. 2007), tendamistat (Meyer and Chilkoti, 1999), fluorescent protein, calmodulin (Trabbic-Carlson et al. 2004) have been successfully purified with ELPs in *E. coli*, enzymes especially oxidoreductases were seldom reported. β-lactamase was recombined with self-cleaving ELPs tag (Fong et al. 2009). The purification folds at room temperature and 37ºC were 15 and 9.9, which were quite close to our results. However the 110 repeats of ELPs tag they applied was much greater, molecular weight of β-lactamase was only 29 kDa. Because of the sensitive and vulnerable characteristics of PDOR, purification protocols were further studied and optimized. This is the first report for enzyme purification within the range of 40-50 kDa using the ELPs tag of only 20 pentapeptide repeats.

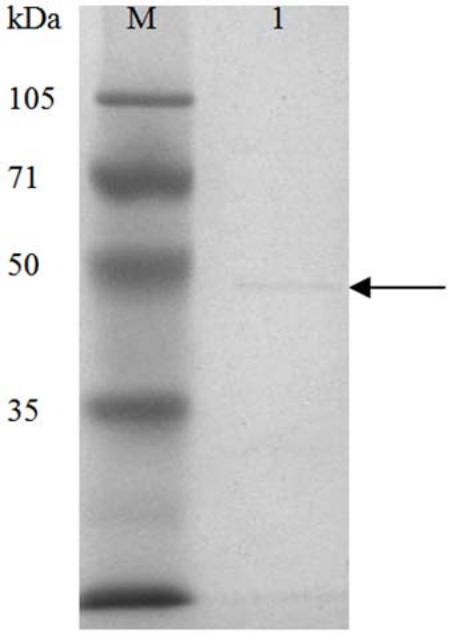

Fig. 5 SDS-PAGE assay of purified PDOR by histidine tag. *Lane M* protein molecular marker; *lane 1* purified PDOR from BL21 (DE3) strain by histidine tag; dilution multiple was 50 for cell-free extract sample, loading amount in each lane was 0.45 μg.

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