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Production and Characterization of the Recombinant *Sphingomonas chlorophenolica* Pentachlorophenol 4-Monooxygenase

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Pentachlorophenol 4-monooxygenase (PCP4MO) from *Sphingomonas chlorophenolica* is a flavoprotein that hydroxylates PCP in the presence of NADPH and oxygen. In order to investigate the structure and function of active site, recombinant PCP4MO (rePCP4MO) was produced in *Escherichia coli* as a glutathione *S*-transferase (GST) fusion protein. Moreover, a tobacco etch virus (TEV) protease cleavage site (EKLYFQG) was introduced into GST-PCP4MO and a his-tagged TEV protease was employed. Hence, a two-step purification protocol was developed which allowed obtaining 15–20 mg of rePCP4MO from 1 L culture. The rePCP4MO revealed identity with native enzyme by SDS–PAGE and N-terminal sequence analyses. Furthermore, a polyclonal PCP4MO antibody was produced with GST-PCP4MO and purified by immunoaffinity chromatography, where both the native and recombinant forms of PCP4MO showed interaction. However, rePCP4MO was identified as apoprotein with no evidence for a typical flavoprotein spectrum. The catalytic activity could be detected in the presence of FAD. The K_m and V_{max} values for PCP were 50 μM and 30 nmol/min/mg, respectively. © 2001 Academic Press

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Pentachlorophenol (PCP) has been used as a pesticide and herbicide for many years (1). Its widespread use has caused contamination of soil, groundwater, and even drinking water. Bioremediation of PCP contamination has therefore become an important focus of research. Several microorganisms have been isolated that are able to degrade PCP (2–6). The biochemistry and genetics of PCP degradation by *Sphingomonas chlorophenolica* sp. ATCC39723 (formerly *Flavobacterium* sp.) have been extensively studied (7–9). The degradation of PCP is initiated by pentachlorophenol 4-monooxygenase (PCP4MO) encoded by the *pcpB* gene. The enzyme is a flavin monooxygenase that converts PCP to tetrachloro-*p*-hydroquinone (TeCH) utilizing O_2 and two equivalents of NADPH. The enzyme not only catalyzes dehalogenation but also replaces hydrogen, nitro, amino, and cyano groups with a hydroxyl group at the *para* position (10). The ability of PCP4MO to degrade high concentration PCP and a broad spectrum of substrates should make it a good candidate for rational enzyme engineering to improve bioremediation potential. However, the three-dimensional structure of PCP4MO has not been solved yet. In fact, little information is presently available on the structure and catalytic mechanism of its active site.

Structural analysis is often hampered by the inability to isolate large quantities of the protein from its native source. Recombinant DNA technology provides a convenient way to obtain large amounts of proteins for biochemical and biophysical characterization of their structure and function. The production of polypeptides in frame with GST allows one-step purification of the fusion protein from crude bacterial extracts by affinity chromatography on a glutathione-agarose or glutathione-sepharose column under nondenaturing conditions (11). GST is subsequently cleaved from the target protein by a site-specific protease such as thrombin, blood coagulation factor Xa, or PreScission protease. In general, a limitation of this system is that some GST fusion proteins are partially or completely insoluble (12). In some cases, the fusion protein is not efficiently eluted from the glutathione-matrix (12) or the cleavage by the protease is not strictly specific (13).

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We describe here expression and purification of recombinant PCP4MO using a modified pGEX expression system, identification of rePCP4MO by SDS–PAGE and immunoblot analyses, N-terminal sequence comparison and characterization of enzyme properties. The rePCP4MO should provide material for 3D-structure analysis by protein crystallography and for more detailed functional analysis by site-directed mutagenesis.

MATERIALS AND METHODS

Construction of plasmid. To construct the expression plasmid, pGEX/pcpB, the DNA fragment encoding PCP4MO was amplified from the plasmid, pCL3, (generously donated by Dr. R. Crawford, University of Idaho, USA) by PCR amplification. Two oligonucleotide primers were designed (5′-GAGATTGGGATCCCCTCGACCTATCCAATCAATGCG-3′ and 5′-CGCACGGAATTCATTAGGCGCACGGCACAAGCATC-3′) which defined the *pcpB* sequence and introduced *Bam*HI and *Eco*RI cleavage sites (underlined) at the 5′ and 3′ end of the PCR product, respectively. PCR amplification was performed using native *Pfu* polymerase (Stratagene, USA). The condition of PCR was essential according to the introduction manual. The PCR product was digested with *Bam*HI and *Eco*RI, and ligated into the *Bam*HI and *Eco*RI sites of pGEX-3X (Pharmacia Biotech, Sweden).

Oligonucleotides coding (5′-GATCCTCGAGAAGCTTTATTTTCAGGGCCG-3′ and 5′-GATCCGGCCCTGAAAATAAAGCTTCTCGAG-3′) for a TEV protease recognition site EKLYFQG (underlined) were mixed in equal amounts and heated at 65°C for 5 min before cooling down to 30°C. Subsequently, the annealed pair of oligonucleotides, containing *Bam*HI cohesive ends, was ligated into the *Bam*HI-digested pGEX/pcpB. The resulting plasmid, pGEX/HW, created an open-reading frame coding for the GST-factor Xa cleavage site-TEV cleavage site-PCP4MO fusion protein.

Expression and purification of GST-PCP4MO. Escherichia coli JM109 cells harbouring the expression plasmids were grown in LB medium containing 100 μg/ml ampicillin overnight at 37°C, and were subcultured in 1 liter of 2 × YT medium supplemented with 2% glucose and ampicillin to an optical density of 0.8–1 at 600 nm. The culture was then induced at 25°C for an additional 3–6 h by adding isopropyl β-D-thiogalactopyranoside (IPTG). The cells were harvested by centrifugation at 5000 rpm and 4°C for 10 min.

The pellets were suspended in 25 ml of STE (20 mM Tris pH 8, 150 mM NaCl, 1 mM EDTA, 1 mM DTT), supplemented with 200 μg/ml of lysozyme, 20 μg/ml DNAase, 1 mM protease inhibitor phenylmethylsulfonyl fluoride (PMSF), and 20 μM FAD. The suspension was incubated for 15 min at 4°C, and Sarkosyl was added to a final concentration of 2.5%. The cells were disrupted by sonication as described previously (14), and the cell debris was removed by centrifugation at 12,000 rpm and 4°C for 20 min. After adding 3% Triton X-100, the supernatant was filtered through a 0.45-μm membrane (Millipore) to prevent clogging of resin. The supernatant was mixed with 4 ml 50% glutathione-sepharose 4B. The mixture was rotated end-over-end at 4°C for 1 h, and packed into a 30-ml poly-PreChromatograph column (Bio-Rad, USA). The column was washed extensively with STE buffer. The fusion protein was competitively eluted by reduced glutathione using elution buffer (100 mM Tris pH 9, 20 mM glutathione).

Purification of recombinant PCP4MO. GST-PCP4MO was cleaved either in eluent or on-column by factor Xa (Pharmacia, Biotech, Sweden) or his-tagged TEV protease (GIBCO-BRL, USA or EMBL, Germany). For cleavage in eluent, glutathione was removed by exchanging buffer with Econo-Pac10DG disposable chromatograph columns (Bio-Rad, USA). Approximately 1 mg of fusion protein was incubated with 10 μg of factor Xa at 25°C or rTEV at 30°C for 1 h. GST and uncleaved fusion protein was removed by rechromatography of the cleavage mixture with glutathione-sepharose. The fraction containing rePCP4MO and rTEV protease was subsequently loaded onto a Ni^{2+}-agarose column to remove rTEV.

The fusion protein was also cleaved by rTEV on-column. Protease rTEV (10–20 μg) was added to 1 ml bed-volume of washed beads, to which the fusion protein had been adsorbed. The slurry was incubated end-over-end at 4°C overnight, and centrifuged (500*g*, 4°C, 5 min). The supernatant was collected, and the protease was removed by passing over the Ni^{2+}-agarose column.

Production and purification of polyclonal antibody against PCP4MO. The antibody production service was purchased from University of Oulu, Finland. Briefly, rabbits were immunized with GST-PCP4MO and the immune sera were collected. IgG-fraction was obtained from serum by precipitation twice with $(NH_4)_2\ SO_4$ added to 33.3% saturation solution and salt was removed by exchange buffer. GST antibody was removed from IgG-fraction by GST-cross-linked glutathione sepharose. Finally, the desired antibody against PCP4MO was affinity-purified with a PCP4MO-coupled HiTrap NHS-activated affinity column (Pharmacia Biotech, Sweden).

Enzyme identification. Recombinant PCP4MO was identified by SDS–PAGE, immunoblotting and N-terminal sequencing analyses. Samples were loaded onto 12% SDS–PAGE and stained with Coomassie blue. Immunoblotting was performed by electroblotting proteins onto a nitrocellulose membrane (Trans-Blot, Bio-Rad, USA) (14). Rabbit anti-PCP4MO (1:1000) or goat anti-GST (1:1000) antibodies was used as primary antibody, and horseradish peroxidase conjugated anti-rabbit IgG or alkaline phosphatase conjugated anti-goat IgG was as probe. The blots were developed by using the chemiluminescent Super Signal detection system (Pierce, USA), or substrate solution containing NBT (nitro blue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3-indolyl phosphate). N-terminal sequencing of rePCP4MO was performed by Dr. Nisse Kalkkinen (University of Helsinki) as previously described (15).

Enzyme assays. Enzyme activity of rePCP4MO was measured by monitoring conversion of PCP into TeCH. The analysis was performed as previously described by HPLC on a LiChroCART 250-4 column (Merck, Germany) (9). Reaction mixture was composed of 100 μM PCP, 100 μM NADPH, 5 μM FAD, and 500 μg enzyme in 2.5 ml 50 mM potassium phosphate (KP_I) pH 7.0 at RT (20–25°C). At interval, aliquots of reaction mixture were withdrawn and acidified by HCl, and centrifuged (13,000 rpm, RT, 10 min). The supernatant was injected onto the column. Concentration of PCP and TeCH was analyzed by comparing their peak areas with that of authentic standards. The Michaelis–Menten kinetic parameters were determined by measuring the initial rates at 5 min in a 500 ml of reaction mixture with 150 μg of rePCP4MO. The initial rates were detected by determining the amount of PCP depleted. The values of K_m and V_{max} for PCP were determined with a fixed concentration of NADPH (100 μM) and a variable concentration of PCP (from 20 to 80 μM).

RESULTS

Purification of GST-PCP4MO fusion protein. GST-PCP4MO was identified as an 89-kDa-protein band on SDS–PAGE, which was verified by immunoblotting with GST antibody. The yield was increased by an increase in the induction time and temperature. However, most of the GST-PCP4MO was found in the insoluble fraction.

The cells were lysed in the presence of detergent Triton X-100, Sarkosyl, guanidinium hydrochloride, or urea. Only Sarkosyl was found to achieve a significant solubilization effect. Most of the insoluble protein was

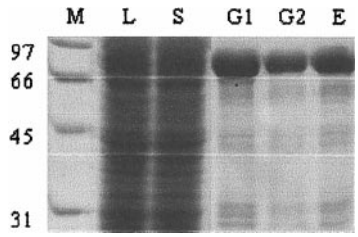

FIG. 1. Purification of GST-PCP4MO fusion protein. Lane L, lysate; S, supernatant; G1, GST-PCP4MO bound beads; G2, beads after elution; E, eluent with elution buffer (100 mM Tris pH 9, 20 mM glutathione); M, marker proteins.

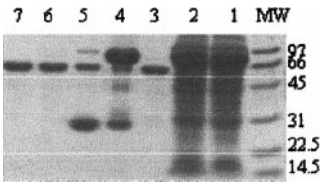

FIG. 3. Purification of recombinant PCP4MO. MW, molecular weight; lane 1, supernatant, L 2, flowthrough; L 3, eluent from glutathione-Sepharose column after digestion by rTEV; L 4, GST-PCP4MO bound glutathione beads; L 5, glutathione beads after digestion; L 6, eluent from Ni^{2+} column, L 7, wash fraction from Ni^{2+} column.

observed to become soluble at Sarkosyl concentrations between 1.5 and 3%. However, an additional problem was encountered in the subsequent binding of the solubilized fusion protein to glutathione-sepharose. Previous studies showed that nonionic detergents are able to sequester the Sarkosyl (12), which is known to be compatible with the binding of fusion protein to glutathione-sepharose (11). We therefore studied whether the addition of non-ionic detergents could enhance the binding of GST-PCP4MO to the column. The amount of bound GST-PCP4MO was shown to be close to saturation with 3–4% Triton X-100.

It was desired to efficiently elute GST-PCP4MO from the glutathione-sepharose column. The elution buffer commonly used contains 10 mM reduced glutathione to competitively elute GST from column. GST-PCP4MO was not, however, efficiently eluted by 10 mM reduced glutathione. Increasing the glutathione concentration, the ionic strength, or the addition of 0.1% Triton X-100 did not significantly increase GST-PCP4MO elution. In contrast, adjusting pH of the elution buffer from 8 to 9 clearly increased the amount of eluted protein. Thus, GST-PCP4MO could be considerably purified using this modified approach (Fig. 1).

Cleavage of GST-PCP-4MO fusion protein. For crystallization, GST must be removed from PCP4MO by a protease. The cleavage efficiency by factor Xa was tested. Figure 2A shows the time course for cleavage of the fusion protein using a ratio of 1:100 for factor Xa to substrate at 25°C. Most of the GST-PCP4MO was cleaved after 2 h incubation. However, the target protein released by factor Xa was not homogeneous. An extra band slightly smaller than PCP4MO appeared. This suggests nonspecific cleavage of PCP4MO, as has been reported elsewhere (13).

A TEV recognition site was inserted immediately following the factor Xa cleavage site. To optimize rTEV protease cleavage, different digestion times and different ratios of rTEV to substrate were tested. Figure 2B shows that most efficient cleavage of GST-PCP4MO occurred after 2 h incubation and was most complete using the ratios 1:125 and 1:100. Complete cleavage of GST-PCP4MO was indicated by the appearance of two protein bands (63 kDa for PCP4MO and 25 kDa for GST). It has been reported that rTEV protease is active over a broad temperature range, but the activity of rTEV was found to be substrate-dependent (16). GST-PCP4MO cleavage by rTEV at 4°C was therefore tested. Recombinant TEV (10–20 μg) was applied to 1 ml bed-volume beads to which GST-PCP4MO was bound. After incubation at 4°C overnight, complete cleavage was observed (Fig. 3). These results indicated

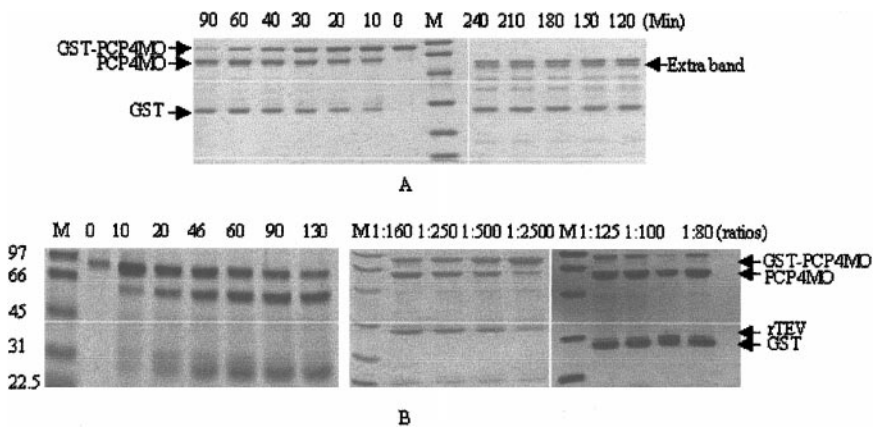

FIG. 2. (A) Cleavage of GST-PCP4MO by factor Xa at 25°C with ratio (1:100) of factor Xa to substrate. (B) Cleavage of GST-PCP4MO by rTEV at 30°C with different ratios of rTEV to substrate for 3 h and different times with the same ratio (1:160).

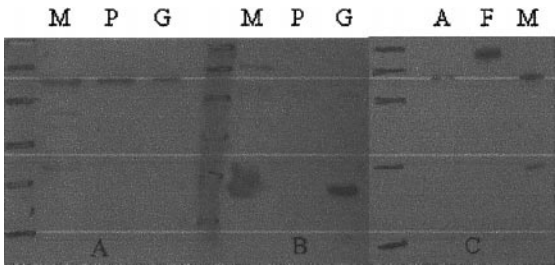

FIG. 4. Immunoblotting with antibody against PCP4MO (A), anti-GST antibody (B), and rabbit anti-GST-PCP4MO serum (C). A, cells of *S. chlorophenolica* sp. ATCC39723; F, GST-PCP4MO fusion protein; M, cleavage mixture of GST-PCP4MO by protease; P, rePCP4MO; G, purified GST.

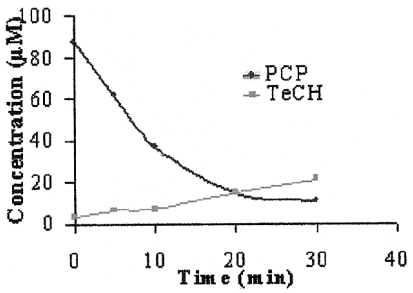

FIG. 5. Enzymatic reaction with rePCP4MO.

that the cleavage of GST-PCP4MO by rTEV was specific and efficient even at a low temperature.

An improved purification procedure. The general purification procedure requires elution of the fusion protein from the glutathione beads and subsequent cleavage of the eluent. Additional chromatography steps were needed to remove uncleaved fusion protein, GST, and protease. The high specificity of the rTEV cleavage for GST-PCP4MO suggested that it might be possible to cleave the fusion protein while bound to the resin. Removal of the his-tagged TEV was achieved by second affinity chromatography with the Ni^{2+} resin. Complete cleavage of GST-PCP4MO by rTEV was observed and a single band of rePCP4MO occurred as seen in Fig. 3 (lanes 6 and 7). The simple two-step purification procedure provides higher yields than multistep protein purification because it minimizes the number of manipulations. As a result, more than 15 mg of pure PCP4MO was obtained from 1 L of bacteria culture.

Recombinant enzyme identification. The SDS–PAGE analysis of purified rePCP4MO revealed a protein of 63 KDa (Fig. 3), which is identical to the native PCP4MO as reported previously (9).

The purified PCP4MO band and the extra band produced by factor Xa were subjected to N-terminal sequencing. The 10 N-terminal amino acid residues of the PCP4MO band were found to be 100% identical to the N-terminal sequence of PCP4MO from *Sphingomonas chlorophenolica* sp. ATCC39723. The protein Accession No. is P42535 in the protein sequence database. The first N-terminal amino acid of the extra band was identified as the 61st amino acid of PCP4MO. This suggests that factor Xa had recognized a secondary cleavage site within the target protein. Database comparison revealed that the nonspecific cleavage of GST-PCP4MO occurred at Ile_{57}-His-Ala-Arg_{60}.

The 11 N-terminal amino acids of PCP4MO band cleavage by rTEV showed 100% identity with the N-terminal sequence of PCP4MO from *Sphingomonas chlorophenolica* sp. ATCC39723, except for the four extra amino acids (Gly-Arg-Ile-Pro-) that were incorporated during the construction of the fusion protein.

A polyclonal antibody against PCP4MO was produced using GST-PCP4MO as the immunogen and subsequently purified from serum by affinity purification. Immunoblot analysis of PCP4MO antibody revealed that it was able to detect both the recombinant PCP4MO and the native enzyme (Fig. 4), suggesting the native structure to be faithfully reproduced in the recombinant protein (despite the four extra amino acids).

Recombinant enzymatic activity. PCP was used as a substrate to monitor the enzymatic activity of the recombinant PCP4MO. Conversion of PCP to TeCH was evident under the reaction conditions used (Fig. 5). The enzyme completely depleted 50 μM PCP but produced only 7.5 μM TeCH within 10 min. The nonstoichiometry of consumption of PCP and formation of TeCH was presented as shown in Fig. 6. This may be due to the instability of TeCH and its facile oxidation to semiquinones and benzoquinones, as described previously (17). The control reactions without enzyme showed no spontaneous formation of TeCH. FAD was found to be necessary for the recombinant enzyme activity, since no TeCH was detected in the absence of FAD (results not shown). Previous studies have demonstrated that PCP4MO is a flavoprotein exhibiting a typical flavoprotein spectrum with a peak at 446 nm

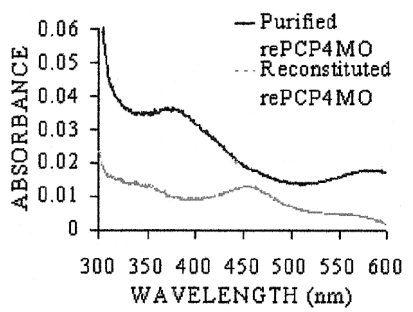

FIG. 6. Absorbance spectrum of purified rePCP4MO and reconstituted rePCP4MO. The rePCP4MO solution contained 7 μM enzyme in 50 mM Tris (pH 8). The reconstituted rePCP4MO solution contained 5.7 μM enzyme in 50 mM KP_i (pH 7.5).

(9). However, the absorption spectrum of rePCP4MO did not show a maximum at 450 nm, but had more absorption in the 380 nm region as well as a small amount of absorption beyond 550 nm (Fig. 6). The result indicated that the rePCP4MO represents an apoprotein, which has to be supplemented by FAD to form a holoprotein. The apoprotein could be reconstituted by incubation of the protein with FAD and dithiothreitol as previously described (18). The reconstituted enzyme did exhibit a peak at 454 nm as shown in Fig. 6.

Recombinant enzyme kinetics. From three independent experiments, the apparent K_m and V_{max} were estimated as 50 ± 7 μM and 30 ± 3 nmol/min/mg, respectively. The catalytic efficiency (K_{cat}/K_m) was 0.7 $s^{-1}mM^{-1}$. In comparison, PCP4MO from *S. chlorophenolica* ATCC 39723 showed K_m and V_{max} for PCP as 30 μM and 16 μmol/min/mg, respectively (9). Using these values, the catalytic efficiency was calculated to be 405 $s^{-1}mM^{-1}$.

DISCUSSION

In this paper, we describe the conditions for high-level production of GST-PCP4MO and methods for the enhancement of its solubility and recovery. The fusion protein GST-PCP4MO was expressed in *E. coli*, however, the protein was poorly soluble and predominantly in inclusion bodies. The insoluble protein was solubilized by using an ionic detergent, Sarkosyl and subsequently bound to a glutathione-sepharose column in the presence of a nonionic detergent, Triton X-100. This might be due to the ability of Sarkosyl to inhibit the coaggregation of protein with bacterial outer membrane components. Furthermore, nonionic detergent Triton X-100 could sequester Sarkosyl and allow the GST-PCP4MO to bind glutathione sepharose, which is consistent with earlier work (12). Elution of GST-PCP4MO was then efficiently carried out by using a high pH elution buffer. Because of the high acidity of glutathione, it might be difficult to maintain a high pH in reduced glutathione elution buffer.

For crystallization, pure and homogenous protein preparation is critical, and the isolation tag (GST) must be removed by a protease. Since PCP4MO was nonspecifically released by factor Xa, a TEV recognition site was inserted into the fusion protein. High specific PCP release and efficient fusion protein cleavage at 4°C were observed, which allowed digestion of GST-PCP4MO on-column.

The biochemical and functional characteristics of rePCP4MO were compared to those of the native PCP4MO by SDS–PAGE, immunoblotting, and N-terminal sequencing analyses, and by measurement of enzyme activity. Both forms of the enzyme had a molecular weight of 63,000, the same N-terminal sequence, and they were detected by the polyclonal PCP4MO antibody.

Concerning enzymatic activity, differences were however observed between the native and recombinant form. The rePCP4MO did not exhibit a typical flavoprotein spectrum and no enzyme activity was detected in the absence of FAD. A previous study (9) indicated that PCP4MO is closely related to external flavoprotein monooxygenases, which contain a flavin, and use aromatic compounds as substrate and NADPH or NADH as reductant. The FAD in PCP4MO is tightly bound, but some loss during purification may occur. Almost total loss of FAD during purifying rePCP4MO was however observed.

The K_m value of rePCP4MO was decreased by one-fold as compared to that of native enzyme. The result indicated that binding efficiency of substrate (PCP) to both forms of enzyme was similar, which further illustrated that their binding pockets were essentially identical. Catalytic efficiency of rePCP4MO was however decreased by 578-fold as compared to the native PCP4MO, indicating that the turnover of substrate to product was reduced. This decrease could be due to a low amount of active enzyme in the reaction mixture. In other words, a majority of the enzyme in the sample was apoprotein without the required cofactor FAD. The concentration of FAD might have been too low to restore the activity completely. For comparison, chlorophenol 4-monooxygenase from *Burkholderia cepacia* AC1100 showed a K_{cat} of 0.306 s^{-1} (19) and TCP-4-monooxygenase from *Azotobacter* sp. Strain GP1 exhibited a K_{cat} of 0.029 s^{-1} (20). Both enzymes can use several chlorinated phenols as substrates and subsequently convert the chlorinated phenols to chloro-*p*-hydroquinone. In addition, both enzymes require FAD, which stimulated the enzyme activity. The same was observed with rePCP4MO with a K_{cat} of 0.035 s^{-1}. The lower activity of these three enzymes seems to support the hypothesis that the apoprotein might not be completely reconstituted by FAD. The above comparisons provide evidence that the activities of functionally similar enzymes vary widely. Recent studies have revealed that the concentration of toxic intermediates is very low *in vivo* (17). The rate of each step in the pathway must be equal to or greater than that of preceeding step in order to avoid the accumulation of intermediates. In the case of PCP4MO which is the first enzyme in the degradation of PCP, the rate of PCP conversion to TeCH could appropriately be controlled to maintain low levels of chlorinated hydroquinone intermediates and thus to avoid damage to the microorganism.

In conclusion, rePCP4MO should provide material for three-dimensional structure analysis and for enzyme mechanism study. The expression and purification system we created here should enable studies of active site residues of PCP4MO by site-directed mutagenesis.

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