Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 6, Issue 10, 2014

Original Article

PURIFICATION AND KINETIC STUDIES OF ORGANOPHOSPHORUS HYDROLASE FROM *B. DIMINUTA*

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Received: 31 Aug 2014 Revised and Accepted: 29 Sep 2014

ABSTRACT

Objective: Extraction and purification of Organophosphorus hydrolase (OPH) enzyme from *Brevundimonas diminuta* and to study kinetic properties of the purified enzyme.

Methods: The enzyme was extracted from bacteria and purified by using a combination of gel filtration and ion-exchange chromatography and the purity of an enzyme was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The activity of the purified enzyme was monitored by enzyme assay and total protein content was determined by using Lowry's method. The kinetic properties of the enzyme were also studied.

Results: A 72 kDa organophosphorus hydrolase (OPH) enzyme was extracted and purified. The purified enzyme was homodimer and showed a single band on SDS-PAGE. The Michaelis constant (K_m) and maximal velocity (V_{max}) values of free OPH enzyme for methyl parathion as substrate was 285.71 µM and 50 µM/min respectively. At optimum pH (7.5) and incubation temperature (35°C), free enzyme showed maximum activity with incubation time of 8 min.

Conclusion: The bacteria contain OPH enzyme with high potential to detoxify OP pesticides, attractive for bioremediation due to good pH & temperature conditions, were also useful in development of bio analytical techniques such as biosensors for OP pesticide detection.

Keywords: Organophosphorus pesticides, *Brevundimonas diminuta*, Organophosphorus hydrolase, Chromatography.

INTRODUCTION

Organophosphorus hydrolase (OPH) also known as parathion hydrolase, paraoxonase, phosphotriesterase, aryldialkylphosphatase, isolated from *Pseudomonas diminuta* or *Flavobacterium* have been one of the most studied enzyme due to its ability to catalyze the hydrolysis of nerve agents and organophosphorus pesticides. The properties of OPH were studied by many researchers, but the primary information related to the structure and function of the enzyme was provided by Raushel and Wild. A study revealed that the expressed form of an enzyme was membrane associated [1]. When the gene responsible for the expression of an enzyme was cloned into other hosts, it expressed the membrane associated enzyme, which makes it difficult for purification. OPH is a homodimer consists of 336 amino-acid residues with a molecular mass of 72 kda [2]. It was found that the mature enzyme was synthesized as a 365 amino acid precursors with 29 amino acids long signal peptide [3]. When this 29 amino acid long signal peptide found in precursor enzyme, responsible for membrane localization, was removed from the clone, then recombinant enzyme can be expressed in the cytoplasm as a mature enzyme that retained activity [4]. The recombinant enzyme has been expressed in different hosts such as insects, insect cells, fungi and *Streptomyces*. The organophosphorus hydrolase is a metalloprotein contains two Zn^{2+} ions at the active site in the native enzyme [5, 6]. A number of different divalent metal ions (Mn^{2+}, Cd^{2+}, Co^{2+}, or Ni^{2+}) can be used as substitute for Zn^{2+} ions [7]. When the enzyme substituted with Co^{2+}ions, it showed the highest activity on organophosphorus compounds used as substrate that contain P-F and P-S bonds.

OPH is able to degrade a wide variety of organophosphorus pesticides due to its hydrolytic activity [8-11]. Due to its bioremediation potential, OPH has been used in industrial, health care and military purposes [12]. It can also be used in bioanalytical techniques for the detection of neurotoxic war agents [13-15] 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) [16]. In the present study, organophosphorus hydrolase enzyme was extracted from *Brevundimonas diminuta* and enzyme was purified to homogeneity by using chromatographic methods and also studied the kinetic parameters of the purified enzyme.

MATERIALS AND METHODS

Chemicals and reagents

Sephadex G-100 from MP Biomedicals, nutrient broth, tris-HCL, EDTA, lysozyme, NaCl, sucrose and sodium hydroxide were purchased from Himedia, India and methyl parathion. All other chemicals were of analytical reagent grade unless otherwise stated. Double distilled water (DDW) was used throughout the experiments.

Instruments and equipments

UV spectrophotometer (Shimadzu corporation, Japan), digital pH meter (EUTECH), refrigerator (LG), microwave oven (LG), rotatory incubator shaker (HICON), magnetic stirrer (HICON), refrigerated centrifuge (SIGMA) and deep freezer (Voltas).

Microorganism

The lyophilized sample of bacteria *Brevundimonas diminuta* (MTCC 3361) was purchased from IMTECH, Chandigarh.

Revival and growth of lyophilized sample of bacteria

Take lyophilized sample of bacteria 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 *Brevundimonas 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 taking OD at 600 nm.

Extraction and purification of OPH from *Brevundimonas diminuta*

B. diminuta cells were harvested by centrifuged at 8000 rpm for 10 min and cells were suspended in 7.5 ml of 50 mM sodium phosphate buffer, pH-8, containing 0.75 M sucrose and incubated at 20 °C for 10

min. 20 µl of 10 mg/ml lysozyme solution was added and incubated for 10 min. Add three times the solution containing 1.5 mM K-EDTA and 10 mg/ml lysozyme to the suspension and incubated for 1 h [17]. Spheroplasts were harvested by centrifuged at 10,000 rpm for 5 min. Spheroplasts pellet was re suspended in 20 ml of 50 mM sodium phosphate buffer (pH-8) containing 500 mM NaCl and incubated on ice for 1 h with constant stirring [18]. 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. The protein content of crude solution was concentrated by using a lyophilizer machine. For purification of OPH enzyme, the lyophilized sample of crude solution was dissolved in minimal volume of Tris-HCl buffer (50 mM, pH-8). The crude enzyme solution was then loaded onto the sephadex G-100 column (2.5×30 cm) and run in 50 mM Tris-HCl (pH-8.0) at a flow rate of 0.5 mL/min. The active fractions were pooled and subjected to ion exchange chromatography on DEAE-Sepharose column (1.5×25 cm) that had been pre-equilibrated with Tris HCl buffer (50 mM, pH 8). The enzyme was eluted with a NaCl gradient solution (.01-1 M) [19]. The eluted fractions were analyzed for purity on a 12.5% SDS-PAGE gel [20].

Enzyme assay and protein estimation

The enzyme assay of purified organophosphorus hydrolase was carried out by following reaction described by Chaudhary et al., 1988 [21] with slight modification: -

$$\text{OP pesticide} + H_2O \xrightarrow[(\lambda = 410\ nm)]{\text{OPH}} \text{4-nitophenol} + \text{diethyl phosphate}$$

The 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. The protein content of enzyme solution was determined using the method described by Lowry *et al.,* (1951) [22].

Kinetic properties of free organophosphorus hydrolase

Effect of pH

The optimum pH of free enzyme was determined by varied the pH of reaction buffer 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.

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.

Effect of time of incubation

Time of incubation was also studied from 2 min to 12 min at a regular interval of 2 min.

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.

Determination of K_m and V_{max}

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]).

RESULTS AND DISCUSSION

Purification of organophosphorus hydrolase from *Brevundimonas diminuta*

Enzyme was purified from crude extract by gel filtration and DEAE-Sepharose chromatography. The purification steps and results were summarized in Table 1. The enzyme was purified 14.15 fold to a specific activity of 20.81 U/mg of protein from the crude enzyme solution with a yield of 19.92%.

Table 1: It shows the purification of OPH.

Fraction	Total volume (ml)	Protein (mg/ml)	Activity (Unit/ml)	Specific activity (Unit/mg)	Total activity (Units)	Purification fold	% yield
Crude	20	0.87	1.28	1.47	25.6	1	100
Sephadex G-100	9	0.073	1.09	14.93	9.81	10.15	38.32
DEAE-Sepharose	5	0.049	1.02	20.81	5.1	14.15	19.92

Kinetic properties of free OPH enzyme

Effect of pH

The activity of free OPH was determined in the pH range of 6.0-10 at a regular increase of 0.5. The optimum pH of free OPH was 7.5 (Figure 1) which was compared with OPH NL01 isolated from *Pseudomonas aeruginosa* NL01 (pH-8.0) [23], His_6-OPH from *E. coli* (pH-10.5) [24], parathion hydrolase from *Streptomyces lividans* (pH-9.0) [25], phosphotriesterase from sf9 cells (pH-9.0) [10], phosphotriesterase from *E. coli* cells (pH-8.0) [7].

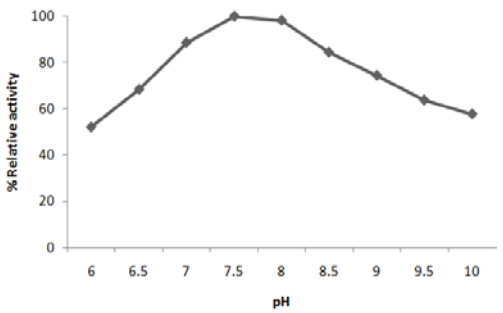

Fig. 1: It shows an effect of pH on the activity of free OPH enzyme.

Effect of incubation temperature

The effect of the temperature on the enzyme activity from 20°C to 50°C was also investigated. The optimum temperature for free OPH was 35°C (Figure 2), which was lower than previously reported method [3, 23-25].

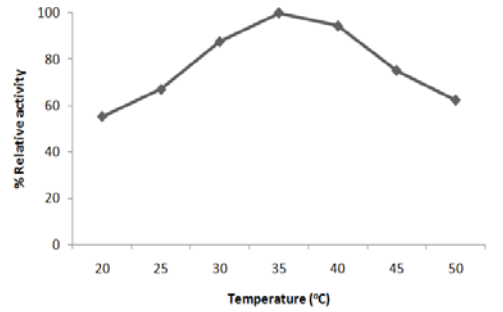

Fig. 2: It shows effect of temperature on the activity of free OPH enzyme.

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 no increase was observed (Figure 3).

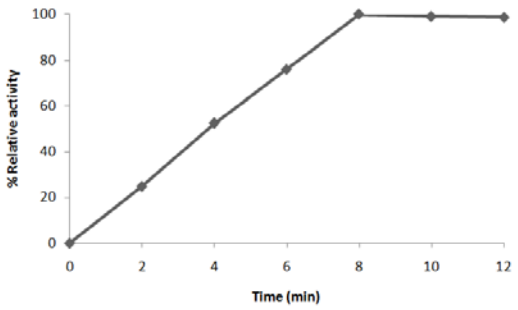

Fig. 3: It shows effect of time of incubation on the activity of free OPH enzyme.

Effect of substrate concentration

Effect of methyl parathion concentration on the activity of free enzyme was studied in the concentration range up to 600 µM. Free OPH enzyme showed a hyperbolic relationship between its activity and methyl parathion concentration up to a final concentration of 500 µM after which no increase was observed (Figure 4).

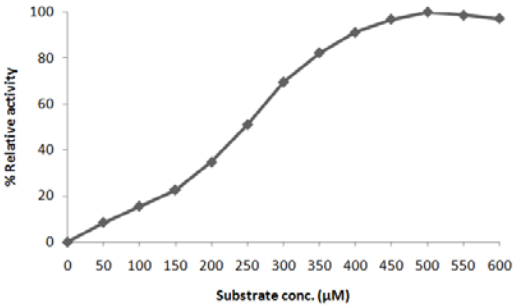

Fig. 4: It shows effect of substrate concentration on the activity of free OPH enzyme.

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 285.71 µM and 50 µM/min, respectively (Figure 5), which were compared with previously reported methods [24, 25].

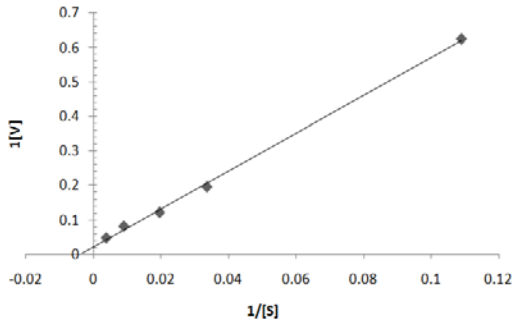

Fig. 5: It shows Lineweaver–Burk plot between 1/[S] and 1/[V] for free OPH enzyme.

CONCLUSION

In this report, the bacteria contain OPH enzyme with high potential to detoxify OP pesticides. The good pH and temperature stability could make the purified enzyme an attractive candidate for bioremediation procedures. In addition, the OPH enzyme due to its high potential to catalyze the hydrolysis of organophosphorus pesticides can be a very useful tool for application in bioanalytical techniques for quantitative determination of OP pesticides [26].

CONFLICT OF INTERESTS

Declared None

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