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Extraction and Purification of Organophosphorus Hydrolase Enzyme from Soil Microorganism *Pseudomonas diminuta*

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ABSTRACT

Synthetic organophosphorus compounds are highly toxic hence, widely used as pesticides, insecticides and chemical warfare agents. Organophosphorus hydrolase (OPH) is an organophosphotriester hydrolysing enzyme; effectively hydrolyse a range of organophosphate esters. The objective of the present study was extraction and purification of OPH enzyme from Pseudomonas *diminuta* bacteria (soil microorganism) and to study kinetic properties of the purified enzyme. Enzyme was extracted and purified from bacteria by ammonium sulphate precipitation and ion exchange chromatography. Purity of an enzyme was determined by sodium dodecyl sulphate-polyacryamide gel electrophoresis (SDS-PAGE). Purified OPH enzyme specific activity was found to be 27.7 fold of 32.8 U/mg protein, molecular weight of 72 Kda and it is a homodimer since it has shown a single band in SDS-PAGE separation. Maximum activity of the free OPH enzyme was found at Optimum pH 7.5 and temperature 35 ^{o}C with the incubation time of 10 min. Michaelis constant (K_m) and maximum velocity (V_{max}) values of free OPH enzyme for methyl parathion as substrate was found to be 286.2 µM and 2.5 µM/min respectively.

Keywords: Organophosphorus compounds, *Pseudomonas diminuta*, Organophosphorus hydrolase (OPH), Chromatography

1. INTRODUCTION

Organophosphorus hydrolase (OPH) is an organophosphotriester hydrolysing enzyme; it is also known as parathion hydrolase, paraoxonase, phosphotriesterase and aryl dialkyl phosphatise[1,2]. OPH enzyme was originally isolated from soil bacteria i.e. Pseudomonas *diminuta* and Flavobacterium species. OPH is a member of the amidohydrolase superfamily and share the same (α–β) 8 barrel structure[3]. It is one of the most studied enzymes, due to its ability to catalyze the hydrolysis of nerve agents and organophosphorus pesticides. OPH requires one or two metal ions for full catalysis i.e. either zinc or cobalt[1,4]. Zinc was found to be the native metal; however, activity can also be supported by other divalent cat ions such as Co^{2+}, Cd^{2+}, Ni^{2+} and Mn^{2+}, where Co^{2+} shows the highest activity[5].

The enzyme is known to have broad substrate specificity and able to hydrolyse almost all of organophosphorus compounds with P–O, P–S, P–F, P–CN bonds[1,6-8]. Due to its bioremediation potential, it has been also used in industrial, health care and military purposes[9]. It can also be used in bioanalytical techniques for the detection of neurotoxic war agents[10-12] and for therapeutic as well as prophylactic purpose by using liposome encapsulated OPH against OP toxicity when used alone or in combination with antidote such as atropine and 2-PAM (Pyridine-2-Aldoxime Methyliodide)[13]. OPH is a homodimeric and metal dependent enzyme consists of 336 amino-acid residues with a molecular weight of 72 Kda[14]. It was found that the mature enzyme was synthesised as a 365 amino acid precursors with 29 amino acids long signal peptide[15].

OPH is able to degrade a wide variety of organophosphorus pesticides due to its hydrolytic activity organophosphorus hydrolase is a cytoplasmic membrane enzyme; hydrolyses the parathion into *p*-nitrophenol (PNP) and diethyl thiophosphoric acid[16]. PNP can be detected by electrochemical and colorimetric methods, which can be exploited to develop a biosensor for detection of organophosphate pesticide. The use of OPH is extremely attractive for the biosensing of OP substances that act as substrates for the enzyme, rather than exerting an inhibitory action. Several types of OPH-based biosensors have been introduced recently, including potentiometric[10,17,18]. In the present study, OPH enzyme was extracted from *pseudomonas diminuta,* purified to homogeneity by chromatographic method and also studied the kinetic parameters of the purified enzyme.

2. MATERIALS AND METHODS

Nutrient broth, tris-HCl, sodium succinate, sodium borate, Folin's Ciocalteus reagent, and sodium potassium tartarate were purchased from Merck, India. Bovine serum albumin

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(BSA), EDTA, lysozyme, protein kinase A, PMSF, NaCl, sodium dodecyl sulphate (SDS), acrylamide, bisacrylamide, TEMED, ammonium persulphate (APS), commassie brilliant blue R-250, sodium hydroxide, sucrose and methyl parathion were purchased from Sigma, Aldrich India.

2.1 Microorganism

Pseudomonas diminuta sp. MTCC 3361 was obtained from Microbial Type Collection Centre, Institute of Microbial Technology, Chandigarh, India, in lyophilised form.

2.2 Revival and Growth of Lyophilised sample of Bacteria

Lyophilised sample of bacteria was taken in a test tube and about 5 ml of nutrient broth (pH-8.0) was added to make a bacterial suspension. The revived bacterial suspension was transferred to the conical flask (250 ml each) containing nutrient broth for culturing the *Pseudomonas diminuta* cells. Incubated the culture medium in a rotator incubator shaker (200 rpm) at 30˚C for 24 hours or overnight and growth of the bacteria was monitored by measuring OD at 600 nm.

2.3 Extraction and Purification of OPH from *Pseudomonas Diminuta*

P. diminuta cells were harvested at 8000 rpm for 10 min at 4°C and the cell pellet obtained was resuspended in 7.5 ml of 50 mM sodium phosphate buffer (pH.8) containing 0.75 M sucrose and incubated on ice for 10 min. Then added 100µl of lysozyme containing 1.5 mM K-EDTA to the suspension and incubated for 1 h [19]. Spheroplasts were removed by centrifuging at 10,000 rpm for 5 min. Spheroplasts pellet was resuspended in 20 ml of 50 mM sodium phosphate buffer (pH-8) containing Protein Kinase A (0.5mg/ml) and incubated on ice for one hour with constant stirring[20]. After incubation, 20µl of 1mM of PMSF was added to inhibit the protein Kinase A activity. This step significantly enhanced the release of membrane-bound OPH enzyme into solution. Spheroplasts were removed by centrifuged at 10,000 rpm for 5 min. The supernatant containing an enzyme was collected and considered as crude enzyme solution. Enzyme activity was monitored by enzyme assay.

For purification of OPH enzyme, the sample of crude enzyme solution was dissolved in a sodium phosphate buffer (pH.8). A solution of ammonium sulphate 40% was then added to the solution in an ice bath while gently stirring for one hour. Then centrifuged for 15 min at 4 °C and 20,000 X g, an insoluble upper layer was appeared and discarded. Subsequently, a solution containing ammonium sulphate (60 percent) was added and again centrifuged; resulting precipitate was dissolved in 50mM sodium phosphate buffer and loaded on a DEAE Sepharose CL-6B resin (Pharmacia, Sweden) in a 300×15 mm column. The column was washed with excess amount of 50 mM sodium phosphate buffer (pH-8) with a flow rate of 1 ml/min, to remove unbounded substances. Bounded proteins were sequentially eluted by concentration gradient of sodium chloride in 50 mM sodium phosphate buffer (pH-8). The fractions (5-6 ml) were collected and monitored for OPH activity. The incubation time was determined for optimum activity. Samples with the highest OPH activity were lyophilised and stored until analysis. The purity of the samples was monitored on 10 percent sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by staining with commassie brilliant blue R-250[21].

2.4 Enzyme Assay and Protein Estimation

Microbial OPH activity was measured spectrophotometrically by monitoring the production of p-nitrophenol. The standard assay reactions containing 0.1 ml of purified OPH solution was mixed with 2.9 ml of 50 mM sodium phosphate buffers (pH.8.0) containing 0.1 ml of 10 mM methyl parathion (as substrate) and incubate for 10 min at 37°C. The activity was assayed by measuring the formation of 4-nitrophenol at 410 nm. One unit of enzymatic activity was defined as the amount of enzyme capable to catalyzing the production of 1 µmol of 4-nitrophenol per minute under the standard conditions[22]. The protein content of enzyme solution was determined using the method described by[23].

2.5 Kinetic Properties of Free Organophosphorus Hydrolase

2.5.1 Effect of pH

The optimum pH for the maximum free enzyme activity was determined in the pH range from 6-10 using the following buffers: sodium succinate (pH- 6.0, 6.5 and 7.0), sodium phosphate (pH- 7.5, 8.0 and 8.5) and sodium borate (pH- 9.0, 9.5 and 10.0) buffers each at a final concentration of 0.1 M [22].

2.5.2 Effect of Temperature

The incubation temperature was determined for optimum activity. The reaction mixture was incubated at different temperatures ranging from 20°C to 50°C at an interval of 5°C [22].

2.5.3 Effect of Time of Incubation

The effect of incubation time was studied from 2 min to 12 min at a regular interval of 2 min[22].

2.5.4 Effect of Substrate Concentration

The effect of substrate (Methyl parathion) concentration on the enzyme activity was studied up to 600 µM in the reaction mixture at an interval of 50 µM [22].

2.5.5 Determination of Km and Vmax

K_m and V_{max} values for free OPH enzyme were also calculated from Lineweaver-Burk plot between reciprocal of substrate concentration (1/[S]) and reciprocal of velocity of the reaction (1/ [V]) [22].

2.5.6 Effect of Metal Ions

The effect of metal ions(2mM) on the enzyme activity was studied in presence of the metal ions such as Zinc(Zn^{2+}) sulphate, Cobalt(Co^{2+}) chloride, Magnesium(Mg^{2+}) sulphate, Calcium(Ca^{2+}) carbonate and Ferric($Fe3^{+}$) chloride[24].

3. RESULTS

3.1 Extraction and Purification of Organophosphorus Hydrolase Enzyme from *Pseudomonas Diminuta*

Organophosphorus Hydrolase (OPH) enzyme was purified from crude extract of *Pseudomonas diminuta* by ammonium sulphate precipitation followed by DEAE-Sepharose ion exchange chromatographic separation. Protein content, activity, specific activity, total activity and percentage yield of

Table 1. Protein content, activity, specific activity, total activity and percentage yield of the crude extract, fraction and purified OPH enzyme

Sample	Total volume (ml)	Protein (mg/ml)	Activity (U/ml)	Specific activity (U/mg protein)	Total activity (U)	Purification factor	% Yield
Crude	74	0.91	1.08	1.18	79.92	1	100
Precipitated fraction	37	0.10	1.28	12.8	47.36	10.84	59.92
Purified OPH	4.5	0.05	1.64	32.8	7.38	27.79	9.2

the crude extract and fractions are as shown in Table 1. The enzyme was purified to 27.79 fold to a specific activity of 32.8 U/mg of protein from the crude enzyme solution with a yield of 9.2 percent.

3.2 Biochemical Characterisation of OPH Enzyme

To test the homogeneity, the purified OPH fractions were subjected to SDS-PAGE, it exhibited enzyme a single band (Lane 1 and 2) indicating apparent homogeneity of purified enzyme (Fig. 1). This protein band matched with that of standard protein of up to 98 KDa (Lane 1) revealing that the enzyme had a molecular weight of 72 KDa.

3.3 Kinetic Properties of Free OPH Enzyme

3.3.1 Effect of pH

The effect of pH on the enzyme activity of free OPH was determined in the pH range of 6.0-10. The maximum enzyme activity of free OPH was observed at pH 7.5 as shown in (Fig. 2).

3.3.2 Effect of Temperature

The effect of the temperature on the enzyme activity from 20 °C to 50 °C was also investigated. Initially as temperature rises there was an increase in the enzyme activity from 30 °C-35 °C, after that there was a decrease in the enzyme activity when temperature was increased from 40 °C - 50 °C. As per our observation, the optimum temperature for free OPH enzyme was 35 °C as shown in Fig. 3.

3.3.3 Effect of Incubation Time

The activity of the free enzyme to incubation time was studied in the time range from 2 min to 12 min at a regular interval of 2 min. The activity was linearly increasing up to a time of 8 min and thereafter constant enzyme activity was observed, as shown in the Fig. 4.

3.3.4 Effect of Substrate Concentration

Effect of methyl parathion concentration on the activity of free enzyme was studied in the concentration range from 50 to 600µM. Free OPH enzyme showed a hyperbolic relationship between its activity and methyl parathion concentration up to a final concentration of 500 µM. Above this concentration there was not much changes in the enzyme activity, as shown in the Fig. 5.

3.3.5 Determination of K_m and V_{max}

The Michaelis constant (K_m) and maximal velocity (V_{max}) values of free OPH enzyme for methyl parathion as substrate were 286.2 µM and 2.5 µM/min, respectively.

3.3.6 Effect of Metal Ions

The effect of Co^{2+}, Ca^{2+}, Mg^{2+} , Zn^{2+} and Fe^{3+} metal ions(2mM) on OPH enzyme activity was studied at pH 7.5 and temperature 35° C. The activity of the enzyme in standard reaction mixture without these cations was taken as 100% and the results are tabulated in the Table 2. The results indicate that, the interference of Ca^{2+}, Mg^{2+} and Zn^{2+} ions does not have the significant effect on activity. Whereas, Co^{2+} ions increases the enzyme activity but Fe^{3+} ion significantly decreases the enzyme activity.

4. DISCUSSION

OPH is a membrane bound protein so its purification requires sophisticated techniques to obtain its pure form. OPH enzyme was purified from crude extract of *Pseudomonas*

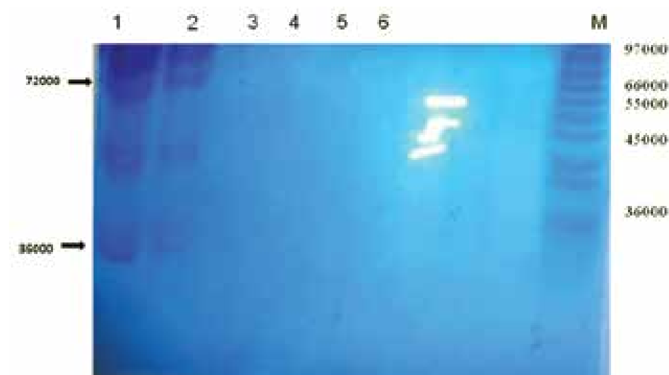

Figure 1. SDS-PAGE analysis of crude (3) and purified OPH fractions (F 1, 2, 3, 4, 5, and 6) are Ion exchange chromatography fractions, M- Protein markers.

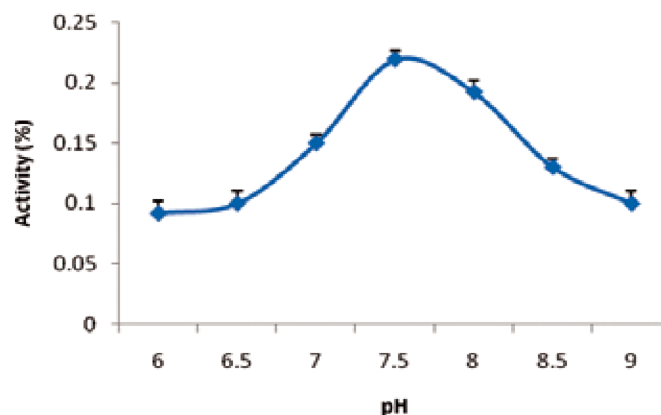

Figure 2. Effect of pH on the activity of free OPH enzyme.

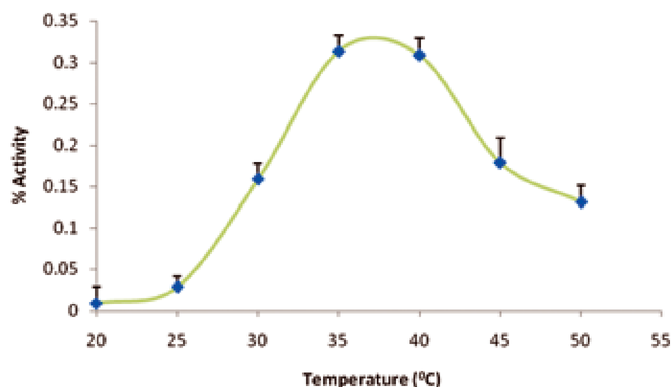

Figure 3. Effect of temperature on the activity of free OPH enzyme.

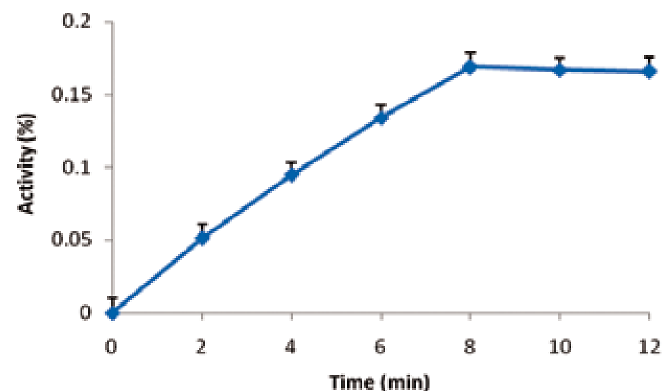

Figure 4. Effect of Incubation time on the activity of free OPH enzyme.

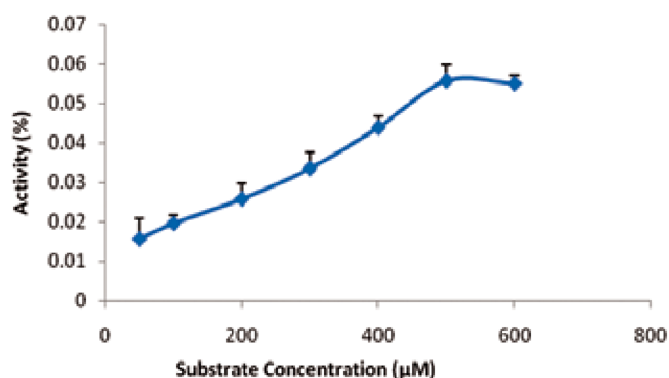

Figure 5. Effect of substrate concentration on the activity of free OPH enzyme.

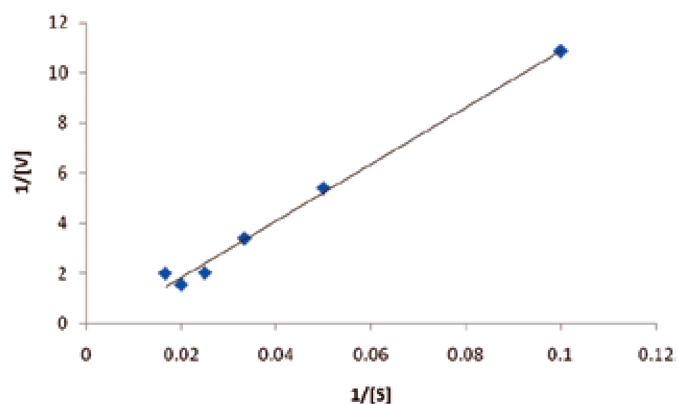

Figure 6. Line-weaver burk plot between [1/s] and 1/[v] for free OPH enzyme.

diminuta by ammonium sulphate precipitation followed by DEAE-Sepharose ion exchange chromatographic separation to obtain a specific activity of 32.8 U/mg of protein, this shows there was a 30 times increase in the enzyme activity. In one of the reported study, OPH was purified to homogeneity from sf9 cells infected with the recombinant baculovirus (A5B) containing the Opd gene from *Pseudomonas diminuta* using a combination of gel filtration and ion exchange chromatographic techniques to increase the specific activity[1]. In order to assess the purity of the fraction, it is subjected to SDS-PAGE analysis. It exhibited a single band indicating apparent homogeneity of purified enzyme. This protein band matched with that of standard protein of upto 98 Kda revealing that the molecular weight of purified enzyme was 72 Kda, which is similar to OPH from *P. diminuta* MG[9] and *Flavobacterium* sp. strain ATCC27551[25].

Kinetic properties of the free OPH enzyme were studied to determine the optimum condition for the maximum activity. Because most biological based assays have the influence of temperature, pH and substrate concentration on the enzyme activity. Free enzyme was showing maximum activity at pH.7.5 and temperature 35°C. Our results are correlated well with the previously reported studies[24,26,27].

Most biological-based assays are influenced by the assay temperature. Since variation in temperature is likely to affect the enzyme activity. The reaction rate initially increases as temperature rises, due to increased kinetic energy of the reacting molecules. Eventually, however, the kinetic energy of the enzyme exceeds the energy barrier for breaking the weak hydrogen and hydrophobic bonds that maintain its secondary-tertiary structure. At this temperature denaturation of the enzyme occurs, with change in the active site structure and with an accompanying loss of catalytic activity. Therefore enzymes exhibit maximum activity at an optimal temperature[28].

Free OPH enzyme showed a hyperbolic relationship between its activity and methyl parathion concentration upto a final concentration of 500µM. Substrate concentration is an important factor to evaluate the free enzyme activity. Decreased enzyme activity was observed even when concentration of enzyme is high but due to lower concentrations of substrate as they don't occupy all the active sites of the enzyme. When the substrate concentration was sufficiently high, we observed increase in the enzyme activity by occupying active site. After some duration if we further increase the substrate concentration there is no change in the enzyme activity this indicates enzyme saturation. Under these conditions, there was no free enzyme present and the concentration of enzyme-substrate complex was the total enzyme concentration present. Michaelis constant (K_m) and maximal velocity (V_{max}) values of free OPH enzyme for methyl parathion as substrate which were compared with previously reported methods[26,27].

5. CONCLUSIONS

Organophosphorus hydrolase (OPH) is one of the most studied enzyme due to its ability to catalyze the hydrolysis of nerve agents and organophosphorus pesticides. It becomes an enzyme of choice in the development biosensor for qualitative

Table 2. Effect of metal ions on the OPH enzyme activity

Metal ions and chemicals	Activity (Percent)
Normal	100
Zn^{2+}	86
Co^{2+}	121
Ca^{2+}	74
Mg^{2+}	81
Fe^{3+}	48

and quantitative determination of OP pesticides. In addition to this, the low Km value and temperature stability could make the purified enzyme an attractive biocatalyst for applied bioremediation and also as a useful tool for the efficient biodegradation bio fertilizers. Future studies are under progress to enhance the stability of the enzyme for the development of biosensors.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest to the materials or techniques employed in this present study.

CONTRIBUTORS

Dr Maheshwari D.T. has received her PhD in Life Sciences from Bharathiar University, Coimbatore, India, in 2013. Presently working as Scientist ‘C’. She has published some research papers in peer reviewed journals in the area of nutrition and biochemistry. Her areas of interests : Biosensors development, biodetection systems, immunology and molecular biology.

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Dr N. S. Kumar, currently working as Scientist ‘F’ and heading the Department of Environmental Protection at DEBEL. He was involved in the design and development of NBC protective equipment, air quality management for submarine (INS Arihant) and NABL Accreditation. He has more than 25 publications in peer reviewed journal and posses some patents.