ISSN 0006-2979, Biochemistry (Moscow), 2006, Vol. 71, No. 2, pp. 167-172. © Pleiades Publishing, Inc., 2006.
Original Russian Text © Yu. A. Votchitseva, E. N. Efremenko, T. K. Aliev, S. D. Varfolomeyev, 2006, published in Biokhimiya, 2006, Vol. 71, No. 2, pp. 216-222.

Properties of Hexahistidine-Tagged Organophosphate Hydrolase

Yu. A. Votchitseva, E. N. Efremenko*, T. K. Aliev, and S. D. Varfolomeyev

Faculty of Chemistry, Lomonosov Moscow State University, 119992 Moscow, Russia;
fax: (7-495) 939-5417; E-mail: efremenko@enzyme.chem.msu.ru

Received January 27, 2005
Revision received February 22, 2005

Abstract—The catalytic properties of organophosphate hydrolase (OPH) containing a hexahistidine tag His_6 (His_6-OPH) and purified to 98% homogeneity were investigated. The pH optimum of enzymatic activity and isoelectric point of His_6-OPH, which were shown to be 10.5 and 8.5, respectively, are shifted to the alkaline range as compared to the same parameters of the native OPH. The recombinant enzyme possessed improved catalytic activity towards S-containing substrates: the catalytic efficiency of methylparathion hydrolysis by His_6-OPH is $4.2 \cdot 10^6$ $M^{-1} \cdot sec^{-1}$, whereas by native OPH it is $3.5 \cdot 10^5$ $M^{-1} \cdot sec^{-1}$.

DOI: 10.1134/S0006297906020088

Key words: organophosphate hydrolase, fusion proteins, hexahistidine tag, organophosphate neurotoxins, substrate specificity

Various organophosphorus pesticides with acute and cumulative neurotoxicity [1-4] pose a real threat to human health. With developments in biotechnology, enzymatic methods are increasingly used for detection and destruction of these compounds, in particular, methods based on the use of organophosphate hydrolase (OPH, aryldialkylphosphatase, phosphotriesterase, EC 3.1.8.1). This enzyme hydrolyzes organophosphorus compounds (OPC) containing P–O, P–F, and P–S bonds in triesters of orthophosphoric acid [5].

The need for industrial production of OPH stems from its possible application as a catalytically active element in biosensor devices designed for detection of OPC in various samples (food, river and waste water) [6, 7]. Methods for biological degradation of OPC (also including those that utilize OPH [8]) are being actively developed worldwide. To facilitate the practical application of these methods, technologically feasible, economic, and highly effective approaches for enzyme production are needed. As a rule, such approaches imply the increase in enzyme synthesis due to the optimization of cultivation conditions of recombinant OPH producers, as well as optimization of protein isolation and purification processes with minimization of the number of steps.

The currently used process for OPH isolation and purification consisting of five consecutive steps [10] is labor-intensive, time-consuming, and thus is not technologically adaptable. Preparation of a genetic construct for expression of recombinant protein containing an additional hexahistidine tag (His_6) allows obtaining highly purified enzyme already after two technological steps using metal chelating chromatography [11, 12]. Such an approach to protein isolation and purification is based on the formation of affinity complexes between protein His_6 tag and metal ions, which are incorporated in the chromatographic support modified with chelating agents [13].

In most cases introduction of a His_6 tag in a protein molecule does not affect its catalytic properties [14-16]. This fact motivates wide practical application of the abovementioned approach for the efficient isolation of recombinant proteins. However, it is known from the literature data that His_6 tag modification can in some cases alter the properties of the target protein, in particular, enzyme catalytic properties [17, 18], solubility [19], and ability to form oligomers [20]. Moreover, the level of modified protein in the active soluble form can decrease due to the formation of inclusion bodies [21].

All these factors listed above defined the design of genetic constructs to achieve a high level of expression of native OPH containing His_6 at the N-terminus of the protein molecule (His_6-OPH) [22-24]. An *Escherichia coli* strain providing the maximal level of soluble His_6-OPH

Abbreviations: OPH) organophosphate hydrolase; OPC) organophosphorus compounds.

* To whom correspondence should be addressed.

accumulation was selected [23]. The modified protein was isolated and purified to 98% homogeneity on Ni-NTA agarose [24].

The present work focuses on the investigation of the following catalytic properties of the His_6-modified protein: substrate specificity, pH optimum, and temperature dependence of enzyme activity. The properties of the native and modified OPH are also compared in this work.

MATERIALS AND METHODS

Materials. The following chemicals were used in this work: paraoxon (diethyl-*p*-nitrophenyl phosphate), parathion (diethyl-*p*-nitrophenyl thiophosphate), imidazole, Ches (2-[N-cyclohexyl-amino]ethanesulfonic acid, Hepes (4-[2-hydroxyethyl]-1-piperazineethansulfonic acid), cobalt chloride hexahydrate, nickel chloride, glycerol, bromophenol blue, Coomassie brilliant blue R-250, ampicillin sodium salt, kanamycin disulfate, egg albumin, sodium dodecyl sulfate (Sigma, USA); methylparathion (dimethyl-*p*-nitrophenyl phosphate) (Fluka, Switzerland); tryptone, yeast extract (Difco, USA); acrylamide, bis-acrylamide, ethylenediaminetetraacetic acid (EDTA) (Merck, Germany); isopropyl-β-D-thiogalactopyranoside (IPTG), protein markers for electrophoresis with molecular weights 14.4, 18.4, 25.0, 35.0, 45.0, 66.2, and 116.0 kD (Fermentas, Lithuania); N,N,N′,N′-tetramethylethylenediamine (TEMED), ammonium persulfate (Bio-Rad, USA). Ni-NTA agarose was used as affinity chromatography support (agarose modified with nitrilotriacetic acid) (Quiagen, USA). All other chemicals were of analytical grade and purchased from Labtekhnika and Khimmed (Russia).

To transform *E. coli* the following genetic constructs were used: pTES-OPH (containing the previously cloned *oph* gene from *Pseudomonas diminuta* VKM B-1297 cells) and pTES-His-OPH (for expression of OPH containing hexahistidine tag at the N-terminus of the protein molecule). The constructs were obtained as described earlier [22, 24]. *Escherichia coli* DH5α and *E. coli* SG13009[pREP4] cells were used for the isolation of native and modified OPH, respectively.

The *E. coli* DH5α cells were cultured at 37°C in LB medium with ampicillin (100 μg/ml), whereas SG13009[pREP4] strain containing pTES-His-OPH plasmid was grown in LB medium with both ampicillin (100 μg/ml) and kanamycin (25 μg/ml). After cultivation for 16 h, the inoculate was introduced into 100 ml of the nutrient medium containing (g/liter): tryptone, 12.0; yeast extract, 24.0; glycerol, 4.0; KH_2PO_4, 6.95; $K_2HPO_4{\cdot}3H_2O$, 12.54 (pH 7.0). When the absorbance reached 0.6 at 540 nm (A_{540}), enzyme expression was induced by addition of IPTG in the medium up to the concentration of 0.25 mM. At the same time, $CoCl_2$ solution was added to give 10^{-4} M concentration of Co^{2+}. The cells were cultivated at 30°C with constant stirring (200 rpm) on a thermostatted Adolf Kuhner AG shaker (Switzerland) for 21-24 h. Then the biomass was separated by centrifugation (20 min at 5000*g*) on a Beckman J2-21 centrifuge (USA).

Native OPH was isolated and purified according to the known procedure [23], which yielded 12 mg of target protein with specific activity of 5820 units/mg from 1 g of cell biomass. The enzyme yield was 69% of the initial OPH quantity produced by the cells.

His_6-OPH was isolated and purified on Ni-NTA agarose. Wet biomass was weighed and resuspended in 50 mM phosphate buffer, pH 8.0, containing 300 mM NaCl to the concentration of 0.2 g/ml. Then the cell suspension was treated with ultrasound (frequency of 44 kHz) 6 times for 45 sec. The resulting cell lysate was separated by centrifugation (30 min at 15,000*g*). An equal volume of Ni-NTA agarose pre-equilibrated in 50 mM phosphate buffer was added to the supernatant. The resulting suspension was packed in a chromatographic column (5 ml) and washed with 50 mM phosphate buffer (pH 8.0) containing 300 mM NaCl and 10 mM imidazole at the flow rate of 0.5 ml/min until the absorbance (A_{280}) decreased to 0.01. The enzyme was eluted by an imidazole concentration gradient (10-500 mM). Imidazole was removed from collected protein fractions by dialysis against 50 mM phosphate buffer, pH 8.0, and after that the fractions were analyzed, and protein concentration and enzymatic activity were determined. As a result, 5.2 mg of highly purified enzyme preparation with average specific activity of 6870 units/mg protein was obtained from 1 g of wet biomass. Enzyme yield was 81.7% of the initial amount of His_6-OPH synthesized by the cells.

SDS-PAGE. The level of OPH and His_6-OPH expression as well as homogeneity of enzyme preparations was estimated by electrophoresis under denaturing conditions in 12% polyacrylamide gel using a Miniprotean II cell (BioRad) followed by Coomassie R-250 staining.

Protein concentration was determined by the Bradford method using the reagent supplied by BioRad.

Protein isoelectric points were determined by analytical isoelectrofocusing (IEF) on a Model 111 Cell instrument (BioRad).

Statistics. All experiments were performed in triplicates, and mean values and standard deviations were calculated in each case.

Determination of OPH and His_6-OPH activities. Enzymatic activity was determined spectrophotometrically using an Agilent 8453-UV instrument (Germany) monitoring the accumulation of hydrolysis product, 4-nitrophenolate anion, at 25°C and 405 nm (ε 17,000 $M^{-1}{\cdot}cm^{-1}$, pH 9.0; ε 18,000 $M^{-1}{\cdot}cm^{-1}$, pH 10.5). To determine the catalytic activity of OPH and His_6-OPH, 50 mM Ches buffer (pH 9.0) and 50 mM carbonate buffer (pH 10.5) were used, respectively. Aqueous

solutions of paraoxon, parathion, and methylparathion (1 mM) were used for the investigation of substrate specificity of the enzymes. The catalytic reaction was initiated by introduction of OPH or His_6-OPH into the cuvette containing buffer and the substrate so that the concentration of each enzyme in the reaction mixture was 10^{-10}-10^{-9} M (E_0).

One unit of enzymatic activity was defined and expressed as the quantity of enzyme that is necessary for hydrolysis of 1 μmol of the substrate per min at 20°C and pH 9.0 (in the case of native OPH) or pH 10.5 (in the case of modified OPH).

The enzymatic reaction rates were calculated using the initial linear regions of the kinetic curves ($v_0 = \tan\alpha$). The maximal velocities of enzymatic reaction (V_{max}) and Michaelis constants (K_m) were determined both by using Lineweaver–Burk coordinates ($1/v_0 - 1[S]$) and from the entire curves of substrate-dependent reaction velocities.

For the investigation of dependence of enzyme catalytic activity on the pH, 50 mM buffers with overlapping pH values were used: Hepes (pH 7.5-8.5), Ches (pH 8.5-10.0), and phosphate-carbonate (pH 9.5-12.0).

To determine the temperature optimum for native and modified enzymes, the cuvette with buffer and substrate was thermostatted for 10 min at selected temperature in the range of 20 to 70°C. The reaction was initiated by addition of OPH or His_6-OPH aliquot (10 μl) in 50 mM phosphate buffer (pH 8.0), and the absorbance of the solution was measured on the Agilent 8454-UV spectrophotometer equipped with a thermostatted cell.

Activities of OPH and His_6-OPH at different pH and temperatures were expressed as a percentage of the maximum value found for each of the enzymes.

For the investigation of dependence of OPH and His_6-OPH enzymatic activities on the nature of the metal ion, the ions were removed from the enzyme active site by treatment with 50 mM EDTA prior to the experiment. The complexing agent was removed by dialysis against 50 mM phosphate buffer (pH 8.0). Then the corresponding apo-enzyme was incubated with 10-fold molar excess of metal ion and enzymatic activity of OPH and His_6-OPH was determined.

RESULTS AND DISCUSSION

Determination of catalytic properties of OPH and His_6-OPH. Figure 1 shows the electrophoretic data for analysis of both OPH and His_6-OPH isolated enzyme fractions. The hexahistidine tag changed the electrophoretic mobility of the enzyme. The band corresponding to His_6-OPH is located considerably higher on electrophoregram than the native OPH band. The decrease in electrophoretic mobility of His_6-OPH was not proportional to the increase in its molecular weight. Such behavior of His_6-OPH is apparently associated with the change

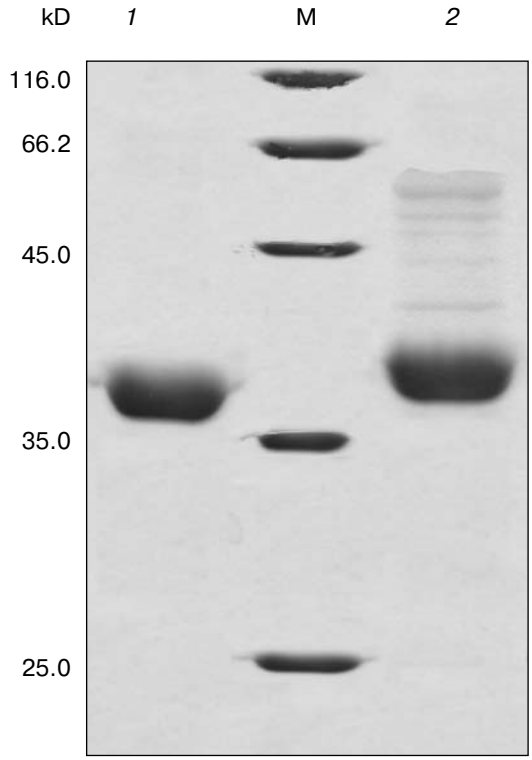

Fig. 1. Electrophoregram of the purified OPH and His_6-OPH preparations: *1*) OPH; *2*) His_6-OPH; M, molecular weight markers.

in total protein charge due to the incorporation of additional amino acid residues in the protein molecular structure.

According to IEF data, the isoelectric point (p*I*) for native OPH is 7.4, and the p*I* for His_6-OPH is shifted to alkaline pH by 1.1 units resulting in p*I* value of 8.5. It can be assumed that introduction of the additional hexahistidine tag in the N-terminus of protein molecule leads to the protonation of nitrogen atoms in the imidazole ring of the histidines and release of hydroxyl ions. As a result, the total protein charge changes, and the p*I* shift for His_6-OPH is observed.

The pH dependence of enzymatic activity, thermostability, and temperature optimum for native OPH and His_6-OPH were studied.

The modified enzyme displayed steady catalytic activity in the wide pH range from 7.0 to 12.0, and the rate of the catalyzed reaction in the end points of the range was 30% of the maximum. The pH dependences of enzymatic activities of OPH and His_6-OPH had similar patterns, where the maximal activity for both enzymes was observed in a rather wide pH range: from 8.5 to 11.0 for native OPH, and from 9.5 to 11.5 for His_6-OPH. Thereby, the optimal pH range for His_6-OPH was shifted to the alkaline range by 0.5-1.0 pH unit as compared with pH optimum for the native enzyme (Fig. 2). It was found

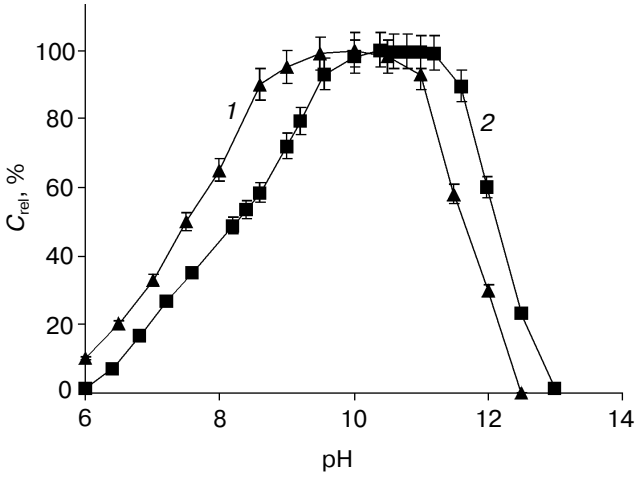

Fig. 2. Dependence of relative enzymatic activity (C_{rel}, % of maximum value) of OPH (*1*) and His_6-OPH (*2*) on the pH of the medium. Substrate: 1 mM paraoxon. Each point represents the average of three measurements.

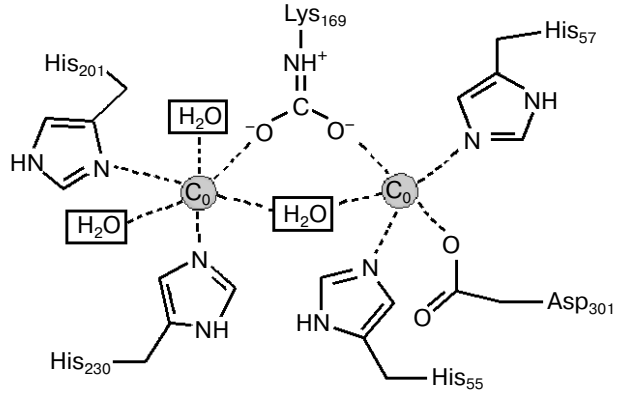

Fig. 3. Structure of the active site of Co^{2+}-containing organophosphate hydrolase.

that the nature of the buffer also has an influence on activity of the investigated enzyme preparations. The enzymes retained their activity up to pH 13.0 in the carbonate buffer, while a dramatic drop in activity at pH above 10.0 was observed for both enzymes when other buffers that maintain pH within the range of 9.5-12.0 were used (Ches, phosphate, and others). This fact confirms the earlier assumption that carbonate buffer causes the formation of carbamylated lysine residue in the enzyme active site (Fig. 3) [25, 26].

Investigation of enzymatic hydrolysis of OPC by OPH and His_6-OPH at different temperatures and pH values revealed a number of general trends (Fig. 4). At pH 8.0, the temperature optimum for both enzymes was within 57-62°C (Fig. 4a). If the OPC hydrolysis reaction was carried out at pH 9.0, the temperature optima for both enzymes were shifted to lower temperatures: the optimal intervals for native and modified OPH were 50-55 and 53-58°C, respectively (Fig. 4b).

Significant difference in the temperature optima for these enzymes was observed at pH 10.5 in the range of 43-47°C for OPH and 47-52°C for His_6-OPH (Fig. 4c). Thus, the tendency in shifting the temperature optima to lower temperature under more alkaline reaction condi-

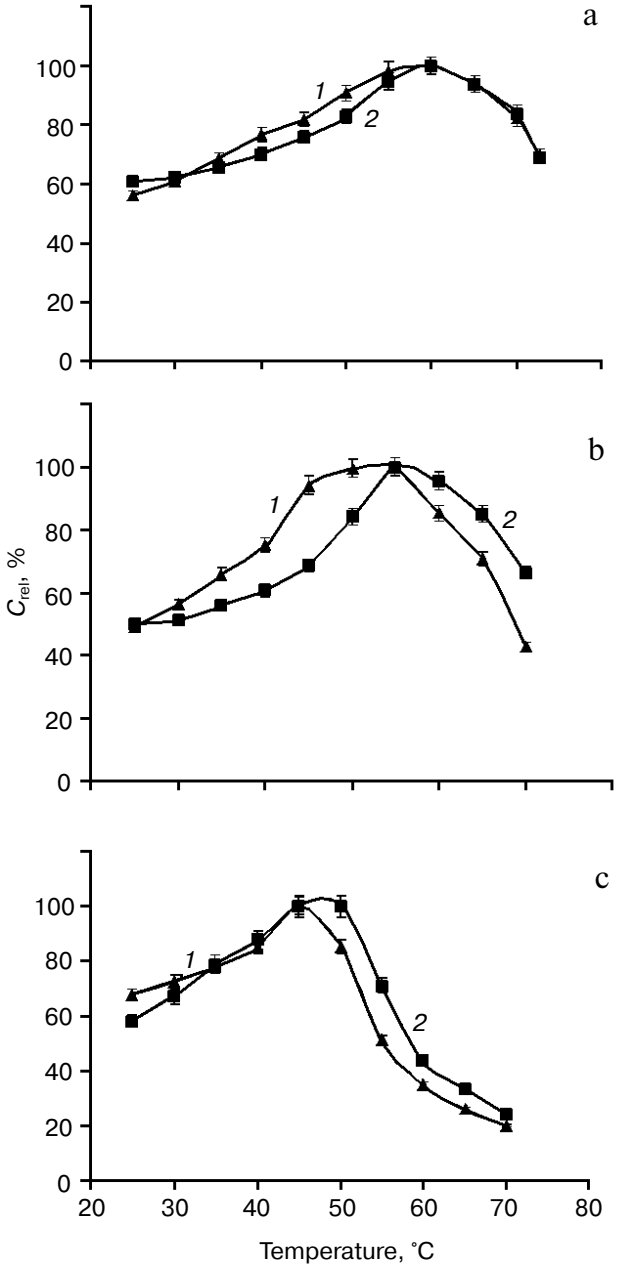

Fig. 4. Dependence of relative enzymatic activity (C_{rel}, % of maximum value) of OPH (*1*) and His_6-OPH (*2*) on temperature at different pH of the medium: a) pH 8.0; b) pH 9.0; c) pH 10.5. Substrate: 1 mM paraoxon. Each point is the average of three measurements.

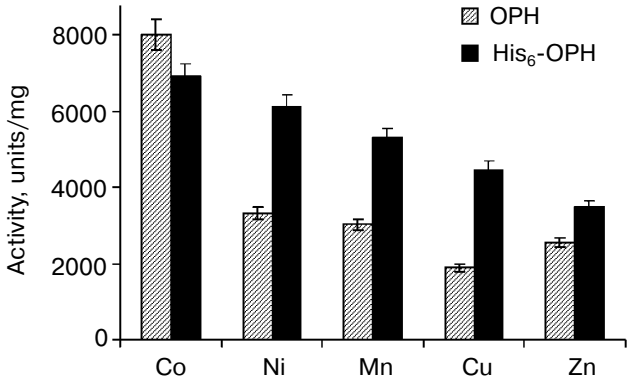

Fig. 5. Histogram showing dependence of OPH and His_6-OPH specific enzymatic activity on the nature of metal ion in the enzyme active site. Substrate, 1 mM paraoxon.

tions was observed. The shift was more pronounced in the case of native OPH.

It is known that OPH is a metalloenzyme that contains two bivalent metal ions in the active site [27]. For this reason, the influence of bivalent ions on the rate of the His_6-OPH catalyzed reaction was investigated. It was found that with variation of metal ion in the enzyme active site the activity of His_6-OPH decreases in the series: $Co^{2+} > Ni^{2+} > Mn^{2+} >> Cu^{2+} > Zn^{2+}$ (Fig. 5). The data are nearly identical with those obtained for native OPH previously, with the exception of that the native enzyme is more active in the presence of Zn^{2+} rather than Cu^{2+}.

Determination of the kinetic parameters for substrate hydrolysis (paraoxon, parathion, and methylparathion) by OPH and His_6-OPH (table) demonstrated that depending on the substrate the catalytic activity of the modified enzyme was 2-10 times higher than the native OPH. This difference was most remarkable when S-containing substrates were used. Thus, in the case of methylparathion, $V_{max}/[E_0]$ increased and K_m decreased simultaneously, whereas in the case of parathion $V_{max}/[E_0]$ value remained virtually unchanged, and the Michaelis constant decreased almost 5-fold. In the case of paraoxon the K_m value was also decreased.

Such improvement of the catalytic properties of the modified enzyme compared to the native one could be due to conformational changes in the structure of the sorption site induced by the N-terminal region of the enzyme molecule, which is spatially close to the C-terminus and located on the opposite side from the active center [26]. Moreover, the change in catalytic properties of the modified enzyme could be caused by emergence of an additional catalytic center formed by coordinated interaction between metal atoms and histidine residues of the genetically introduced affinity tag sequence. Such metallocomplex could potentially be formed and have influence on OPC hydrolysis by His_6-OPH. A number of examples confirming the possibility of OPC hydrolysis upon the action of various metallocomplexes [28-30], especially those containing histidine residues [29, 30], are known.

According to available literature, the influence of hexahistidine tag on catalytic properties of metalloenzymes has not been studied before. It is likely that the meager number of publications in this field is associated with the fact that genetic modification of metalloenzymes is undesirable due to the possible formation of strong coordination bonds between metal ions in the chelating support and the metalloenzyme active site. As a result, modified protein can be eluted from the column only under harsh conditions using eluent containing high imidazole concentrations (up to 1 M) or eluent with acidic pH (3.0-4.0), which can affect the activity of metalloenzymes [13]. Moreover, in the vast majority of cases, hexahistidine tag is incorporated into the protein structure only to simplify the isolation procedure, and authors do not compare the native and recombinant enzyme properties.

The results of the present work pose a question regarding the influence of enzyme structure on catalytic acceleration mechanism. The reasons for altered His_6-OPH properties compared to the native enzyme will be further investigated.

Kinetic constants for OPC hydrolysis by OPH and His_6-OPH

Substrate	$V_{max}/[E_0]$, sec^{-1}		K_m, μM		$V_{max}/([E_0]\cdot K_m)$, $M^{-1}\cdot sec^{-1}$	
	Enzyme					
	OPH	His_6-OPH	OPH	His_6-OPH	OPH	His_6-OPH
Paraoxon	4900 ± 100	5100 ± 100	16 ± 0.5	10 ± 0.5	$(3.0 \pm 0.2) \times 10^8$	$(5.1 \pm 0.3) \times 10^8$
Parathion	620 ± 40	530 ± 30	60 ± 1.0	15 ± 0.5	$(8.8 \pm 0.4) \times 10^6$	$(5.0 \pm 0.3) \times 10^7$
Methylparathion	75 ± 5	310 ± 20	210 ± 10	73 ± 1.0	$(3.5 \pm 0.2) \times 10^5$	$(4.2 \pm 0.2) \times 10^6$

Catalytic characteristics of His_6-OPH revealed in this work evidence enzyme stabilization under alkaline hydrolysis conditions compared to the native OPH. Moreover, the modified enzyme has improved catalytic efficiency towards the number of known substrates (widely used organophosphorus pesticides). The possibility of simplification of enzyme isolation procedure by using metal chelating chromatography, yielding the enzyme with improved properties, can provide a basis for wide application of polyhistidine tagged OPH.

REFERENCES

1. Alavanja, M. C., Hoppin, J. A., and Kamel, F. (2004) *Ann. Rev. Pub. Health*, **25**, 155-197.
2. Eskenazi, B., Bradman, A., and Castorina, R. (1999) *Environ. Health Perspect.*, **107**, 409-419.
3. Fukushima, M., and Yamaguchi, Y. (1994) *Kankyo Gijutsu*, **23**, 74-76.
4. Kwong, T. C. (2002) *Ther. Drug Monit.*, **24**, 144-149.
5. Efremenko, E. N., and Sergeeva, V. S. (2001) *Izvest. Akad. Nauk*, *Ser. Khim.*, **10**, 1743-1749.
6. Mulchandani, P., Chen, W., and Mulchandani, A. (2001) *Environ. Sci. Technol.*, **35**, 2562-2565.
7. Simonian, A. L., Efremenko, E. N., and Wild, J. R. (2001) *Anal. Chim. Acta*, **444**, 179-186.
8. McDaniel, C. S. (2004) USA Patent Appl. 20040109853.
9. Aubert, S. D., Li, Y., and Raushel, F. M. (2004) *Biochemistry*, **43**, 5707-5715.
10. Omburo, G. A., Kuo, J. M., Mullins, L. S., and Raushel, F. M. (1992) *J. Biol. Chem.*, **267**, 13278-13283.
11. Nilsson, J., Stahl, S., Lundeberg, J., Uhlen, M., and Nygren, P. A. (1997) *Protein Expr. Purif.*, **11**, 1-16.
12. Chaga, G. S. (2001) *J. Biochem. Biophys. Meth.*, **49**, 313-334.
13. Terpe, K. (2003) *Appl. Microbiol. Biotechnol.*, **60**, 523-533.
14. Kowolik, C., and Hengstenberg, W. (1998) *Eur. J. Biochem.*, **257**, 389-394.
15. Chen, H.-M., and Chen, K.-T. (2000) *Enz. Microbiol. Technol.*, **27**, 219-226.
16. Hung, M., Gibbs, C., and Tsiang, M. (2002) *Antiviral. Res.*, **56**, 99-114.
17. Goel, A., Colcher, D., Koo, J., Booth, B., Pavlinkova, G., and Batra, S. (2000) *Biochim. Biophys. Acta*, **1523**, 13-20.
18. Yang, S., Park, N., Lee, T., and Cha, J. (2002) *Biotechnol. Appl. Biochem.*, **35**, 199-203.
19. Woestenenk, E. A., Hammarstrom, M., van den Berg, S., Hard, T., and Berglund, H. (2004) *J. Struct. Funct. Genom.*, **5**, 217-229.
20. Mohanty, A. K., and Wiener, M. C. (2004) *Protein Exp. Purif.*, **33**, 311-325.
21. Sachdev, D., and Chirgwin, J. M. (1998) *Biochem. Biophys. Res. Commun.*, **244**, 933-937.
22. Varfolomeev, S. D., Efremenko, E. N., Aliev, T. K., and Votchitseva, Yu. A. (2002) RF Patent No. 2232807.
23. Efremenko, E. N., Votchitseva, Yu. A., Aliev, T. K., and Varfolomeyev, S. D. (2004) in *Biocatalytic Technology and Nanotechnology* (Zaikov, G. E., ed.) Nova Science Publishers, Inc., N. Y., pp. 65-71.
24. Efremenko, E. N., Votchitseva, Yu. A., Aliev, T. K., and Varfolomeev, S. D. (2003) Application for RF Patent No. 2003136646.
25. Hong, S.-B., Kuo, J. M., Mullins, L. S., and Raushel, F. M. (1995) *JACS*, **117**, 7581-7582.
26. Benning, M. M., Kuo, J. M., Raushel, F. M., and Holden, H. M. (1995) *Biochemistry*, **34**, 7973-7978.
27. Kuo, J. M., Chae, M. Y., and Raushel, F. M. (1997) *Biochemistry*, **36**, 1982-1988.
28. Deal, K., and Burstyn, J. (1996) *Inorg. Chem.*, **35**, 2792-2798.
29. Vichard, C., and Kaden, T. A. (2002) *Inorg. Chim. Acta*, **337**, 173-180.
30. Hay, R. W., and Govan, N. (1998) *Transition Met. Chem.*, **23**, 721-725.