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# Crystallization and preliminary X-ray studies of methyl parathion hydrolase from *Pseudomonas* sp. WBC-3

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Methyl parathion hydrolase (MPH) from *Pseudomonas* sp. WBC-3, an enzyme that catalyzes the degradation of methyl parathion (*O*,*O*-dimethyl *O*-*p*-nitrophenyl phosphorothioate; MP), has been purified and crystallized by the hanging-drop vapour-diffusion method. The crystals were grown at 291 K using a precipitant solution consisting of 30% PEG 400, 0.1 *M* sodium acetate pH 4.6, 0.1 *M* $CdCl_2$. MPH is a zinc-containing enzyme judged by inductively coupled plasma mass-spectrometric (ICP-MS) analysis. Multiple-wavelength anomalous dispersive X-ray data were collected at 2.5 Å resolution from a single crystal on beamline 41XU at SPring-8. The crystal belongs to space group $P4_32_12$, with unit-cell parameters $a$ = 84.94, $b$ = 84.94, $c$ = 200.38 Å, $\alpha = \beta = \gamma = 90^\circ$. The asymmetric unit contains two molecules and has a solvent content of ∼52%. Crystal structure determination is in progress.

## 1. Introduction

Organophosphate pesticides, a group of highly toxic agricultural chemicals including parathion (*O*,*O*-diethyl *O*-*p*-nitrophenyl phosphorothioate) and methyl parathion (*O*,*O*-dimethyl *O*-*p*-nitrophenyl phosphorothioate; MP), have been used worldwide for the control of a wide range of insect species. However, there are environmental problems caused by the extensive use of organophosphate pesticides (approximately $3 \times 10^7$ kg $y^{-1}$). Thousands of cases of pesticide poisoning occur each year, causing about 800 deaths annually (Dave *et al.*, 1994), in addition to numerous poisoning events of animals. Biodegradation of organophosphate agents is one of the main ways to solve the problem (Munnecke & Hsieh, 1974; Serdar *et al.*, 1982; Chaudhry *et al.*, 1988; Rani & Lalithakumari, 1994; Harper *et al.*, 1988; Hayatsu *et al.*, 2000).

Some microorganisms have been reported to utilize parathion (Sethunathan & Yoshida, 1973; Munnecke & Hsieh, 1974; Serdar *et al.*, 1982; Mulbry *et al.*, 1986; Mulbry & Karns, 1989) and its analogue methyl parathion (Laveglia & Dahm, 1977; Chaudhry *et al.*, 1988; Ou & Sharma, 1989; Rani & Lalithakumari, 1994; Cui *et al.*, 2001). Organophosphorus hydrolase (OPH; EC 3.1.8.1) from *Pseudomonas diminuta* GM (Dumas *et al.*, 1989) has been studied more intensively (Omburo *et al.*, 1993; Benning *et al.*, 1994, 1995, 2001; Kuo & Raushel, 1994; Banzon, Kuo, Miles *et al.*, 1995; Banzon, Kuo, Fisher *et al.*, 1995; Vanhooke *et al.*, 1996; Hong & Raushel, 1996; Kuo *et al.*, 1997) than methyl parathion hydrolase (MPH) from *Pseudomonas putida* (Rani & Lalithakumari, 1994) and *Plesiomonas* sp. M6 (Cui *et al.*, 2001).

A methyl parathion-degrading bacterial strain, *Pseudomonas* sp. WBC-3, was recently isolated from soil near the Sanonda Pesticides Manufacturing Company, Hubei, China. The strain can use methyl parathion as a sole C/N source to grow and can degrade *p*-nitrophenol, the product of methyl parathion, completely (Chen *et al.*, 2002). The *mpd* gene encoding methyl parathion hydrolase from *Pseudomonas* sp. WBC-3 has been determined (Liu *et al.*, unpublished work) and the complete gene sequence is now available (GenBank, AY251554). The methyl parathion hydrolase has a molecular weight of 34.4 kDa and the amino-acid sequence deduced from the nucleotide sequence shows 99.7 and 98.5% identity to those from *Pseudomonas putida* and *Plesiomonas* sp. M6, respectively. The *mpd* gene lacks sequence homology with the *opd* gene that encodes parathion hydrolase from *P. diminuta* (Dumas *et al.*, 1989) and *Flavobacterium* sp. (Mulbry & Karns, 1989).

Crystallizing the MPH protein and solving its structure will result in a better understanding of the catalytic mechanism, especially the functional and structural differences between MPH and OPH, and allow MPH to be engineered in order to enhance its activity and enlarge the substrate range. Here, we report the crystallization and preliminary X-ray diffraction studies of MPH from *Pseudomonas* sp. WBC-3.

## 2. Materials and methods

### 2.1. Expression and purification of MPH

A single colony of *Pseudomonas* sp. WBC-3 was cultured overnight at 303 K in 20 ml Luria–Bertani medium containing 100 µg ml$^{-1}$ ampicillin. This 20 ml culture was transferred to 1 l fresh LB medium and the cells were grown with shaking for 48 h at 303 K. The cells were harvested by centrifugation at 5000*g* for 10 min at 277 K. The OD at the time of harvest was 0.6. Following harvesting, the cell pellets were resuspended in buffer *A* (10 m*M* $KH_2PO_4$ pH 7.0) and then disrupted by sonicating 200 times for 4 s periods at 6 s intervals. Cell debris was removed by centrifugation of lysates at 15 000*g* for 30 min at 277 K and the supernatant was stored at 277 K for purification.

All the following purification steps were performed at 289 K. The clear supernatant was loaded onto a 16 ml CM-Sepharose Fast Flow Column (Pharmacia) previously equilibrated with buffer *A*. The protein was then eluted using a linear gradient of NaCl from 0 to 0.56 *M* in buffer *A* at a flow rate of 1 ml min$^{-1}$. The fractions containing the highest MPH activity were collected and concentrated by centrifugation on a 5K ultrafiltration membrane. Further purification was achieved by gel-filtration chromatography on Superdex G75 (Pharmacia) pre-equilibrated with buffer *B* (20 m*M* Tris–HCl pH 7.6 and 80 m*M* NaCl). The target protein was eluted from the column with buffer *B* and the peak fractions were combined and concentrated to 40–50 mg ml$^{-1}$ using a 5K ultrafiltration membrane and were immediately used for crystallization. The homogeneity of the protein was assessed by 12% SDS–PAGE (Laemmli, 1970). The yield was approximately 40 mg protein from a 1 l culture.

### 2.2. Crystallization

Preliminary crystallization experiments were carried out at 291 K in 16-well plates with Crystal Screen reagent kits I and II (Hampton Research) using the hanging-drop vapour-diffusion method (Jancarik & Kim, 1991). Drops consisting of 1.5 µl protein solution and an equal volume of reservoir solution were equilibrated against 200 µl reservoir solution. The protein concentration was 40–50 mg ml$^{-1}$ prior to mixing with the reservoir solution. The protein sample was centrifuged at 13 000*g* for 13 min to clarify the solution before initiating any crystal trials.

### 2.3. Data collection and processing

Crystals were picked up from the hanging drop with a nylon crystal-mounting loop (Hampton Research) and then flash-frozen prior to data collection at 105 K. The freezing solution was identical to the reservoir solution but with the addition of 30% glycerol as a cryoprotectant. Multiple-wavelength anomalous dispersion (MAD; Hendrickson *et al.*, 1990) data were collected on beamline 41XU at SPring-8, Japan. Data processing was performed using the program *DENZO* and data sets were scaled and merged using *SCALEPACK* (Otwinowski & Minor, 1997).

## 3. Results and discussion

The purity of the protein was confirmed to be more than 99% as evaluated by SDS–PAGE analysis and it was therefore deemed suitable for use in crystallization trials. Rhombic shaped crystals appeared from condition No. 12 of Crystal Screen II (30% PEG 400, 0.1 *M* sodium acetate pH 4.6, 0.1 *M* $CdCl_2$) after two months. The crystals were suitable for X-ray diffraction (Fig. 1).

X-ray diffraction data were collected at beamline 41XU using an X-ray CCD detector (MAR CCD 165) system. Since MPH was judged to be a zinc-containing enzyme by inductively coupled plasma mass-spectrometric (ICP-MS) analysis (data not shown), the multiple-wavelength anomalous dispersion (MAD) method was considered. The results of X-ray absorption fine structure (XAFS) spectroscopy gave the peak at 9.6655 keV ($\lambda_1$; 1.2828 Å) and the edge at 9.6619 keV ($\lambda_2$; 1.2832 Å) (Fig. 2). MAD data were collected from a single crystal at peak ($\lambda_1$; 1.2828 Å), inflection ($\lambda_2$; 1.2832 Å) and remote ($\lambda_3$; 0.9000 Å) wavelengths to 2.5, 2.5 and 2.6 Å resolution, respectively. 135 images were collected to maximum resolutions of 2.5, 2.5 and 2.3 Å, respectively, from the zinc-containing crystal (Fig. 3). The crystal belonged to space group $P4_32_12$, with

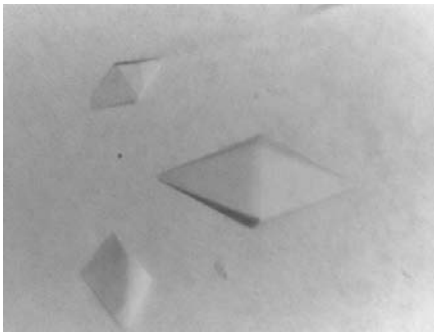

**Figure 1**
Crystals of MPH (the size of a single crystal is about 0.2 × 0.1 × 0.1 mm).

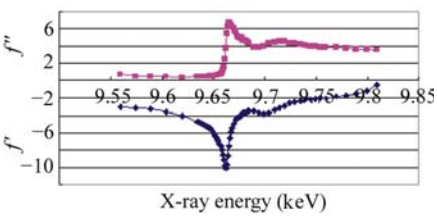


**Figure 2**
The results of XAFS spectroscopy: Zn *K* edge in MPH.

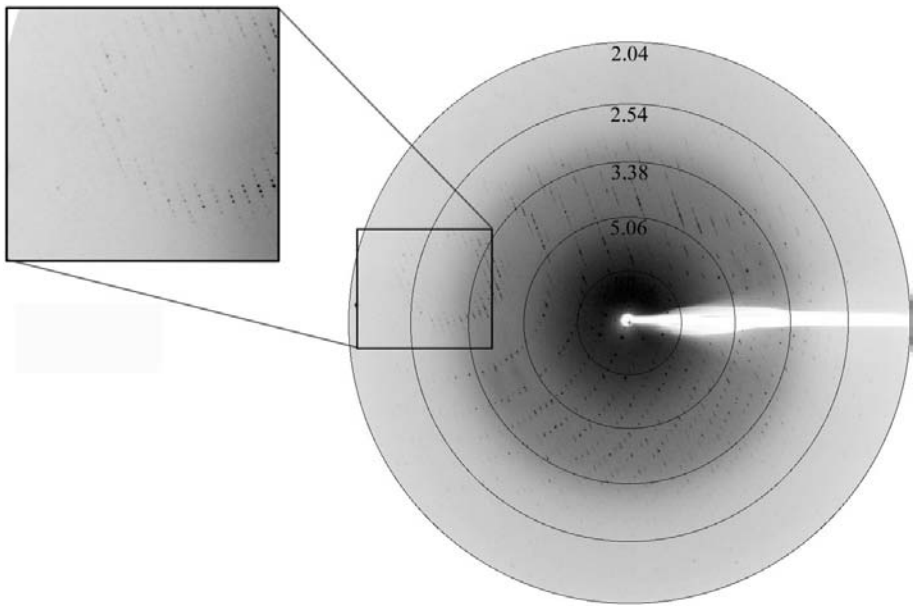


**Figure 3**
A typical X-ray diffraction pattern from a crystal of the MPH showing spots to 2.3 Å resolution. X-ray diffraction data were collected at beamline BL41XU of SPring-8 (Hyogo, Japan). The crystal-to-detector distance used was 135 mm and the 2$\theta$ angle was fixed at 0°, with a 0.8° oscillation angle and a 5 s exposure time for each frame. The edge of the detector corresponds to 2.04 Å resolution.

**Table 1**
Crystallographic parameters and data-collection statistics.

All data were collected on beamline 41XU at SPring-8, Japan. Values in parentheses are for the highest resolution shell.

| | Peak | Inflection | Remote |
|---|---|---|---|
| Space group | $P4_32_12$ | | |
| Unit-cell parameters (Å, °) | $a$ = 84.94, $b$ = 84.94, $c$ = 200.38, $\alpha = \beta = \gamma$ = 90 | | |
| Wavelength (Å) | 1.2828 | 1.2832 | 0.9000 |
| Resolution (Å) | 50–2.50 (2.59–2.50) | 50–2.50 (2.59–2.50) | 50–2.30 (2.38–2.30) |
| Completeness (%) | 99.7 (99.6) | 99.8 (100.0) | 95.9 (96.0) |
| Matthews coefficient (Å$^3$ Da$^{-1}$) | 2.6 | 2.6 | 2.6 |
| Molecules per AU | 2 | 2 | 2 |
| Solvent content (%) | 52.0 | 52.0 | 52.0 |
| Total observations | 275957 | 275466 | 180629 |
| Unique reflections | 26289 | 26253 | 32143 |
| Redundancy | 10.5 (10.7) | 10.5 (10.9) | 5.6 (4.7) |
| Average $I/\sigma(I)$ | 8.2 (6.4) | 8.2 (6.3) | 7.7 (2.5) |
| $R_{\rm merge}$† (%) | 10.8 (47.8) | 10.4 (47.6) | 8.0 (38.0) |

† $R_{\rm merge} = 100 \times \sum |I_i - \langle I \rangle| / \sum I_i$, where $I_i$ is the intensity of the observation.

unit-cell parameters $a$ = 84.94, $b$ = 84.94, $c$ = 200.38 Å, $\alpha = \beta = \gamma = 90^\circ$. The value of the Matthews coefficient (Matthews, 1968) was 2.6 Å$^3$ Da$^{-1}$ for two molecules in the asymmetric unit, giving an estimated solvent content of 52%. Statistics of the data collection from the crystals are summarized in Table 1. Structure determination of MPH is currently under way.

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