Applied Microbiology and Biotechnology
https://doi.org/10.1007/s00253-020-11008-w

ENVIRONMENTAL BIOTECHNOLOGY

A novel biosensor for the detection of organophosphorus (OP)-based pesticides using organophosphorus acid anhydrolase (OPAA)-FL variant

Monika Jain[1] · Priyanka Yadav[1] · Bhavana Joshi[1] · Abhijeet Joshi[1] · Prashant Kodgire[1]

Received: 11 March 2020 / Revised: 28 October 2020 / Accepted: 8 November 2020

Abstract

Indiscriminate use of organophosphorus (OP)-based insecticides is a great concern to human health because of bioaccumulation-induced health hazards. Potentially fatal consequences and limited treatment methods of OP poisoning necessitate the need for the development of reliable, selective, cost-effective, and sensitive methods of OP detection. To tackle this issue, the development of effective devices and methods is required to sensitively detect as well as degrade OPs. Enzymatic sensor systems have gained popularity due to high catalytic activity, enhanced detection limits, and high sensitivity with the environmentally benign operation. Organophosphorus acid anhydrolase (OPAA) from *Alteromonas* sp. JD6.5 is capable of hydrolyzing the P-F, P-O, P-S, and P-CN bonds, in OPs, including nerve agents of the G/V-series. Several mutants of OPAA are reported which have greater activity against various OPs. In this study, recombinant expression of the OPAA-FL variant in *Escherichia coli* was performed, purified, and subsequently tested for activity against ethyl paraoxon. OPAA-FL variant showed its optimum activity at pH 8.5 and 50 °C. Colorimetric and fluorometric assays were used for estimation of ethyl paraoxon based on *p*-nitrophenol and fluorescein isothiocyanate (FITC) fluorescence intensity, respectively. Colorimetric and fluorometric assay estimation indicates that ethyl paraoxon can be estimated in the linear range of 0.01 to 1 mM and 0.1 to 0.5 mM, with LOD values 0.04 mM and 0.056 mM, respectively. Furthermore, the OPAA-FL variant was immobilized into alginate microspheres for colorimetric detection of ethyl paraoxon and displayed a linear range of 0.025 to 1 mM with a LOD value of 0.06 mM.

Key points

- *Biosensing of paraoxon with purified and encapsulated OPAA-FL variant.*
- *Colorimetric and fluorometric biosensing assay developed using OPAA-FL variant for paraoxon.*
- *First report on alginate encapsulation of OPAA-FL variant for biosensing of paraoxon.*

Keywords Organophosphorus acid anhydrolase (OPAA)-FL variant · Acetylcholinesterase (AChE) · Paraoxon · Organophosphorus compounds (OPs)

Introduction

An increase in the global population has led to greater demand for food supplies, which eventually demands increased production from existing cultivable land. In recent times, massive agricultural expansion has necessitated an increase in pesticide and insecticide use in order to improve the productivity of the crops. According to a recent report, globally every year, over a few billion pounds of pesticides are used (Hoppin 2014). These commonly used pesticides are classified mainly into three categories: organochlorines, carbamates, and organophosphates. Due to their low-cost synthesis and high efficacy, organophosphates are widely used. OPs are also known as phosphotriesters because of the presence of the three phosphodiester bonds. Structurally, OPs have alkyl or aryl groups that either are bonded to phosphorous via oxygen or sulfur

✉ Abhijeet Joshi
abhijeet.joshi@iiti.ac.in

✉ Prashant Kodgire
pkodgire@iiti.ac.in

[1] Department of Biosciences and Biomedical Engineering, Indian Institute of Technology Indore, Khandwa Road, Indore, India

Published online: 16 November 2020

linkage, or are directly bonded to the phosphorus. The leaving group attached to the phosphorus may be an aromatic, halogen, aliphatic, or heterologous cyclic group (Jain et al. 2019). If the central phosphorus atom is bound to sulfur, then the OPs are called as thions and if the central atom is bound to the oxygen, then such OPs are called as oxons (Peter et al. 2010). These OPs are extensively used as pesticides for the last several decades. It has been reported that persistent exposure to the OPs leads to the accumulation at the depot sites, from where they are released into the blood in a sustained manner and consequently, they reach the nervous system and their target, the AChE enzyme. These compounds inactivate a variety of serine hydrolases (lipases and esterases) that are important for the proper functioning of the human body (Mangas et al. 2017).

Currently, OP-based pesticides constitute between 33 and 60% in the developed, as well as in the developing countries (Atwood and Paisley-Jones 2008). These hazardous OPs exhibit severe toxicity via various modes, such as skin absorption, inhalation, or ingestion. The use of OPs has been of serious concern, as these OPs have a propensity to block the active site of important enzymes in neurons, such as acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), which break down and remove acetylcholine from the nerve synapse. Furthermore, the lower biodegradability of OPs as compared to newer pesticides brings further complexity for the environmental scientists. OPs in the blood can enter tissues and subsequently bind to AChE. Such binding of OPs to AChE prevents its normal function, i.e., breakdown of acetylcholine in the neuromuscular junctions. Thus, the inability to break down acetylcholine in the neuromuscular junctions can lead to severe neurological problems leading to convulsions, tremors, and even death (Hertz-Picciotto et al. 2018). Though OP poisoning is traditionally treated using a combination of atropine and pralidoxime (Basher et al. 2013), these medications have enormous side effects (Eddleston 2018; Cornelissen et al. 2020). Moreover, these drugs are not as effective in patients with prolonged exposure due to an effect known as the "aging effect," in which a non-enzymatic loss of alkyl chain from the phosphate group in the OP compound converts the inhibited/blocked AChE into a non-reactivatable form (Masson et al. 2010). Incidentally, poisoning by OPs has a high mortality rate of up to 10–20% (Eddleston 2000; Liu et al. 2012) and therefore is a serious cause of concern for healthcare professionals and clinicians (Kang et al. 2009). Thus, measures to facilitate a fast, accurate, and convenient detection of OPs in humans, as well as other environmental samples, are necessary (Jain et al. 2019).

Initially, separation methods like chromatography were used for pesticide analysis in the environment; however, these techniques prevented adequate monitoring and had several limitations (Van Dyk and Pletschke 2011). An alternative method of pesticide detection was promoted many years back using enzymes. The main enzymes that were utilized at that time were AChE and BChE, using which the first enzymatic sensors for the detection of organophosphates were developed (Kulys and D'Costa 1991; Marty et al. 1992). Both enzymes play a key role in the functioning of the nervous system (Van Dyk and Pletschke 2011). The OPs and nerve agents were detected based on the inactivation of the enzymes in proportion to the OPs and nerve agents present. Based on this property of the OPs, various inhibition-based biosensors have been designed for the accurate detection of the OPs (Pundir and Chauhan 2012; Pohanka 2013). Nevertheless, AChE and BChE are also inhibited by various chemical agents apart from nerve agents and OPs, such as hypochlorite, detergents, carbamates, heavy metals, fluoride, and nicotine, and therefore, one cannot rely on the detection of the inhibition-based biosensors using AChE and BChE (Karami et al. 2016). Additionally, it involves the prior cleanup procedure of the instrument and requires a tedious process of extraction, thereby making it not so suitable for the detection of the OPs and nerve agents on a daily basis. Thus, there is a need for the development of a biosensor that is rapid, selective, and sensitive for the detection of OPs as well as nerve agents. In recent times, enzymatic sensors based on the hydrolysis of OPs have emerged as important candidates for enzymatic detection of OP compounds. The first enzymatic sensor using the principle of hydrolysis of OP compounds was developed by Wild and group in 1996 (Rainina et al. 1996).

OPAA (EC 3.1.8.2) is one of the promising enzymes which can hydrolyze hazardous OPs (Li et al. 2016). The wild-type OPAA is reported to act on various OPs, including nerve agents of G-series (Jeong et al. 2012), and OPAA variants are reported to act on V-series nerve agents as well (Daczkowski et al. 2015). The OPAA enzyme isolated from an aerobic, short-rod, gram-negative bacterium *Alteromonas* sp. has drawn a keen focus due to its efficiently high expression in *Escherichia coli* as well as its high activity against nerve agent GD (DeFrank and Cheng 1991). In the year 1993, Luqipei and group determined OPAA in blood using a colorimetric method to develop a pioneered approach; OPAA was encapsulated in erythrocytes. OPAA activity was determined with paraoxon as a substrate. The activity was reported based on the amount of liberation of *p*-nitrophenol and diethyl phosphoric acid formed during the reaction. The detection limit was reported to be 0.01 mM of *p*-nitrophenol (Pei et al. 1993).

Earlier in 2003, Mello and group built a fluorescence-based biosensor in which they developed a Langmuir and Langmuir-Blodgett (LB) monolayer film of OPAA. One layer of OPAA was labeled with fluorescein isothiocyanate (FITC), a fluorescent probe. It was deposited onto the quartz slide and tested against diisopropyl fluorophosphate (DFP) as a sensor. The stability of the LB film as a sensor showed the potential of the system as a biosensor, and a clear pronounced response was

reported (Sarita V. Mello et al. 2003). Two protons and an alcohol are generated upon hydrolysis of the OP compound, which are often electroactive or chromophoric (Pedrosa et al. 2010; Kirsch et al. 2013). The two protons produced reduce the pH of the environment and thus drop the intensity of the FITC (Choudhary et al. 2019). In 2004, Simonian and his group invented a new approach of using a multi-enzyme strategy for the detection of various classes of neurotoxins based on the substrate specificity of the enzymes. OPAA was used in a modified pH-sensitive field-effect transistor. Sensor response of DFP was reported and linear order kinetics was reported between 12.5 and 500 μM concentration of DFP whereas barely any measurable signals were reported in the case of paraoxon (Simonian et al. 2004). Reports about the use of OPAA for the detection of paraoxon are meager in literature.

Here, we studied a variant of OPAA-FL expressed in *E. coli* and purified to study its kinetics against ethyl paraoxon. The colorimetric and fluorometric detection method was employed for the measurement of ethyl paraoxon based on the production of *p*-nitrophenol and the interaction of protons produced in the catalytic reaction with fluorescein isothiocyanate (FITC). Additionally encapsulated OPAA-FL variant matrices were developed using alginate microspheres and combined with the above optical measurements to quantify the hydrolysis of paraoxon (Mulchandani et al. 2001).

Materials and methods

Ethyl paraoxon, methyl parathion, oligonucleotide primers, $MnCl_2$, $CaCl_2$, sodium alginate, and FITC-dextran were purchased from Sigma-Aldrich. All the reagents and solvents were of HPCL grade. Tris base, Bis-Tris propane, LB broth, and kanamycin were purchased from HiMedia. Ni-NTA columns were purchased from GE Healthcare.

Cloning of OPAA-FL variant in a pET expression vector

The wild-type gene coding for OPAA was initially cloned from *Alteromonas* sp. JD6.5 (Cheng et al. 1996). Subsequently, using site-directed mutagenesis, several mutants of OPAA were reported which have greater activity against various OPs. One of those mutants, FL, having unique Y212F and V342L mutations, shows 5–10-fold increased catalytic activity on nerve agents soman and GP (Bae et al. 2018). The amino acid sequence of the OPAA-FL variant is shown in Fig. S1.

In order to enhance the expression and simplify the purification, the variant of the OPAA-FL gene was cloned downstream of the strong promoter (T7) into the expression vector pET28. OPAA-FL variant gene was amplified from the pSE420-OPAA-FL vector using primers PK702F and PK706R (Table S1), and *Pfu* DNA polymerase. The OPAA-FL variant gene was digested with *Nde*I and *Bam*HI, respectively, and cloned into the pET28 vector. The cloning was performed in such a way that the recombinant protein codes for an N-terminal His-tag (Fig. S2a). Cloning of the OPAA-FL variant in pET28 was confirmed by restriction analysis followed by DNA sequencing. The resultant recombinant plasmid was then transformed into *E. coli* Rosetta (DE3) cells.

Expression and purification of recombinant OPAA-FL variant in *E. coli*

A single colony of *E. coli* Rosetta (DE3) cells transformed with the pET-OPAA-FL vector was inoculated into LB broth containing 10 μg/ml kanamycin, and cells were grown overnight at 37 °C. The secondary culture of the cells was done in a fresh LB broth and cells incubated on an incubator shaker at 37 °C on the incubator shaker at 220 rpm until the OD reaches 0.8. The culture was then induced with 1 mM IPTG and 1 mM $MnCl_2$ and incubated at 20 °C for 20 h on the incubator shaker at 180 rpm. The cells were harvested by centrifuging at 8000 rpm for 10 min. The pellet obtained was then washed and suspended into the 50 mM Tris buffer, pH 7.4, with 10% glycerol. To lyse the cells, 400 μg/ml lysozyme was added to the resuspended cells and incubated at 30 °C for 20 min followed by sonication and centrifugation of cell lysate at 14,000 rpm for 10 min. Cell lysates were prepared and separated as supernatant and pellet to evaluate the amount of protein present in the soluble form. Induction of recombinant OPAA-FL variant protein was confirmed on a 12% SDS-PAGE gel. The results of the expression of the OPAA-FL variant in both pellet and supernatant are shown in Fig. S2b. Most of the expression of the OPAA-FL variant protein was observed in the soluble fraction (Fig. S2b, lane 2).

Since the OPAA-FL variant carries an N-terminal His-tag, we performed affinity purification of recombinant OPAA-FL variant protein on a Ni-nitrilotriacetic acid affinity chromatography column at 4 °C. The input sample was prepared in the equilibration buffer (50 mM Tris-Cl buffer, pH 8.0, 500 mM NaCl, and 10 mM imidazole) and loaded on the pre-equilibrated column at a flow rate of 1 ml/min. Subsequently, the column was washed with a wash buffer comprising 50 mM Tris-Cl, pH 8.0, and 500 mM NaCl, with 50 mM imidazole. The sample was eluted using an elution buffer comprising 50 mM Tris-Cl, pH 8.0, and 500 mM NaCl, with 150 mM imidazole. Purification of recombinant OPAA-FL variant protein was confirmed on a 12% SDS-PAGE gel. Figure S2c shows that the OPAA-FL variant protein was able to bind to the Ni-NTA resin, as most of the protein was absent in the flow-through (Fig. S2c, lane 2). We were able to purify more than 95% pure OPAA-FL variant protein (Fig. S2c, lane 6).

Activity assay of the recombinant OPAA-FL variant

To know whether the purified OPAA-FL variant protein is indeed enzymatically active, we performed the OPAA-FL variant enzyme assay with ethyl paraoxon and methyl parathion. A total of 1 μl of cell lysate or purified OPAA-FL variant protein was mixed with 50 mM Bis-Tris propane buffer, pH 8.5, 1 mM methyl parathion, and 1 mM $MnCl_2$, and the final volume was made up to 250 μl with SDW and the reaction was incubated at 50 °C for 15 min. Similarly to check the activity of the OPAA-FL variant on ethyl paraoxon, 1 μl of cell lysate or purified OPAA-FL variant protein was mixed with Bis-Tris propane buffer, pH 8.5; 1 mM $MnCl_2$ was used at 50 °C for 15 min. OPAA-FL variant enzyme activity was calculated by measuring the increase in absorbance of *p*-nitrophenol formed at 400 nm using an extinction coefficient of 17,000 M/cm for *p*-nitrophenol (UV-Vis spectrum shown in Fig. S3). Specific activities were expressed as units (micromoles of substrate hydrolyzed per minute) per milligram of total protein.

The kinetic parameters of purified and encapsulated OPAA-FL variant with ethyl paraoxon were determined using substrate concentrations of 0.1 to 9 mM and 0.25 to 9 mM, respectively.

Encapsulation of OPAA-FL variant in alginate microspheres

Purified OPAA-FL variant protein was encapsulated into the sodium alginate by using $CaCl_2$ as a cross-linker using an ultrasonic atomizer (Sonozap 2005, USA). A total of 0.7 to 0.8 mg/ml of purified OPAA-FL variant protein was mixed with sodium alginate (0.7% w/v) and sprayed at 0.3 ml/min flow rate using a syringe pump (Multi-phaser™, model NE-300, New Era pump system) through an ultrasonic atomizer at frequency 130 kHz and 3.5 W of power and collected in $CaCl_2$ collection bath kept on a magnetic stirrer at 1400 rpm. OPAA-FL variant encapsulated alginate microspheres were centrifuged at 7000*g* for 15 min and washed thrice with 40 ml sterile distilled water.

Characterization of microspheres and encapsulation efficiency

The morphology of the microspheres was recorded by FE-SEM (FE-SEM, Supra55 Zeiss, Germany). Briefly, a small fraction of microspheres was evenly sprayed into a thin layer onto the glass slide. The drop casted on a glass film was allowed to dry in the presence of silica gel. The film so formed was coated with sputtered copper using Q150R rotary-pumped sputter coater/carbon coater (FEI Talos 200S, Thermo Fischer Scientific, USA) and observed under FE-SEM. OPAA-FL variant protein encapsulated into the alginate microsphere was estimated by 12% SDS-PAGE gel. Microspheres were boiled at 95 °C for 15 min and then loaded on the SDS-PAGE along with the input and the first wash. The encapsulation efficiency for OPAA-FL variant protein in the alginate microspheres was quantified by calculating band intensities of encapsulated protein with reference to the input sample on a 12% SDS-PAGE. Intensities of protein bands were quantified using ImageJ Gel Analysis Program.

Biosensing studies of paraoxon

Colorimetric biosensing of paraoxon was performed by mixing OPAA-FL variant protein or protein-containing alginate microspheres with Bis-Tris propane buffer, pH 8.5; 1 mM $MnCl_2$ was used at 50 °C for 15 min or 30 min, respectively. OPAA-FL variant enzyme activity was calculated by exposing different concentrations of paraoxon (0.01–1 mM) and measuring the increase in absorbance of *p*-nitrophenol formed at 400 nm using the extinction coefficient 17,000 M/cm for *p*-nitrophenol (Hill et al. 2000). Fluorometric biosensing assays comprised of OPAA-FL variant protein and FITC-dextran (1 mg/ml) were mixed in ratio 1:1 (w/w). Subsequently, 1 mM $MnCl_2$ and paraoxon (0.1 mM–0.5 mM) were added to the mixture of OPAA-FL variant protein and FITC-dextran and the volume was made up to 1 ml. The reaction mixture was incubated for 5 min at 50 °C and the decrease in the fluorescence intensity was recorded. Fluorescence excitation of FITC was performed at 488 nm and emission was measured at 519 nm.

Statistical analysis

The statistical analysis was performed by one-way ANOVA and calculated by GraphPad Prism (****$p < 0.0001$, ***$p < 0.0002$, **$p < 0.0021$, *$p < 0.0332$, ns $p < 0.123$, error bars, standard deviation (S.D.)).

Results

OPAA has been demonstrated as an effective and active catalyst for the hydrolysis of OPs and nerve agents, due to which it has attracted widespread attention for the purpose of both detection and remediation of OPs (Iyer and Iken 2015). Figure 1 shows the diagrammatic representation of one such method of using the OPAA-FL variant enzyme for the detection of ethyl paraoxon. OPAA, in presence of $MnCl_2$ (Vyas et al. 2010), hydrolyzes ethyl paraoxon into diethyl phosphoric acid and *p*-nitrophenol, a yellow-colored product, which can be detected colorimetrically (Fig. 1). This reaction also releases two protons, which eventually decreases pH and in conjunction with a pH-sensitive fluorophore like FITC can be used for indirect fluorometric detection of OP substrates.

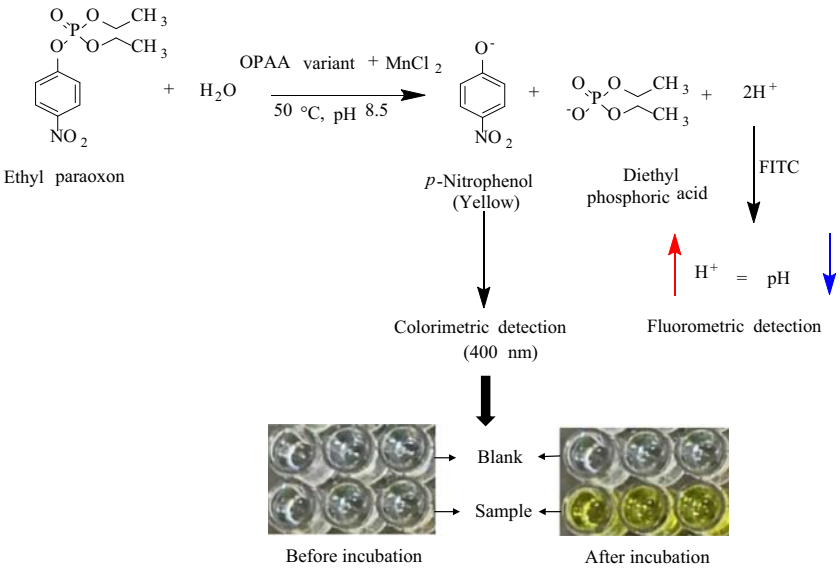

Fig. 1 The reaction of the OPAA-FL variant with ethyl paraoxon: Sensing scheme of fluorometric and colorimetric detection of OP compound using OPAA-FL variant

Cloning, expression, and purification of recombinant OPAA-FL variant

A gene for the OPAA-FL variant cloned into a pET expression vector and subsequently transformed into expression host *E. coli* Rosetta (DE3) cells. *E. coli* Rosetta (DE3) cells with the OPAA-FL variant expression vector were induced with 1 mM IPTG, and the recombinant OPAA-FL variant protein was affinity purified on a Ni-NTA resin. Specific activities of various samples of OPAA-FL variant protein with ethyl paraoxon are mentioned in Fig. 2a. As expected, induced cell lysate showed several folds higher specific activity as compared with the un-induced cell lysate. Furthermore, purified recombinant OPAA-FL variant protein demonstrated higher specific activity as compared to that in the induced cell lysate. The specific activity in the induced cell lysate was around 0.826 U/mg, and purified OPAA-FL variant showed even higher specific activity, that is, 1.96 U/mg.

Similarly, to know whether the purified protein is indeed enzymatically active against other OPs, we performed an OPAA-FL variant enzyme assay with methyl parathion. To perform OPAA-FL variant assay, 1 μl of cell extract or purified protein was mixed with 50 mM Bis-Tris propane buffer, pH 8.5, 1 mM methyl parathion, and 1 mM $MnCl_2$ and final volume was made up to 250 μl with SDW and reaction mixtures were incubated at 50 °C for 15 min. OPAA-FL variant activity was calculated by measuring the absorbance of *p*-nitrophenol produced due to the hydrolysis of methyl parathion at 400 nm (ε400 = 17,000/M/cm for *p*-nitrophenol). Specific activities were expressed as units (micromoles of substrate hydrolyzed per minute) per milligram of total protein.

Specific activities of various samples of OPAA-FL variant protein with methyl parathion are mentioned in Fig. 2b. Surprisingly, we observed almost identical specific activity in both un-induced and induced cell lysates. However, the purified recombinant OPAA-FL variant protein demonstrated

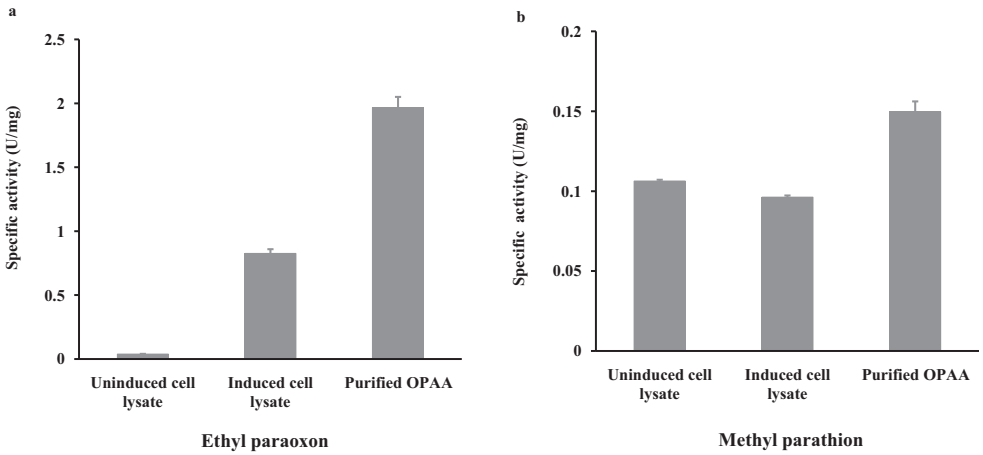

Fig. 2 Colorimetric activity assay. OPAA-FL variant with 1 mM substrate in presence of Bis-Tris propane buffer, pH 8.5, at 50 °C for 15 min. **a** 1 mM of ethyl paraoxon. **b** 1 mM of methyl parathion. The values are an average of duplicate samples and error bars represent standard deviation. At least three independent experiments were performed, and a representative result is shown

a higher specific activity as compared to that in the induced cell lysate. The specific activity in the induced cell lysate was around 0.096 U/mg, and the purified OPAA-FL variant showed a higher specific activity of 0.149 U/mg.

Considering the relatively low specific activities observed for the methyl parathion substrate with the OPAA-FL variant as compared to the ethyl paraoxon substrate, further experiments were carried out using ethyl paraoxon as a substrate to develop a working biosensor. With this in mind, the kinetic parameters for ethyl paraoxon as a substrate using purified as well as encapsulated OPAA-FL variant have been reported in Figs. S4 and S5, respectively, and in Table S2.

Effect of pH and temperature

As we observed a significant specific activity with substrate ethyl paraoxon, the effect of pH and temperature on the activity of the OPAA-FL variant was examined. To know the optimum temperature for the OPAA-FL variant with ethyl paraoxon, we tested its activity at various temperatures between 20 and 60 °C for 15 min, in 50 mM Bis-Tris propane buffer, pH 8.5, 1 mM ethyl paraoxon, and 1 mM $MnCl_2$. Similar to earlier reports (DeFrank and Cheng 1991), OPAA-FL variant activity was gradually increasing until 50 °C, and any further increase in the temperature led to a decrease in the specific activity. Our experiments suggested that 50 °C is the optimum temperature for the OPAA-FL variant with ethyl paraoxon (Fig. 3a).

To know the optimum pH for OPAA-FL variant with ethyl paraoxon, we tested its activity at various pH between 6 and 10, at 50 °C for 15 min, in either 50 mM phosphate buffer (pH 6.0, 6.5, 7.0, 7.5, and 8.0, respectively) or 50 mM Bis-Tris propane buffer (pH 8.5, 9.0, and 10.0, respectively), 1 mM ethyl paraoxon, and 1 mM $MnCl_2$. We observed optimum activity at pH 8.5 for OPAA-FL variant (Fig. 3b).

Encapsulation of OPAA-FL variant in alginate microspheres

Typically, encapsulation of proteins in polymers is performed to maintain the activity and the function of the protein over a prolonged time, to reduce the chances of interference, and to increase the sensitivity of the biosensor response (Yap et al. 2014). We encapsulated the OPAA-FL variant into the sodium alginate by using $CaCl_2$ as a cross-linker (Fig. 4a). Alginate was selected for encapsulation of OPAA-FL variant as it is a natural polymer with numbers of favorable properties such as biocompatibility and ease of gelation, and moreover, it enables the protein to retain its activity for a longer period of time while maintaining its structure and function (Lee and Mooney 2012). Subsequently, we estimated the encapsulation efficiency of the OPAA-FL variant into the alginate microsphere, by boiling the alginate microspheres and running these on 12% SDS-PAGE, along with input and wash controls (Fig. 4b). In general, we observed a 25–30% encapsulation efficiency. After confirming the encapsulation of OPAA-FL variant protein in the alginate microsphere, we further performed the surface characterization using scanning electron microscopy (SEM) (Fig. 4c–e). SEM analysis revealed the spherical-shaped microspheres ranging from 0.1 to 0.8 μm.

Colorimetric biosensing of ethyl paraoxon

The biosensing activity of the OPAA-FL variant with ethyl paraoxon was evaluated colorimetrically using substrate concentrations ranging from 0.005 to 1 mM (Fig. 5). A linear range of 0.01 to 1 mM for the detection of ethyl paraoxon was observed with a LOD of 0.04 mM. The sensitivity of such estimation was found to be 1.38 a.u./mM. For encapsulated OPAA-FL variant with ethyl paraoxon as the substrate, a linear range of 0.025 to 1 mM was observed (Fig. 6). The LOD of biosensing was 0.06 mM, and the sensitivity was 1.79 a.u./mM.

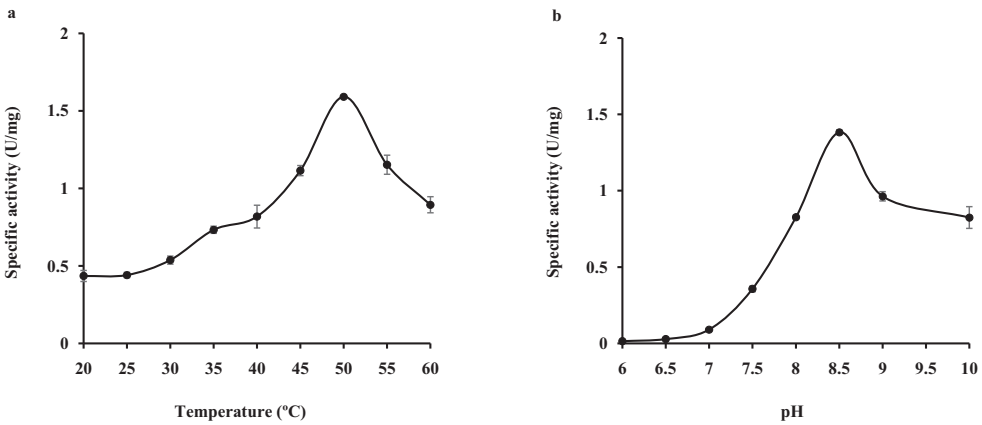

Fig. 3 Effect of temperature and pH on the OPAA-FL variant. **a** Effect of temperature on the specific activity of the OPAA-FL variant with ethyl paraoxon. **b** Effect of pH on the specific activity of OPAA-FL variant ethyl paraoxon. The values are an average of duplicate samples and error bars represent standard deviation. At least three independent experiments were performed, and a representative result is shown

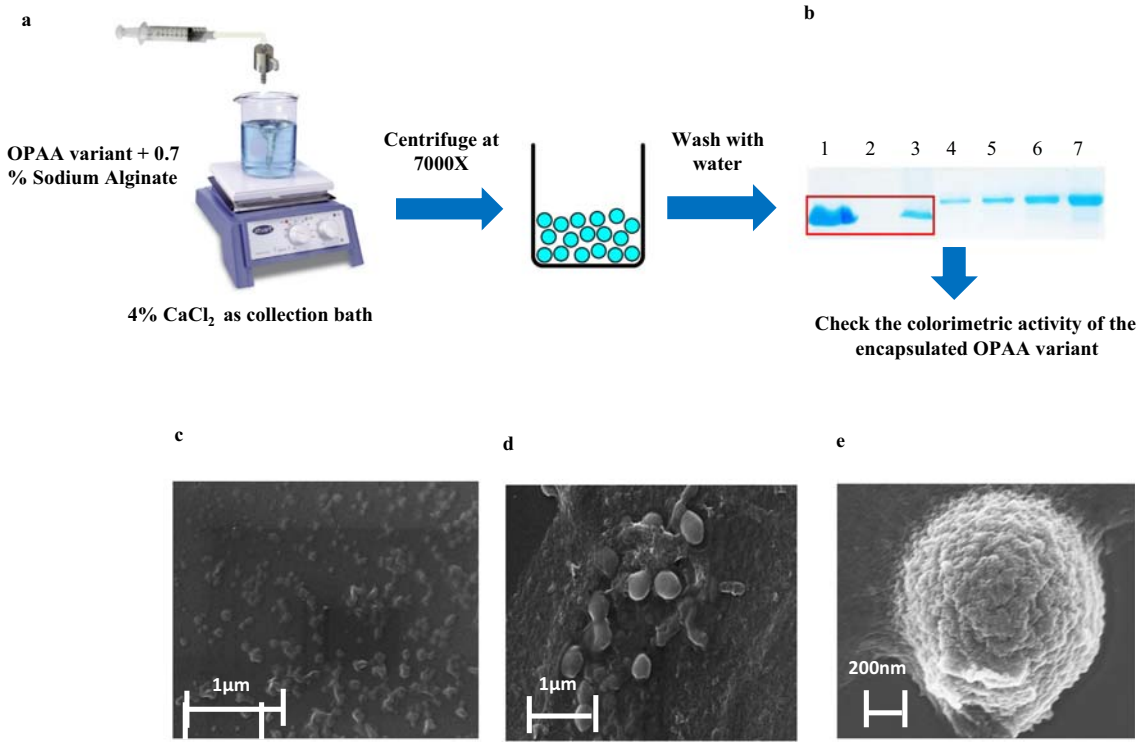

Fig. 4 Encapsulation of the OPAA-FL variant. **a** Schematic representation of immobilization procedure for the OPAA-FL variant using an atomizer. **b** Analysis of OPAA-FL variant–loaded alginate microspheres using SDS-PAGE. Lane 1, input; lane 2, unloaded OPAA-FL variant in wash 1 after buffer exchange; lane 3, encapsulated OPAA-FL variant; lanes 4 to 7, 0.5, 1, 2, and 4 µg of BSA. **c**–**e** Characterization of microspheres: SEM images of OPAA-FL variant–loaded alginate microspheres with 0.7% alginate. **c** At × 6.32 K magnification, **d** at × 32.0 K magnification, and **e** at × 57.95 K magnification

Fluorescent biosensing of ethyl paraoxon

OPAA-FL variant on hydrolysis of OPs release *p*-nitrophenol as well as two protons. The protons released result in lowering the pH of the reaction mixture. A pH-sensitive fluorescent probe like FITC can be efficient in detecting such a reaction and indirectly quantify paraoxon. Fluorescence intensity of FITC decreases as H^+ concentration of the reaction mixture increases, lowering pH. FITC has known to exhibit a linear range of pH determination between pH 4 and 8. This property

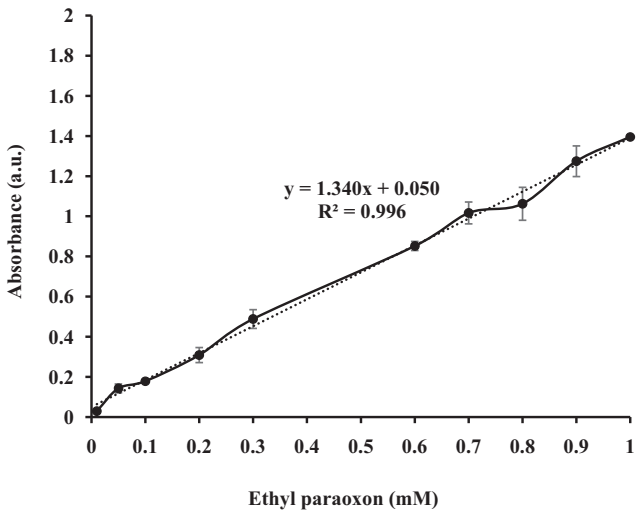

Fig. 5 Colorimetric biosensing of ethyl paraoxon using the OPAA-FL variant enzyme. The values are an average of duplicate samples and error bars represent standard deviation. At least three independent experiments were performed, and a representative result is shown

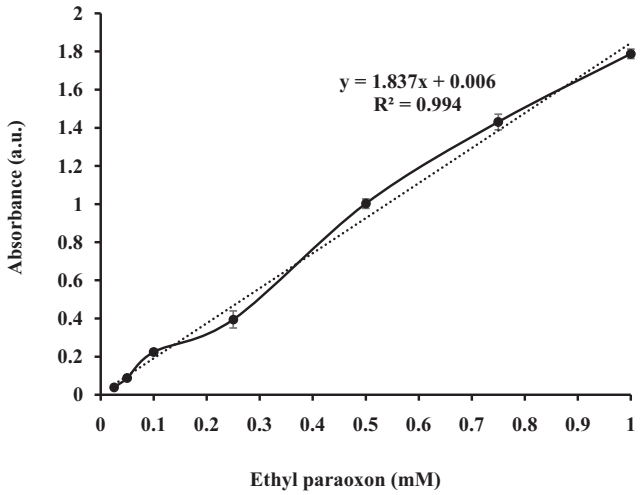

Fig. 6 Colorimetric biosensing of ethyl paraoxon using the encapsulated OPAA-FL variant enzyme. The values are an average of duplicate samples and error bars represent standard deviation. At least three independent experiments were performed, and a representative result is shown

of FITC is exploited in most of the enzymatic reactions especially those producing and utilizing protons. The ionization of FITC in basic pH leads to an increase in fluorescence intensity and vice versa. The effects of ionization produced also are affected by the buffering of biological and environmental samples. Generally, the buffer system is responsible for maintaining the conditions under which the enzyme can express maximum activity and stability. A high buffer concentration leads to high buffering capacity, which can suppress pH changes in the medium and hence suppresses the sensitivity of the sensor (Głąb et al. 1994), thus explaining the better results observed for the fluorometric sensing of ethyl paraoxon with 0 mM buffer.

To know optimum buffer and reaction conditions for fluorometric detection of OPs using the OPAA-FL variant, we initially tested a decrease in fluorescence values of FITC in different buffer concentrations. The reaction mixture was prepared with 1 mM $MnCl_2$, 1 mM ethyl paraoxon, OPAA-FL variant, FITC-dextran, and sterile distilled water up to 1 ml. We used 0 mM, 25 mM, and 50 mM activity buffer (Bis-Tris propane), respectively, and measured loss in the fluorescence values (Fig. 7). Interestingly, the maximum decrease in the fluorescence values was observed in the absence (0 mM) of activity buffer (p value = 0.0058), indicating that higher buffering capacity of reaction inhibits the loss in fluorescence values.

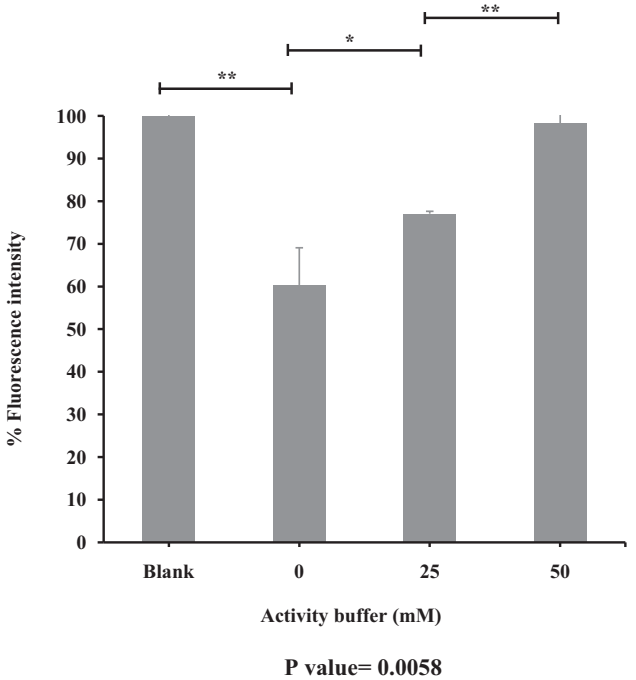

Fig. 7 Optimization of concentration of Bis-Tris propane buffer for the OPAA-FL variant fluorescence assay. 0 mM, 25 mM, and 50 mM Bis-Tris propane buffer were used at 50 °C, pH 8.5, for 5 min with ethyl paraoxon. The values are an average of duplicate samples and error bars represent standard deviation. At least three independent experiments were performed, and a representative result is shown. The statistical analysis was performed by one-way ANOVA and calculated by GraphPad Prism (****p < 0.0001, ***p < 0.0002, **p < 0.0021, *p < 0.0332, ns p < 0.123, error bars, standard deviation (S.D.))

Subsequently, to identify an optimum ratio of OPAA-FL:FITC-dextran that gives a higher decrease in fluorescence values, we analyzed six ratios, namely, OPAA-FL:FITC-dextran 0.25:1, 0.5:1, 0.75:1, 1:1, 3:1, and 5:1, respectively (Fig. 8a–f). We tested a relative decrease in fluorescence values at three different concentrations of ethyl paraoxon, that is, 0.1 mM, 0.5 mM, and 1 mM, respectively. Incidentally, we observed that the OPAA-FL:FITC-dextran ratio 1:1 gives a higher and consistent decrease in the fluorescence values of FITC (p = < 0.0001) (Fig. 8d), possibly due to optimal labeling of FITC and protein (Chakraborty et al. 2015; Breen et al. 2016). In fact, with increasing the concentration of ethyl paraoxon, the fluorescence intensity of FITC decreased faster confirming that the higher number of H^+ released at higher substrate concentrations.

The fluorescence-based biosensing activity of the OPAA-FL variant with different concentrations of ethyl paraoxon showed that a linear range in 0.1 to 0.5 mM of paraoxon (Fig. 9). The LOD of biosensing was 0.056 mM, and the sensitivity was 95%/mM. Incidentally, the encapsulated OPAA-FL variant microspheres did not show the expected biosensing response in the fluorescence assay.

Discussion

There is a need for the development of an enzyme-based biosensor which is a safe, non-corrosive, cheaper, more user-friendly, rapid, stable, sensitive, selective, and environmentally friendly technology. Microbial enzymes have attracted widespread attention not only because of economic and environmental considerations, among other factors. OP-degrading enzymes have attracted widespread attention because of the toxicity of pesticides and the use of nerve agents as chemical warfare agents. Through our study, we present an OPAA-FL variant enzyme-based biosensor which is safe, user-friendly, and environmentally benign for the detection of paraoxon. This work provides information about the cloning, expression, purification, and encapsulation of the OPAA-FL variant into alginate, and development of a colorimetric as well as a pH-based biosensor.

Out of 517 amino acids of OPAA from *Alteromonas* sp., only 440 are found to affect the activity of the enzyme. Truncation of 77 amino acids from the C-terminal is found to not affect the activity of the enzyme (DeFrank and Cheng 1991). Before 2015, there was no report of OPAA action against V-type nerve agents and there were no previous reports of its engineering. OPAA structure was engineered by Steven P. Harvey's group to improve the catalytic efficiency of the enzyme. Using site-directed mutagenesis, they created few mutations in the small pocket in order to increase the

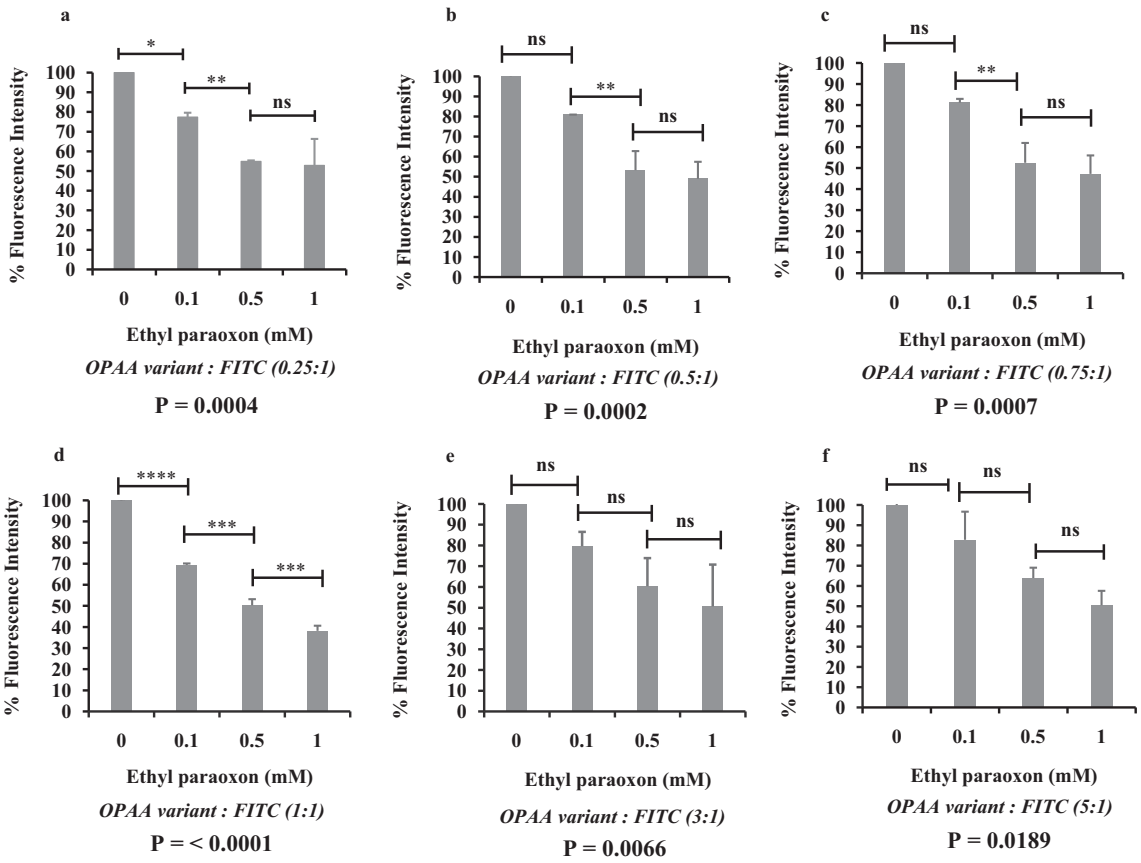

Fig. 8 Optimization of the ratio of the OPAA-FL variant protein:FITC-dextran in the fluorescence assay. Three ratios of the OPAA-FL variant protein:FITC-dextran **a** 0.25:1, **b** 0.5:1, **c** 0.75:1, **d** 1:1, **e** 3:1, and **f** 5:1 were analyzed using 1 mM ethyl paraoxon at 50 °C, for 5 min without activity buffer. The values are an average of duplicate samples and error bars represent standard deviation. At least three independent experiments were performed, and a representative result is shown. The statistical analysis was performed by one-way ANOVA and calculated by GraphPad Prism (****$p < 0.0001$, ***$p < 0.0002$, **$p < 0.0021$, *$p < 0.0332$, ns $p < 0.123$, error bars, standard deviation (S.D.)).

specific activity of the enzyme. Four mutants of OPAA, namely, F (Y212F), FL (Y212F and V342L), FLY (Y212F, V342L, and I215Y), and FLYD (Y212F, V342L, I215Y, and H343D), were produced (Daczkowski et al. 2015), of which OPAA FL variant was reported to display a 20-fold increase in catalytic efficiency for the hydrolysis of Russian VX over wild-type OPAA, whereas the FLY mutant of OPAA so produced displayed more than 30-fold enhancement in the catalytic activity on VR racemic. In the published literature, the OPAA enzyme is reported to hydrolyze OP compounds with P-F, P-O, P-S, and P-CN bonds. A representative comparison table of OPAA-FL with P-F and P-O bond containing OPs, and OPAA WT with P-O bond containing compounds, as well as a comparison of OPAA FL and wild-type OPAA for the hydrolysis of P-S bond containing Russian VX is given in Tables S3 and S4, respectively. A group of scientists in the year 2000 proved that by encapsulating OPAA hydrolyzing enzyme in conjugation with 2-PAM and atropine in lysosome to provide 23 LD50s of protection against DFP (Petrikovics et al. 2000). Our report attempts to evaluate the OPAA-FL variant as a potential candidate for the enzymatic hydrolysis of paraoxon, and it is expected that the studies would aid efforts towards developing effective sensors and/or mutant enzymes for the purpose of detection and/or degradation of organophosphates, especially ones with the P-O bond such as paraoxon.

In the current study, the OPAA-FL variant gene was cloned into the pET28 expression vector with N-terminal His-tag. The recombinant OPAA-FL variant was expressed in *E. coli* cells, then purified and tested for its activity against methyl parathion and paraoxon. The specific activity of the OPAA-FL variant using colorimetric assay was observed to be 1.96 U/mg against paraoxon with an incubation time of 15 min, which is around 1.2-fold higher when compared to previously reported OPAA from marine bacterium *Pseudoalteromonas* sp. SCSIO 04301 (1.6 U/mg) by Xiao et al. (2017). In 2017, Xiao and his group showed that OPAA obtained from the sediment of coastal areas of south China, specifically the bacterium *Pseudoalteromonas* sp. SCSIO 04301 (GIMCC 1.828), expressed activity against methyl paraoxon and

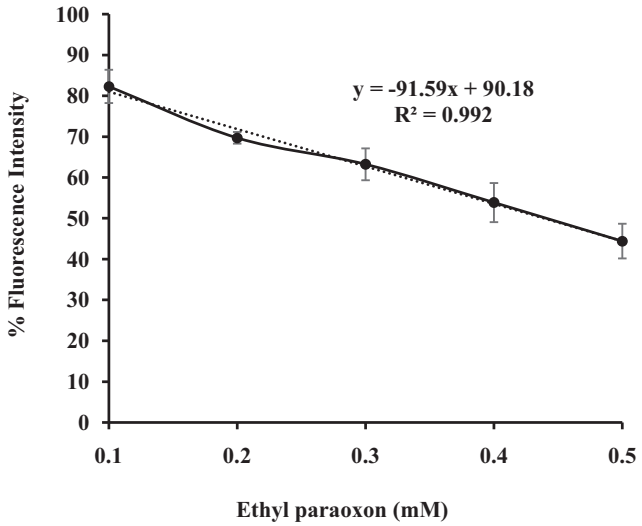

Fig. 9 Biosensing of ethyl paraoxon using the OPAA-FL variant via the fluorescence assay. The 1:1 ratio of OPAA-FL variant and FITC-dextran was used with 1 mM paraoxon as the substrate at 50 °C, for 3 min without activity buffer. The fluorescence intensity of FITC decreased linearly with an increase in the concentration of ethyl paraoxon. The values are an average of duplicate samples and error bars represent standard deviation. At least three independent experiments were performed, and a representative result is shown

paraoxon. They reported a specific activity of OPAA against methyl paraoxon of approximately 0.0314 U/mg and specific activity against paraoxon of about 1.604 U/mg.

The activity was further validated using fluorometric assay using pH-sensitive fluorophore, FITC. For the OPAA-FL variant, we observed the fluorescence intensity of FITC dropped within a response time of 3 min giving a linear range of detection between 0.1 and 0.5 mM and detection limit 0.056 mM. The protein was encapsulated into alginate using an ultrasonic atomizer. The bead morphology was observed by SEM. The average size of the microspheres so formed was found to be around 0.2 μm. Microspheres were further used for the detection of the OPs using a colorimetric assay.

In the colorimetric and fluorescence assays, a buffer solution of Bis-Tris propane was used, which acts as an activity buffer to provide optimal conditions for the maximum enzyme activity (Bisswanger 2014). Colorimetric estimation using the OPAA-FL variant provided a linear range of 0.01–1 mM whereas encapsulated OPAA-FL variant provided a linear range of 0.025–1 mM. The results indicate that both OPAA-FL variant and encapsulated OPAA-FL variant colorimetric estimations exhibit a limit of detection of ethyl paraoxon of 0.04 mM and 0.06 mM, respectively, with ethyl paraoxon as the substrate. Thus, encapsulation results in a decrease in the linear range however with a slight improvement in the sensitivity. In the case of the fluorometric method, the linear range was found to be 0.1–0.5 mM with a similar limit of detection of 0.056 mM. Incidentally, the encapsulated OPAA-FL variant microspheres did not show the expected biosensing response in the fluorescence assay. This could be because of the interference of alginate in the enzymatic reaction or pH response.

The detection methods for OPs commonly used so far are costly and have several limitations for infield analysis. Recombinant expression of organophosphorus acid anhydrolase (OPAA)-FL variant and deployment of OPAA-FL variant into a biosensing assay can help quantify OPs. This report describes the utilization of the OPAA-FL variant for the development of biosensing assay for ethyl paraoxon using a colorimetric and fluorometric estimation. OPAA-FL variant was successfully expressed and purified from *E. coli* Rosetta (DE3) cells. The purified OPAA-FL variant showed higher specific activity than the induced cell lysate and exhibited an optimum temperature of 50 °C and optimum pH of 8.5. Colorimetric estimation of ethyl paraoxon via the *p*-nitrophenol method showed a capability of detection to an extent of 0.01–1 mM. OPAA-FL variant was also immobilized in alginate microspheres using a novel technique of ultrasonic atomization to enhance stability, activity, and biosensing. After encapsulation of the OPAA-FL variant, the microspheres also can detect ethyl paraoxon at similar detection limits. Fluorometric detection of ethyl paraoxon, on the other hand, showed detection capability in the range of 0.1–0.5 mM after a short incubation time of 3 min. Both colorimetric and fluorometric methods can form the basis of new OPAA-FL variant–based optical biosensors and their transformation into point-of-care devices for in-field analysis of insecticides and pesticides in a cost-effective manner. This study, therefore, establishes the use of OPAA variants towards detection of common organophosphate pesticides and paves the way for the research community to develop sensitive, low-cost, and portable point-of-care devices for the timely and accurate detection of these toxic compounds.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00253-020-11008-w.

Acknowledgments The authors would like to thank Prof. S. Harvey, US Army Edgewood Biological Center, MD, USA, for a kind gift of the pSE420-OPAA-FL vector. A. Joshi would like to thank the SERB-Early career award. M. Jain thanks the Ministry of Education (MoE), Government of India, for the Ph.D. fellowship, and B. Joshi would like to acknowledge the DST-INSPIRE fellowship (IF170325).

Authors' contributions P. Kodgire, A. Joshi, and M. Jain designed the experiments; M. Jain and P. Yadav performed the experiments and data analysis; B. Joshi assisted in the experiments and data analysis; P. Kodgire, A. Joshi, and M. Jain wrote the manuscript.

Funding Funding for this work was provided by the Department of Biotechnology, Government of India, for a research grant (BT/PR20319/BBE/117/189/2016 and BT/PR24652/NER/95/795/2017).

Compliance with ethical standards

Competing interests The authors declare that they have no competing interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

Atwood D, Paisley-Jones C (2008) US EPA - Pesticides Industry Sales and Usage 2008 - 2012

Bae SY, Myslinski JM, McMahon LR, Height JJ, Bigley AN, Raushel FM, Harvey SP (2018) An OPAA enzyme mutant with increased catalytic efficiency on the nerve agents sarin, soman, and GP. Enzym Microb Technol 112:65–71. https://doi.org/10.1016/j.enzmictec.2017.11.001

Basher A, Rahman SH, Ghose A, Arif SM, Faiz MA, Dawson AH (2013) Phase II study of magnesium sulfate in acute organophosphate pesticide poisoning. Clin Toxicol 51:35–40. https://doi.org/10.3109/15563650.2012.757318

Bisswanger H (2014) Enzyme assays. Perspect Sci 1:41–55. https://doi.org/10.1016/j.pisc.2014.02.005

Breen CJ, Raverdeau M, Voorheis HP (2016) Development of a quantitative fluorescence-based ligand-binding assay. Sci Rep 6:1–9. https://doi.org/10.1038/srep25769

Chakraborty S, Hu S-Y, Wu S-H, Karmenyan A, Chiou A (2015) The interaction affinity between vascular cell adhesion molecule-1 (VCAM-1) and very late antigen-4 (VLA-4) analyzed by quantitative FRET. PLoS One 10:e0121399. https://doi.org/10.1371/journal.pone.0121399

Cheng TC, Harvey SP, Chen GL (1996) Cloning and expression of a gene encoding a bacterial enzyme for decontamination of organophosphorus nerve agents and nucleotide sequence of the enzyme. Appl Environ Microbiol 62:1636–1641

Choudhary S, Joshi B, Pandey G, Joshi A (2019) Application of single and dual fluorophore-based pH sensors for determination of milk quality and shelf life using a fibre optic spectrophotometer. Sensors Actuators B Chem 298:126925. https://doi.org/10.1016/j.snb.2019.126925

Cornelissen AS, Klaassen SD, van Groningen T, Bohnert S, Joosen MJA (2020) Comparative physiology and efficacy of atropine and scopolamine in sarin nerve agent poisoning. Toxicol Appl Pharmacol 396:114994. https://doi.org/10.1016/j.taap.2020.114994

Daczkowski CM, Pegan SD, Harvey SP (2015) Engineering the organophosphorus acid anhydrolase enzyme for increased catalytic efficiency and broadened stereospecificity on Russian VX. Biochemistry 54:6423–6433. https://doi.org/10.1021/acs.biochem.5b00624

DeFrank JJ, Cheng TC (1991) Purification and properties of an organophosphorus acid anhydrase from a halophilic bacterial isolate. J Bacteriol 173:1938–1943. https://doi.org/10.1128/jb.173.6.1938-1943.1991

Eddleston M (2000) Patterns and problems of deliberate self-poisoning in the developing world. QJM 93:715–731. https://doi.org/10.1093/qjmed/93.11.715

Eddleston M (2018) Are oximes still indicated for acute organophosphorus insecticide self-poisoning? J Med Toxicol 14:1–2

Głąb S, Koncki R, Kopczewska E, Wałcerz I, Hulanicki A (1994) Urea sensors based on PVC membrane pH electrode. Talanta 41:1201–1205. https://doi.org/10.1016/0039-9140(94)80092-8

Hertz-Picciotto I, Sass JB, Engel S, Bennett DH, Bradman A, Eskenazi B, Lanphear B, Whyatt R (2018) Organophosphate exposures during pregnancy and child neurodevelopment: recommendations for essential policy reforms. PLoS Med 15:e1002671. https://doi.org/10.1371/journal.pmed.1002671

Hill CM, Wu F, Cheng TC, Defrank JJ, Raushel FM (2000) Substrate and stereochemical specificity of the organophosphorus acid anhydrolase from *Alteromonas* sp. JD6.5 toward p-nitrophenyl phosphotriesters. Bioorg Med Chem Lett 10:1285–1288. https://doi.org/10.1016/S0960-894X(00)00213-4

Hoppin JA (2014) Pesticides and respiratory health: where do we go from here? Occup Environ Med 71:80–81

Iyer R, Iken B (2015) Protein engineering of representative hydrolytic enzymes for remediation of organophosphates. Biochem Eng J 94:134–144

Jain M, Yadav P, Joshi A, Kodgire P (2019) Advances in detection of hazardous organophosphorus compounds using organophosphorus hydrolase based biosensors. Crit Rev Toxicol:1–24. https://doi.org/10.1080/10408444.2019.1626800

Jeong YS, Choi SL, Kyeong HH, Kim JH, Kim EJ, Pan JG, Rha E, Song JJ, Lee SG, Kim HS (2012) High-throughput screening system based on phenolics-responsive transcription activator for directed evolution of organophosphate-degrading enzymes. In: Protein engineering, design and selection. pp 725–731

Kang EJ, Seok SJ, Lee KH, Gil HW, Yang JO, Lee EY, Hong SY (2009) Factors for determining survival in acute organophosphate poisoning. Korean J Intern Med 24:362–367. https://doi.org/10.3904/kjim.2009.24.4.362

Karami R, Mohsenifar A, Mesbah Namini SM, Kamelipour N, Rahmani-Cherati T, Roodbar Shojaei T, Tabatabaei M (2016) A novel nanobiosensor for the detection of paraoxon using chitosan-embedded organophosphorus hydrolase immobilized on Au nanoparticles. Prep Biochem Biotechnol 46:559–566. https://doi.org/10.1080/10826068.2015.1084930

Kirsch J, Siltanen C, Zhou Q, Revzin A, Simonian A (2013) Biosensor technology: recent advances in threat agent detection and medicine. Chem Soc Rev 42:8733–8768

Kulys J, D'Costa EJ (1991) Printed amperometric sensor based on TCNQ and cholinesterase. Biosens Bioelectron 6:109–115. https://doi.org/10.1016/0956-5663(91)87034-9

Lee KY, Mooney DJ (2012) Alginate: properties and biomedical applications. Prog Polym Sci 37:106–126

Li P, Moon SY, Guelta MA, Harvey SP, Hupp JT, Farha OK (2016) Encapsulation of a nerve agent detoxifying enzyme by a mesoporous zirconium metal-organic framework engenders thermal and long-term stability. J Am Chem Soc 138:8052–8055. https://doi.org/10.1021/jacs.6b03673

Liu SH, Lin JL, Weng CH, Yang HY, Hsu CW, Chen KH, Huang WH, Yen TH (2012) Heart rate-corrected QT interval helps predict mortality after intentional organophosphate poisoning. PLoS One 7. https://doi.org/10.1371/journal.pone.0036576

Mangas I, Estevez J, Vilanova E, França TCC (2017) New insights on molecular interactions of organophosphorus pesticides with esterases. Toxicology 376:30–43. https://doi.org/10.1016/j.tox.2016.06.006

Marty J-L, Sode K, Karube I (1992) Biosensor for detection of organophosphate and carbamate insecticides. Electroanalysis 4:249–252. https://doi.org/10.1002/elan.1140040217

Masson P, Nachon F, Lockridge O (2010) Structural approach to the aging of phosphylated cholinesterases. Chem Biol Interact 187:157–162. https://doi.org/10.1016/j.cbi.2010.03.027

Mello SV, Mabrouki M, Cao X, Leblanc RM, Cheng T-C, DeFrank JJ (2003) Langmuir and Langmuir−Blodgett films of organophosphorus acid anhydrolase. https://doi.org/10.1021/BM025775

Mulchandani A, Chen W, Mulchandani P, Wang J, Rogers KR (2001) Biosensors for direct determination of organophosphate pesticides. Biosens Bioelectron 16:225–230. https://doi.org/10.1016/S0956-5663(01)00126-9

Pedrosa VA, Paliwal S, Balasubramanian S, Nepal D, Davis V, Wild J, Ramanculov E, Simonian A (2010) Enhanced stability of enzyme organophosphate hydrolase interfaced on the carbon nanotubes. Colloids Surf B: Biointerfaces 77:69–74. https://doi.org/10.1016/j.colsurfb.2010.01.009

Pei L, Mcguinn GD, Petrikovics I, Pu L, Cannon EP, Way JL (1993) Determination of organophosphorus acid anhydrase in blood. Toxicol Mech Methods 3:261–267. https://doi.org/10.3109/15376519309068443

Peter JV, Jerobin J, Nair A, Bennett A, Samuel P, Chrispal A, Abraham OC, Mathews KP, Fleming JJ, Oommen A (2010) Clinical profile and outcome of patients hospitalized with dimethyl and diethyl organophosphate poisoning. Clin Toxicol 48:916–923. https://doi.org/10.3109/15563650.2010.528425

Petrikovics I, Cheng TC, Papahadjopoulos D, Hong K, Yin R, DeFrank JJ, Jaing J, Song ZH, McGuinn WD, Sylvester D, Pei L, Madec J, Tamulinas C, Jaszberenyi JC, Barcza T, Way JL (2000) Long circulating liposomes encapsulating organophosphorus acid anhydrolase in diisopropylfluorophosphate antagonism. Toxicol Sci 57:16–21. https://doi.org/10.1093/toxsci/57.1.16

Pohanka M (2013) Cholinesterases in biorecognition and biosensors construction: a review. Anal Lett 46:1849–1868

Pundir CS, Chauhan N (2012) Acetylcholinesterase inhibition-based biosensors for pesticide determination: a review. Anal Biochem 429:19–31

Rainina EI, Efremenco EN, Varfolomeyev SD, Simonian AL, Wild JR (1996) The development of a new biosensor based on recombinant *E. coli* for the direct detection of organophosphorus neurotoxins. Biosens Bioelectron 11:991–1000. https://doi.org/10.1016/0956-5663(96)87658-5

Simonian AL, Flounders AW, Wild JR (2004) FET-based biosensors for the direct detection of organophosphate neurotoxins. Electroanalysis 16:1896–1906. https://doi.org/10.1002/elan.200403078

Van Dyk JS, Pletschke B (2011) Review on the use of enzymes for the detection of organochlorine, organophosphate and carbamate pesticides in the environment. Chemosphere 82:291–307

Vyas NK, Nickitenko A, Rastogi VK, Shah SS, Quiocho FA (2010) Structural insights into the dual activities of the nerve agent degrading organophosphate anhydrolase/prolidase. Biochemistry 49:547–559. https://doi.org/10.1021/bi9011989

Xiao Y, Yang J, Tian X, Wang X, Li J, Zhang S, Long L (2017) Biochemical basis for hydrolysis of organophosphorus by a marine bacterial prolidase. Process Biochem 52:141–148. https://doi.org/10.1016/j.procbio.2016.10.008

Yap WT, Kelsey Song W, Chauhan N, Nina Scalise P, Agarwal R, Miller SD, Shea LD (2014) Quantification of particle-conjugated or particle-encapsulated peptides on interfering reagent backgrounds. Biotechniques 57:39–44. https://doi.org/10.2144/000114190