

# An isofenphos-methyl hydrolase (Imh) capable of hydrolyzing the P–O–Z moiety of organophosphorus pesticides containing an aryl or heterocyclic group

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**Abstract** Organophosphorus pesticide (OP) hydrolases play key roles in the degradation and decontamination of agricultural and household OPs and in the detoxification of chemical warfare agents. In this study, an isofenphos-methyl hydrolase gene (*imh*) was cloned from the isocarbophos-degrading strain of *Arthrobacter* sp. scl-2 using the polymerase chain reaction method. Isofenphos-methyl hydrolase (Imh) showed 98% sequence identity with the isofenphos hydrolase from *Arthrobacter* sp. strain B-5. Imh was highly expressed in *Escherichia coli* BL21 (DE3), and the His<sub>6</sub>-tagged Imh was purified (1.7 mg/ml) with a specific activity of 14.35 U/mg for the substrate isofenphos-methyl. The molecular mass of the denatured Imh is about 44 kDa, and the isoelectric point (pI) value was estimated to be 3.4. The optimal pH and temperature for hydrolysis of isofenphos-methyl were pH 8.0 and 35 °C, respectively. The secondary structure of Imh shows that Imh is a metallo-dependent hydrolase, and it was found that Imh was completely inhibited by the metalloprotease

inhibitor 1,10-phenanthroline (0.5 mM), and the catalytic activity was restored by the subsequent addition of Zn<sup>2+</sup>. Interestingly, Imh had a relatively broader substrate specificity and was capable of hydrolyzing 12 of the tested oxon and thion OPs with the P–O–Z moiety instead of the P–S(C)–Z moiety. Furthermore, it was found that the existence of an aryl or heterocyclic group in the leaving group (Z) is also important in determining the substrate specificity. Among all the substrates hydrolyzed by Imh, it was assumed that Imh preferred P–O–Z substrates still with a phosphamide bond (P–N), such as isofenphos-methyl, isofenphos, isocarbophos, and butamifos. The newly characterized Imh has a great potential for use in the decontamination and detoxification of agricultural and household OPs and is a good candidate for the study of the catalytic mechanism and substrate specificity of OP hydrolases.

**Keywords** Organophosphate detoxification · Organophosphorus pesticide hydrolase · Isofenphos-methyl hydrolase (*imh*) · Substrate specificity

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## Introduction

Synthetic organophosphorus pesticides (OPs) are widely used to control major insect pests in agriculture. Most of these OPs are esters or thioesters of phosphoric acid (P=O bond; oxon OPs) and thiophosphoric acid (P=S bond; thion OPs). The leaving groups of OPs are usually attached to the phosphorus center by an oxygen (P–O bond), such as in the case of parathion, chlorpyrifos, isofenphos-methyl, isocarbophos, profenofos, triazophos, and fenamiphos, or a sulfur (P–S bond), such as in the case of methidathion, dimethoate, phorate, and omethoate (Munnecke 1976). It has been estimated that more than 100 OPs are used, accounting for

~38% of the total pesticide usage in the world (Singh 2009). Continuous and excessive use of OPs has led to the contamination of many terrestrial and aquatic ecosystems (Cisar and Snyder 2000; McConnell et al. 1999; Tse et al. 2004). OPs are toxic to mammals and other nontarget animals, and the toxic effects of OPs on invertebrates, vertebrates, and wildlife are well documented. OPs inhibit the activity of the key acetylcholine esterase (AChE) of the nervous system by covalently binding to its active site and then inhibiting the breakdown of acetylcholine, which results in various hazardous effects (Singh 2009).

Use of microbial cells and enzymes in detoxification and decontamination of OPs is considered a viable and environment-friendly approach. Several microorganisms have been identified to have the capacity to hydrolyze and thus detoxify OPs (Singh 2009; Zhang et al. 2005). Several OP hydrolases, systematically called phosphotriesterases (PTEs; EC 3.1.8.1) that are able to hydrolyze OPs have been purified and characterized from microorganisms, and the genes encoding these hydrolases have been cloned. The organophosphorus hydrolase (OPH) from *Brevundimonas diminuta* (*Pseudomonas diminuta*) and *Flavobacterium* spp. has been widely studied (Dumas et al. 1989; Mulbry and Karns 1989; Serdar and Gibson 1985). OPH is a member of the amidohydrolase superfamily, which has broad substrate specificity and can hydrolyze the P–O, P–F, and P–S bonds of OPs. Another OP hydrolase, arylalkylphosphatase (AdpB), was identified in *Nocardia* sp. strain B-1. AdpB possesses very different physical properties from those of OPH, including the lack of a requirement for metal ions (Mulbry 1992). A third OP hydrolase, organophosphorus acid anhydrolase (OPAA), was characterized in *Alteromonas undina* and *Alteromonas haloplanktis* (Cheng et al. 1993; Cheng et al. 1996; DeFrank and Cheng 1991). This hydrolase belongs to the dipeptidase family, and its hydrolysis rates of P–O bonds of phosphinate compounds are lower than those of the P–F bonds of nerve agents. The methyl-parathion hydrolase (Mph), which was first characterized in *Plesiomonas* sp. strain M6 in our laboratory, is a member of the  $\beta$ -lactamase superfamily. Mph is capable of hydrolyzing methyl-parathion, parathion, malathion, phoxim, chlorpyrifos, and fenitrothion (Cui et al. 2001; Liu et al. 2005). A dimethoate-degrading enzyme that can degrade the P–S bond of dimethoate, formothion, and malathion was purified from *Aspergillus niger* ZHY256 (Liu et al. 2001). In 2002, a variant of OPH (OpdA) was purified in *Agrobacterium radiobacter* (Horne et al. 2002a; Horne et al. 2003). In the same year, another OP hydrolase, whose coding gene is hydrolysis of coroxon (*hoca*), was characterized in *Pseudomonas monteilii* strain C11. This hydrolase has a broad substrate specificity across oxon and thion OPs (Horne et al. 2002b). In 2004, a new organophosphorus hydrolase, which is coded by the *ophc2* gene

and shares 46.4% similarity with the *mph* gene, was characterized in *Pseudomonas pseudoalcaligenes* strain C2-1 (Wu et al. 2004). In 2006, a novel fenitrothion hydrolase (FedA and FedB) was reported in *Burkholderia* sp. strain NF100 (Tago et al. 2006). Recently, a new PTE with hydrolytic activity against *p*-nitrophenyl butanoate, bis-*p*-nitrophenyl-phosphate, and several additional OPs was also reported from archaeon *Sulfolobus acidocaldarius* (Porzio et al. 2007).

In addition to these OP hydrolases, an isofenphos hydrolase was identified in *Arthrobacter* sp. strain B-5 in 1997 (Ohshiro et al. 1997), which shares 58.1% identity with AdpB from *Nocardia* sp. strain B-1 (Ohshiro et al. 1999; Ohshiro et al. 1997). To the best of our knowledge, this hydrolase is the only enzyme reported to hydrolyze isofenphos. It was found that the aryl phosphoester bond of isofenphos is cleaved to produce *O*-ethyl isopropyl phosphoramidothioate and isopropyl salicylate. However, other OPs such as butamifos, amiprofos-methyl, and acephate were not hydrolyzed very efficiently by the hydrolase (Ohshiro et al. 1997). In general, the substrate range and the rule for the substrate specificity of the isofenphos hydrolase have not been studied extensively.

In our previous study, a highly effective isocarbophos-degrading *Arthrobacter* sp. strain scl-2 was isolated from the isocarbophos-polluted soil (Li et al. 2009). It was able to utilize isocarbophos as its sole carbon and phosphorus sources for growth and hydrolyze several other OPs such as profenofos, isofenphos-methyl, and isofenphos. In this study, the isofenphos-methyl hydrolase gene (*imh*) was cloned from the strain of *Arthrobacter* sp. scl-2, and the characteristics of the isofenphos-methyl hydrolase (Imh) were systematically studied. The substrate range and the rules for substrate specificity of Imh were also investigated.

## Materials and methods

### Chemicals and strains

All OPs (>98% purity) used in this study were purchased from the Pesticide Research Institute (Shanghai, China). For proper use, OPs were dissolved in organic solvents (e.g., methanol or acetone) as standard stock solutions. Diethyl pyrocarbonate (DEPC), phenylmethanesulfonyl fluoride (PMSF),  $\beta$ -mercaptoethanol, Triton X-100, and 1,10-phenanthroline were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents used in this study were of analytical reagent grade. *Arthrobacter* sp. strain scl-2 (deposited in China Center for Type Culture Collection under the collection number CCTCC AB 2011023) and *Escherichia coli* species were grown on Luria–Bertani medium (LB) at 30 °C and 37 °C, respectively. Minimal

salt medium (MSM; 1.5 g K<sub>2</sub>HPO<sub>4</sub>, 0.5 g KH<sub>2</sub>PO<sub>4</sub>, 0.2 g MgCl<sub>2</sub>, 1.0 g NaCl, 1.0 g NH<sub>4</sub>NO<sub>3</sub> per liter of water, pH 7.0) supplemented with OPs was used to assay the degrading capacity for the OPs. Medium was solidified with 2.0% agar (Difco) when appropriate. Antibiotics were added, when required, at the following concentrations: ampicillin, 100 µg/ml, and kanamycin, 50 µg/ml.

#### Cloning of the *imh* gene

Genomic DNA was extracted from strain scl-2 using the high-salt method described previously (Sambrook and Russell 2001). Plasmid isolation, polymerase chain reaction (PCR) product purification, and gel extraction were performed using kits according to the manufacturer's protocols (Takara, Dalian, China). The *imh* gene was cloned from strain scl-2 using a PCR-based technique. Because strain scl-2 had a relative broader substrate range, several primer pairs for amplification of OP hydrolase genes, such as the organophosphate degrading gene (*opd*), methyl-parathion degrading gene (*mpd*), organophosphate hydrolase gene (*oph*), and organophosphorus acid anhydrolase gene (*opaA*), were designed and tried to amplify the target OP hydrolase gene (Table 1). Only the primer pairs (11F/11R and 12F/12R) designed specifically for the isofenphos hydrolase gene (*oph*) from *Arthrobacter* sp. strain B-5 resulted in positive amplification. The PCR conditions were as follows: 5 min at 95 °C, followed by 30 cycles consisting of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1.5 min and a final extension step of 10 min at 72 °C. The amplified fragment was subcloned into the pMD18-T vector (Takara) and then transformed into the competent *E. coli* DH5α cells. The positive clone identified by its ability to form a hydrolytic halo on LB agar containing

200 µg/ml isofenphos-methyl (or isocarbophos) was selected and then sequenced by Invitrogen (Shanghai, China). The nucleotide, the deduced amino acid sequences, and the molecular mass were determined using OMIGA software (Oxford Molecular Ltd, UK). BlastP was used to deduce the amino acid identity (<http://www.ncbi.nlm.nih.gov/Blast>). Solvent accessibility and the secondary structure of the protein was predicted by the modeling server JPred (Cuff et al. 1998). Multiple protein sequence alignments were constructed using CLUSTAL X (Kumar et al. 2004). Phylogenetic analysis of the protein sequences was performed using the software MEGA 3.1 (Kumar et al. 2004). Distances (distance options according to the Kimura two-parameter mode) were calculated, and clustering was performed with the neighbor-joining method (Kumar et al. 2004).

#### Expression and purification of the Imh

The restriction sites *Nde*I and *Hind*II were incorporated into the PCR primer pair 12F/12R to facilitate directional cloning of the PCR product. The *imh* gene, without its translation stop codon, was amplified and inserted into the *Nde*I/*Hind*II sites of the expression vector pET29a (+) (Novagen, Darmstadt, Germany) to generate the recombinant plasmid pET-*imh*. Imh was overexpressed in *E. coli* BL21 (DE3) using the His-Bind protein fusion and purification system. The *imh* gene was expressed at different temperatures and induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Harvested cells were then washed twice with sterile phosphate-buffered saline (PBS) (20 mM; pH 7.5) and disrupted by sonication (Auto Science, UH-650B ultrasonic processor, Tianjin Automatic Science Instrument Co., Ltd; 30% intensity) for 5 min. Cell debris and insoluble proteins were removed by centrifugation

**Table 1** The primer pairs used to amplify the OP hydrolase genes in this study

Gene	Primer <sup>a</sup> (forward [F], reverse [R])	Sequence (5'–3')	Gene	Primer (forward [F], reverse [R])	Sequence (5'–3')
<i>mpd</i> (methyl-parathion degrading gene)	1F	cgccttgatggtgggtgag	<i>opaA</i> (organophosphorus acid anhydrolase gene)	7F	tctatgccctgtggact
	1R	cgtcgatgctgagctggttc		7R	tgttgccgtaagcgtgtg
	2F	atgccctgaagaaccgc		8F	aactgctgtatcgctgag
	2R	cacttggggttgacgaccga		8R	atgtgggaagaacgtgga
<i>opd</i> (organophosphate degrading gene)	3F	tggttcgacccgccacttc	<i>hocA</i> (hydrolysis of coroxon gene)	9F	aacctggaagtgggaaga
	3R	cttctagacaatcgactg		9R	gcgttagggcctctgtgat
	4F	aaaggctgtgagaggattc		10F	cagcccatcaatcaccca
	4R	tagctcgaaaacccgaacag		10R	cgttattgtttccaga
<i>ophc2</i> (organophosphorus hydrolase gene 2)	5F	gctctgtccaccgattg	<i>oph</i> (isofenphos hydrolase gene)	11F	gcttgccatccggtgctgc
	5R	ggcgacaacctggtctga		11R	gccagatgctgacctgctt
	6F	gctgagcaccgattgtc		12F	catatgatgcatcactgttttctc
	6R	accgattgaacaggttaagag		12R	aagcttcccgcgaccgccgatg

<sup>a</sup> Two primer pairs were designed for each gene

(12,000g for 20 min at 4 °C). The supernatant was loaded onto a His-Bind resin (Novagen). The target fusion protein was eluted with 100 mM imidazole in 20 mM Tris-HCl (pH 6.5) and 50 mM NaCl. After eluting the nontarget proteins with 25 mM imidazole in 20 mM Tris-HCl (pH 6.5) and 50 mM NaCl, the enzyme was dialyzed against PBS (50 mM; pH 8.0) for 24 h and concentrated using an Amicon ultrafiltration tube. The protein concentration was quantified using the Bradford method, and bovine serum albumin was used as the standard. The molecular mass of the denatured Imh was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the Laemmli method (Laemmli 1970). The protein was stained with Coomassie brilliant blue G250 (Amresco, USA). The *pI* of Imh was estimated by PAGE with 6.25% Ampholine (pH 3.0–10.0) (GE Healthcare, Sweden) in a gel rod (0.5 × 1.0 cm) using a kit for Isoelectric Focusing Calibration (Pharmacia LKB Biotechnology) according to the supplier's recommendation.

#### Determination of the Imh activity

For hydrolysis activity assays, 50 μl Imh was mixed with 0.2 mM substrate in 1 ml of PBS (50 mM; pH 8.0), and the reaction mixture was incubated at 35 °C for 30 s to 5 min depending on the substrate. One unit of enzyme activity was defined as the amount of enzyme required to catalyze the formation of 1 μmol product or hydrolysis of 1 μmol substrate per minute. For kinetic studies, stock solutions of each substrate were appropriately diluted into at least five different concentrations around the dissociation constant ( $K_m$ ) values. Initial reaction velocities were measured at various concentrations of the substrate and were then fitted to the Lineweaver-Burk transformation of the Michaelis-Menten equation. Kinetic analyses by curve fitting were performed with SigmaPlot software.

#### Effect of temperature and pH on Imh activity

The pH range of Imh was determined by incubating 50 μl Imh with 0.2 mM isofenphos-methyl (the best substrate) in 1 ml reaction buffer at 35 °C for 30 s at pH values ranging from 4.0 to 10.0. For pH stability determination, Imh was preincubated at different pH values (ranging from 4.0 to 10.0) for 4 h, and then the remaining activity was assayed as described above. The reaction buffers were citric acid-NaOH buffer (pH 4.0–5.0), PBS (pH 6.0–8.0), Tris-HCl buffer (pH 9.0), and glycine-NaOH buffer (pH 10.0), respectively. The optimal temperature was determined analogously by incubating 50 μl Imh with 0.2 mM isofenphos-methyl in 1 ml PBS (50 mM; pH 8.0) for 30 s at different temperatures ranging from 4 °C to 70 °C. To determine the thermostability, Imh was preincubated in a

water bath at different temperatures (4 °C–70 °C) for 1 h, and then the remaining activity was determined.

#### Effect of metal ions and chemical agents on Imh activity

The effects of potential inhibitors or activators on Imh were determined by the addition of various metal salts (0.5 and 2.5 mM  $\text{Cd}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Li}^+$ ,  $\text{Al}^{3+}$ , and  $\text{Hg}^{2+}$ ) and chemical agents (0.5 and 2.5 mM 1,10-phenanthroline, 10 mM Triton X-100, SDS, and Tween 80; 0.5 mM PMSF, DEPC, and  $\beta$ -mercaptoethanol) to the enzyme in 0.1 M Tris-HCl buffer (pH 8.0). The mixture was preincubated for 30 min at 35 °C, and then the isofenphos-methyl hydrolyzing activity of Imh was then assayed and expressed as the percentage of activity obtained in the absence of the added compound (Li et al. 2009). The apoenzyme of Imh was prepared by the metal ion chelators. Imh (50 μl) was dissolved in 4 ml Tris-HCl (0.1 M; pH 8.0). The enzyme solution was dialyzed against the same buffer containing 1 mM 1,10-phenanthroline at 4 °C for 24 h and then dialyzed against deionized water (pH 7.0) for 24 h, to remove 1,10-phenanthroline- $\text{Zn}^{2+}$  and excess 1,10-phenanthroline. The apoenzyme thus prepared was completely inactive. Different divalent metal ions ( $\text{Cd}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Mg}^{2+}$ , and  $\text{Hg}^{2+}$ ) were added to the apoenzyme to a final concentration of 1 and 5 mM, respectively, and then the mixture was dialyzed against deionized water to remove excess metal ions. The activity of Imh was then assayed to see whether these metal ions restored the activity or not. The metals in the purified Imh were also analyzed by inductively coupled plasma-atomic emission spectrometry (ICP-AES) (Optima 2100DV; PerkinElmer). The purified Imh was first dialyzed to remove nonbinding metals for 24 h and then digested with  $\text{HNO}_3$ . Glassware, test tubes, and tips were pretreated by  $\text{HNO}_3$  to prevent metal ion contamination.

#### The substrate specificity of Imh

The ability of Imh to hydrolyze different OPs was analyzed. Enzymatic activities for parathion and methyl parathion were measured using a spectrophotometer to monitor the production of *p*-nitrophenol at 405 nm (Dumas et al. 1989). Isofenphos-methyl, isocarbophos, isofenphos, triazophos, profenofos, butamifos, and phosalone were analyzed by high-performance liquid chromatography (HPLC) (Ettan LC; Amersham Biosciences, Uppsala, Sweden) and detected by a UV-900 detector at 245 nm (Li et al. 2009; Yang et al. 2011; Li et al. 2011; Gui et al. 2008; Jonsson et al. 2001; Sanchez et al. 2004). Fenitrothion, phoxim, and methidathion were detected at 270 nm (Hong et al. 2007; Shen et al. 2010), and chlorpyrifos was detected at 230 nm (Li et al. 2007). Malathion, dimethoate, omethoate, sulfo-

tep, trichlorfon, phorate, methamidophos, acephate, dichlorvos, and monocrotophos were analyzed by GC (Shimadzu GC-14B) coupled with a nitrogen phosphorus detector. The temperatures of the injector, detector, and column were set at 230 °C, 250 °C, and 200 °C, respectively, for the detection of acephate, dimethoate, sulfotep, monocrotophos, methamidophos, and omethoate (Li et al. 2010; Wang et al. 2010); at 230 °C, 250 °C, and 170 °C, respectively, for trichlorfon and dichlorvos (Yu et al. 2008); at 280 °C, 320 °C, and 170 °C, respectively, for phorate (Zhang et al. 2011); and at 280 °C, 300 °C, and 230 °C, respectively, for malathion (Fu et al. 2008; Tse et al. 2004).

Nucleotide sequence accession number

The nucleotide sequence of the *imh* gene was deposited in the GenBank database under accession number GQ365912.

Results

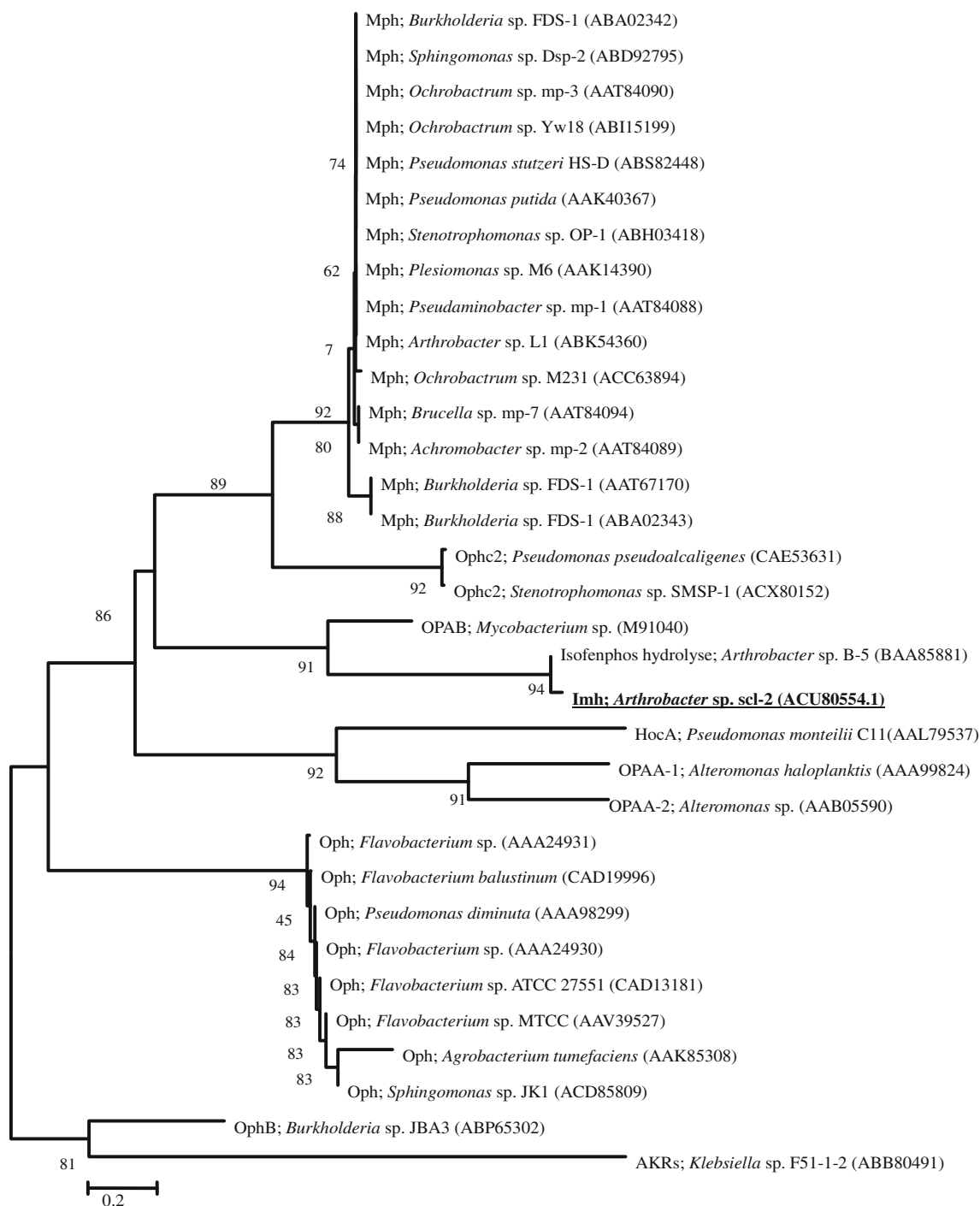
Cloning of the *imh* gene

A 1.3-kb fragment was amplified from *Arthrobacter* sp. strain scl-2 using the primer pair 12F/12R that was designed based on the sequence of the isofenphos hydrolase gene (Ohshiro et al. 1999). The amplified fragment was subcloned into the linear vector pMD18-T

and transformed into the competent *E. coli* DH5 $\alpha$ . The ability of the transformants to hydrolyze isofenphos-methyl was indicated by the presence of a hydrolytic halo around the colony on LB agar containing 200  $\mu$ g/ml isofenphos-methyl (or isocarbophos), and then the hydrolytic ability in liquid medium was further confirmed by HPLC as described previously (Li et al. 2009). The positive clone was sequenced, and the sequence was blasted in the NCBI database. The *imh* gene was found to share 99% sequence similarity with the isofenphos hydrolase gene from *Arthrobacter* sp. strain B-5 (GenBank accession no. AB007293). At the amino acid level, Imh shares 98% identity with the isofenphos hydrolase, with only eight different amino acids (Fig. 1). The BlastP search showed that Imh contains the putative conserved domain of the metallo-dependent hydrolase superfamily (also called the amidohydrolase superfamily). The secondary structure of Imh, as predicted by the modeling server JPred, also shows that Imh has the characteristic fold of metallo-dependent hydrolase (Fig. 1). Imh is also similar to several other hydrolases, including the AdpB from *Nocardia* sp. strain B-1 (58% identity), the amidohydrolase from *Streptosporangium roseum* strain DSM 43021 (46% identity), and the organophosphate acid anhydrase from *Streptomyces sviveus* strain ATCC 29083 (44% identity). Phylogenetic analysis showed that Imh and the isofenphos hydrolase evolved separately from OPH and Mph (Fig. 2).

**Fig. 1** Alignment of the two OP hydrolases from *Arthrobacter* sp. strains scl-2 and B-5. The amino acid residues that differ between these two hydrolases are boxed. The secondary structure was predicted using the modeling server JPred and is shown in the alignment. Helices are indicated with an H, and strands (extended) are indicated by an E. The potential metal ligands are marked with asterisks





**Fig. 2** Phylogenetic tree of the OP hydrolases from different bacterial strains constructed using the neighbor-joining method. The sequences of OP hydrolases were downloaded from the NCBI database.

Statistically significant bootstrap values are shown at the nodes and are expressed as a percentage from 500 replications. The sequence accession numbers of the OP hydrolases follow the host strain

### Expression and purification of Imh

When *E. coli* strain BL21 (DE3) (pET29a-imh) was grown at 37 °C to an  $OD_{600\text{ nm}}=0.6$  and then induced for 16 h by IPTG, the expression of Imh was optimal. Then Imh was purified from the crude extract using Ni-

nitrotriacetic acid affinity chromatography. The concentration of purified Imh was 1.7 mg/ml. The purified Imh gave a single band on SDS-PAGE gel, and the molecular mass of the denatured Imh was approximately 44 kDa, which is in good agreement with the molecular mass deduced from the amino acid sequence of the His6-

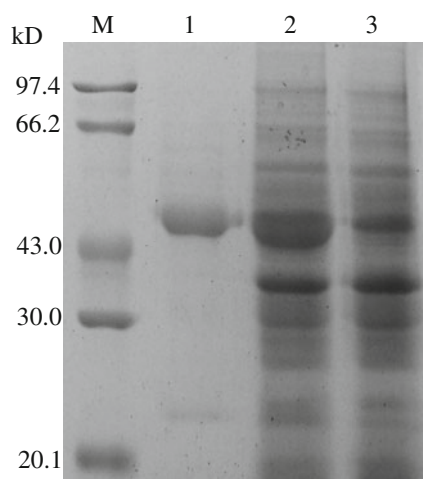
tagged Imh (44.51 kDa) (Fig. 3). The *pI* value of Imh was estimated to be 3.4. The specific activity of the purified Imh for the substrate isofenphos-methyl was 14.35 U/mg.

#### Effect of temperature and pH on Imh activity

The optimal pH and temperature for Imh hydrolysis of isofenphos-methyl were 8.0 °C and 35 °C, respectively (Fig. 4). Imh was fairly stable from 4 °C up to 45 °C and had 55% residual activity at 50 °C. Imh was completely inactivated at 60 °C (Fig. 4a). Imh was very stable at pHs ranging from 6.0 to 8.0 and retained more than 80% of the original activity after preincubation in that pH range for 4 h (Fig. 4b).

#### Effect of metal ions and chemical agents on Imh activity

The surfactants SDS and Triton X-100, the Ser protease inhibitor PMSF, and the His modifier DEPC showed a little inhibition of Imh (Table 2). Imh was seriously inhibited by many metal ions ( $\text{Cu}^{2+}$ ,  $\text{Al}^{3+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Hg}^{2+}$ , and  $\text{Ni}^{2+}$ ). Imh was completely inhibited by the metalloprotease inhibitor 1,10-phenanthroline (0.5 mM), but its activity was restored by the subsequent addition of  $\text{Zn}^{2+}$ . However, subsequent addition of divalent cations such as  $\text{Cd}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Hg}^{2+}$ , and  $\text{Mg}^{2+}$  did not restore the catalytic activity. The ICP-AES analysis showed that purified Imh contained the metal  $\text{Zn}^{2+}$ . The ICP-AES results agreed with the metal removal and reconstitution results indicating that Imh contains the zinc metal center.



**Fig. 3** SDS–PAGE of the overexpressed and purified His-tagged Imh in *E. coli* BL21 (DE3) (pET29a-imh). Lane M, size standards (molecular weights indicated in kDa); lane 1, purified Imh; lane 2, lysate of *E. coli* (pET29a-imh) cells induced by IPTG for 16 h; lane 3, lysate of *E. coli* (pET29a-imh) cells without induction

#### Substrate specificity of Imh

The kinetic constants of purified Imh toward various OPs are listed in Table 3. Imh showed the highest catalytic activity against isofenphos-methyl, isofenphos, isocarbofos, and butamifos. The catalytic activities of these OP substrates by Imh decrease as follows: isofenphos-methyl > isofenphos > isocarbofos > butamifos > profenofos > phoxim > pyridaphenthion > chlorpyrifos > parathion > triazophos > fenitrothion > parathion-methyl. The catalytic efficiency ( $k_{\text{cat}}/K_{\text{m}}$ ) is considered a measurement of the enzyme's specificity. Of these substrates, the results clearly show that isofenphos-methyl is the most preferred substrate. All of these Imh substrates have a P–O–Z moiety (Z represents the leaving group), regardless of whether they are a thion (P=S) or an oxon (P=O) (Fig. 5a). However, some OPs with the P–O–Z moiety, such as monocrotophos and dichlorvos, were not hydrolyzed. By comparing the structure of these substrates, it was found that monocrotophos and dichlorvos lack an aryl or a heterocyclic group in the leaving group, even though there is an unsaturated bond present. These results indicate that the existence of an aryl or heterocyclic group in the leaving group also determines the substrate range of Imh.

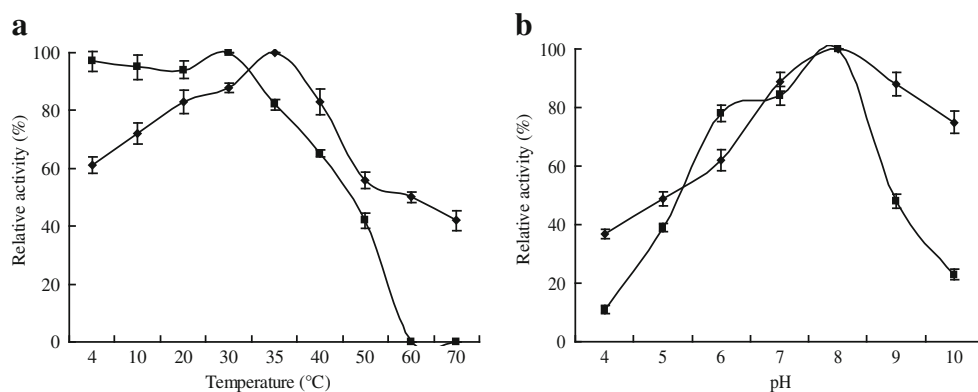
OPs with a P–S–Z moiety, such as phosalone, methidathion, malathion, dimethoate, omethoate, phorate, acephate, and methamidophos (Fig. 5b), were not found to be hydrolyzed, even though there is a heterocyclic group present (e.g., methidathion and phosalone). In addition, OPs with a P–C–X moiety, such as trichlorfon, were not hydrolyzed either.

#### Discussion

OPs are widely used to control the major insect pests in agriculture. The use of enzymes for detoxification and decontamination of OPs is considered a viable and environment-friendly approach. Although many OP hydrolases have been characterized, including the well-studied hydrolases OPH, AdpB, OPAA, and Mph (Fig. 2), isofenphos hydrolase from *Arthrobacter* sp. strain B-5 is the only hydrolase reported that can hydrolyze isofenphos (Ohshiro et al. 1999; Ohshiro et al. 1997). We have experimentally confirmed that Mph could not hydrolyze isofenphos (data not shown). Isofenphos has a special structure and belongs to the family of OPs that contain a P–O–Z moiety and a phosphamide (P–N) bond.

Ohshiro et al. (1997) reported that isofenphos was the only OP among the tested organophosphorus compounds that was efficiently hydrolyzed by the isofenphos hydrolase and that other OPs with a P–N bond, such as butamifos, amiprofos-methyl, and acephate, were not efficiently

**Fig. 4** Effects of temperature (a) and pH (b) on the Imh activity (◆) and stability (■). Isufenphos-methyl was used as the substrate in all reactions. The values are shown as a percentage of the maximum activity (100%)



hydrolyzed. It was also reported that diazinon, isoxathion, fenitrothion, and parathion were not efficiently hydrolyzed. Actually, we argue that butamifos and amiprofos-methyl belong to the OPs family that contains the P–O–Z moiety and that the acephate belongs to OPs family that contains the P–S–Z moiety, even though they all have a P–N bond. It was surprising that the isofenphos hydrolase could not efficiently hydrolyze butamifos, amiprofos-methyl, diazinon, isoxathion, fenitrothion, or parathion (all contain the P–O–Z moiety). We did not test whether or not Imh hydrolyzed amiprofos-methyl, diazinon, and isoxathion because we did not have these OPs. However, we confirmed that Imh could hydrolyze butamifos, fenitro-

thion, and parathion. Therefore, we can conclude prudently that Imh has a relatively broader substrate range than isofenphos hydrolase from *Arthrobacter* sp. strain B-5. Whether the different substrate ranges of these two hydrolases are due to those eight amino acid residues needs further investigation.

The predicted secondary structure of Imh, the inactivation of Imh by metal-chelating metalloprotease inhibitor, the catalytic activity recovery after subsequent addition of  $Zn^{2+}$ , and the ICP-AES analysis all indicate that Imh is a metallo-dependent hydrolase. The superfamily of metallo-dependent hydrolases is a large group of proteins that show conservation in their three-dimensional fold (triosephosphate isomerase barrel; TIM barrel) and in the details of the active site. Most members have a conserved metal binding site involving four histidines and one aspartic acid residue (Benning et al. 2000; Omburo et al. 1992). From the alignment of Imh and other metallo-dependent hydrolases, it was deduced that one of the divalent cations is bound to two histidine residues (His61 and His63) and an aspartic acid (Asp309), whereas the other divalent cation is bound to two histidines (His196 and His216) (Fig. 1).

The substrate range and specificity of Imh was tested with a small substrate library using oxon and thion OPs with P–O–Z and P–S(C)–Z moieties (shown in Table 3). In our study, it was found that Imh could hydrolyze the phosphoester (P–O) bond of 12 of the tested OPs. Imh had an affinity for and catalytic activity on both the commonly occurring P=O-containing OPs and the bigger P=S-containing OPs. Imh did not catalyze the cleavage of P–S and P–C bonds (Table 3). It is hard to determine the rules for the substrate preference of ligands (R1 and R2). However, among the substrates hydrolyzed by Imh, Imh seemed to prefer substrates still with a P–N bond, such as isofenphos-methyl, isofenphos, isocarbophos, and butamifos.

The effect of the leaving group on the substrate specificity was also investigated. A variety of leaving groups were found to be cleaved from the phosphorus center, although the rates of hydrolysis were different. The facts that all the leaving groups of the substrates have an

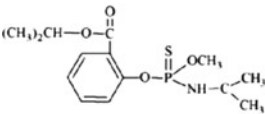
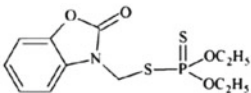
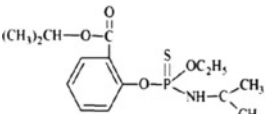
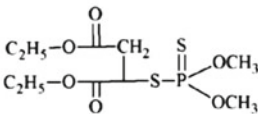
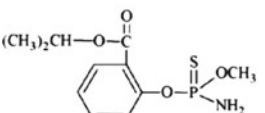
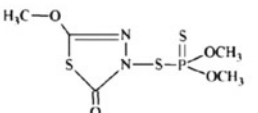
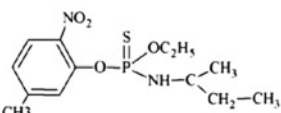
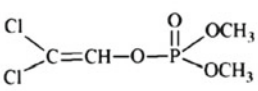
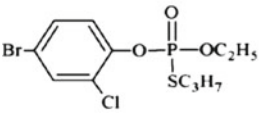
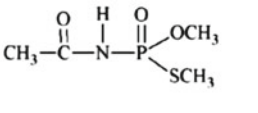
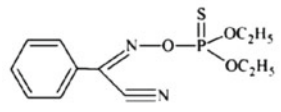
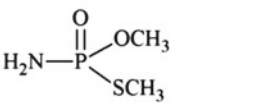
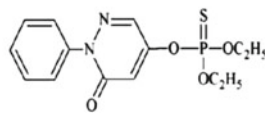
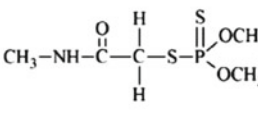
**Table 2** Effect of metal ions and chemical agents on Imh activity

	Relative activity (%) at concentration (mM) of:		
	0.5	2.5	10
ZnCl <sub>2</sub>	93	101	–
CuCl <sub>2</sub>	67	23	–
CoCl <sub>2</sub>	121	89	–
CdCl <sub>2</sub>	64	57	–
LiCl	82	78	–
AlCl <sub>3</sub>	42	45	–
HgCl <sub>2</sub>	54	43	–
FeSO <sub>4</sub>	134	114	–
CaCl <sub>2</sub>	87	72	–
NiCl <sub>2</sub>	56	50	–
MgCl <sub>2</sub>	102	98	–
1,10-Phenanthroline	0	0	–
PMSF	81	–	–
DEPC	87	–	–
β-Mercaptoethanol	104	–	–
Triton X-100	–	–	80
SDS	–	–	67
Tween 80	–	–	55

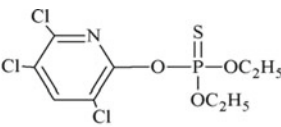
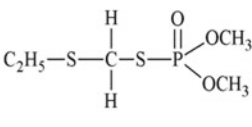
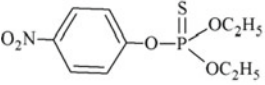
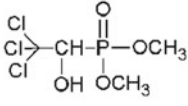
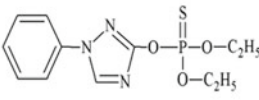
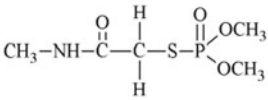
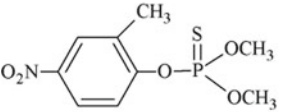
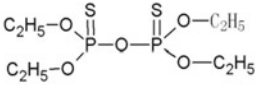
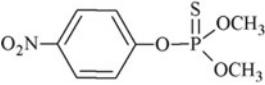
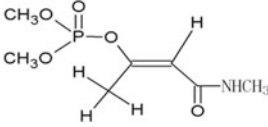
A reaction with no additive was assigned 100% activity. All other reaction rates are relative to this standard



**Table 3** The kinetic constants of OPs hydrolyzed by Imh

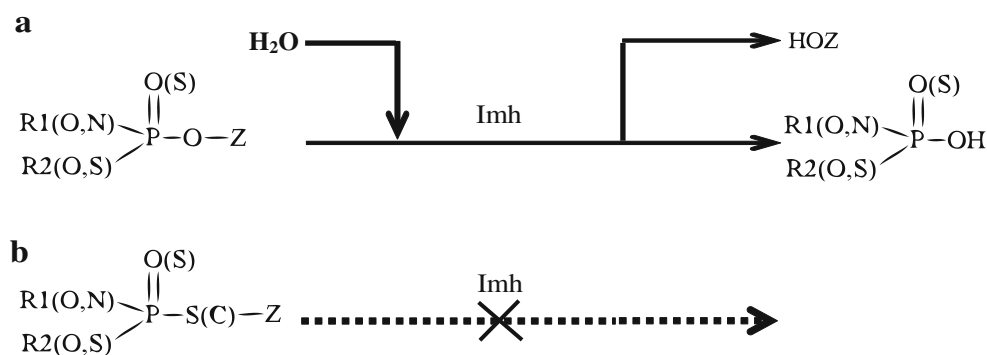
OPs/Structures	$K_m$	$k_{cat}$	$k_{cat}/K_m$	OPs/Structures	$K_m$	$k_{cat}$	$k_{cat}/K_m$
	( $\mu\text{M}$ )	( $\text{s}^{-1}$ )	( $\text{s}^{-1}\cdot\mu\text{M}^{-1}$ )		( $\mu\text{M}$ )	( $\text{s}^{-1}$ )	( $\text{s}^{-1}\cdot\mu\text{M}^{-1}$ )
			<sup>1)</sup>				<sup>1)</sup>
Isofenphos-methyl 	0.81	71.32	88.05	Phosalone 	NA	NA	NA
Isofenphos 	0.93	68.43	73.58	Malathion 	NA	NA	NA
Isocarbophos 	1.07	64.31	60.10	Methidathion 	NA	NA	NA
Butamifos 	2.31	56.18	24.32	Dichlorvos 	NA	NA	NA
Profenofos 	8.40	55.86	6.65	Acephate 	NA	NA	NA
Phoxim 	17.7	32.07	1.81	Methamidophos 	NA	NA	NA
Pyridaphenthion 	21.3	30.63	1.44	Dimethoate 	NA	NA	NA

**Table 3** (continued)

Chlorpyrifos	27.4	10.56	0.39	Phorate	NA	NA	NA
							
Parathion	28.3	3.83	0.14	Trichlorfon	NA	NA	NA
							
Triazophos	30.8	2.45	0.08	Omethoate	NA	NA	NA
							
Fenitrothion	185	0.23	0.001	Sulfotep	NA	NA	NA
							
Methyl parathion	260	0.08	0.0003	Monocrotophos	NA	NA	NA
							

These OPs are not hydrolyzed by Imh

NA, not applicable



**Fig. 5** OPs with a P–O–Z (**a**) instead of a P–S(C)–Z moiety (**b**) were hydrolyzed by Imh. The existence of an aryl or heterocyclic group in the leaving group (Z group) was necessary for hydrolysis by Imh. The double-bonded atom to phosphorous may be oxygen (oxon OPs) or sulfur (thion OPs) (Singh 2009). R1 and R2 can be alkyl groups that

are attached to a phosphorus atom either directly (phosphinates) or through an oxygen (phosphates) or a sulfur (phosphorothioates) atom (Sogorb and Vilanova 2002). In phosphoramidates, at least one of the R groups is attached to –NH<sub>2</sub>

aryl or heterocyclic group and that monocrotophos and dichlorvos were not hydrolyzed by Imh, even though they contain a P–O–Z moiety, indicate that the existence of an aryl or heterocyclic group in the leaving group also determines substrate specificity. The common reaction mechanism of a metallo-dependent hydrolase is deprotonation of a water molecule for a nucleophilic attack on the substrate by the metal ions (such as zinc and cobalt) (Aubert et al. 2004). The substrate specificity and reaction mechanisms of different metallo-dependent hydrolases vary, owing to differences in sequence and the length of the  $\beta$ - or  $\alpha$ -loops that comprise the substrate-binding loops (in particular, loops 1, 7, and 8) (Chen-Goodspeed et al. 2001; Di Sioudi et al. 1999; Gopal et al. 2000; Hong and Raushel 1999; Jackson et al. 2009). The active site of metallo-dependent hydrolases are able to tolerate some structural flexibility in the size and shape of substrates that are turned over (Raushel 2002). It was deduced that the aryl or heterocyclic group in the leaving group may play an important role in the stereochemical and steric selection of substrates by Imh.

In general, the newly characterized Imh has a great potential for use in the decontamination and detoxification of agricultural and household OPs. Furthermore, Imh is a good candidate for the study of the catalytic mechanism and substrate specificity of OP hydrolases. In the future, we are interested in reshaping the active site to expand the substrate specificity of Imh by rationally restructuring the substrate-binding subsites.

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