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ORIGINAL PAPER

Application of methyl parathion hydrolase (MPH) as a labeling enzyme

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Abstract Methyl parathion hydrolase (MPH) is an enzyme that catalyzes the degradation of methyl parathion, generating a yellow product with specific absorption at 405 nm. The application of MPH as a new labeling enzyme was illustrated in this study. The key advantages of using MPH as a labeling enzyme are as follows: (1) unlike alkaline phosphatase (AP), horseradish peroxidase (HRP), and glucose oxidase (GOD), MPH is rarely found in animal cells, and it therefore produces less background noise; (2) its active form in solution is the monomer, with a molecular weight of 37 kDa; (3) its turnover number is 114.70±13.19 s^{-1}, which is sufficiently high to yield a significant signal for sensitive detection; and (4) its 3D structure is known and its C-terminal that is exposed to the surface can be easily subjected to the construction of genetic engineering monocloning antibody–enzyme fusion for enzyme-linked immunosorbent assay (ELISA). To demonstrate its utility, MPH was ligated to an single-chain variable fragment (scFv), known as A1E, against a white spot syndrome virus (WSSV) with the insertion of a [–(Gly–Ser)$_5$–] linker peptide. The resulting fusion protein MPH-A1E possessed both the binding specificity of the scFv segment and the catalytic activity of the MPH segment. When MPH-A1E was used as an ELISA reagent, 25 ng purified WSSV was detected; this was similar to the detection sensitivity obtained using A1E scFv and the HRP/Anti-E Tag Conjugate protocol. The fusion protein also recognized the WSSV in 1 μL hemolymph from an infected shrimp and differentiated it from a healthy shrimp.

Keywords Methyl parathion hydrolase · Labeling enzyme · Single-chain variable fragment · Fusion protein · White spot syndrome virus

Introduction

Enzymes offer sensitive and specific methods for quantitative measurement of many substances. Enzyme kits, enzyme-linked immunosorbent assay (ELISA), enzyme-labeled gene probes, enzyme sensors, and enzyme-labeled nanoparticles are being widely used in clinical diagnosis, bioprocess control, environmental monitoring, quarantine, and life-science research, etc. [1–7]. The most commonly used labeling enzymes are horseradish peroxidase (HRP) and alkaline phosphatase (AP), as documented in many reports. A few other enzymes such as glucose oxidase (GOD) and β-galactosidase (β-GAL) are also commonly used. In most enzyme-labeling systems, enzymes are chemically coupled with biomolecules (known as detection molecules) such as antigens or antibodies and with DNA probes for application in immunoassays and gene assays, respectively [8]. Generally, chemical coupling involves the cross-linking of an

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enzyme and a detection molecule by using bifunctional reagents (e.g., glutaraldehyde and periodate) [9, 10] or the introduction of free SH groups on to the surface of enzyme proteins and antigens or antibodies, which leads to the formation of bioconjugates through S–S bonding [11, 12]. However, most chemical coupling procedures are random reactions that often produce many undesired conjugates through self-coupling and inappropriate coupling; these conjugates might block the active sites of conjugate partners, which may consequently influence the specificity and sensitivity of the assays [8, 13, 14].

The application of genetic engineering to the construction of bioconjugates overcomes the limitations of chemical methods. Homogeneity, controlled stoichiometry ratio, reproducibility, and simple production methods are the desirable features of bioconjugates generated by antigen and enzyme sequences fused in-frame [14]. Pure fusion structures can be easily obtained through gene expression in *Escherichia coli* or yeast cells and subsequent purification. The basic requirement for a successful fusion is that the native steric structure of the enzyme protein should not have its C or N-terminal (which forms the link with the fusion partner) buried inside the protein. Formation of dimeric and tetrameric structures is another difficulty associated with fusion protocols. Aggregation of enzyme proteins greatly enhances the risk of changes in protein conformation; these changes, when they occur, result in loss of the biological functions of the proteins.

In many species, the existence of AP and peroxidases or their substrates eliminates the possibility of using these enzymes in immunohistochemistry and in-situ hybridization, and in ELISA and immunodot blot. For example, shrimp endogenous peroxidase and AP were found to produce false-positive results when ELISA and immunodot blot assay were used to detect the WSSV in shrimp tissue by using antibodies conjugated with AP and HRP [15, 16]. In such cases, GOD would be a good candidate for a labeling enzyme. However, it is a dimeric glycoprotein and is likely to be expressed in eukaryotic cells. Thus, exploration of new labeling enzymes is required for widening and facilitating the application of enzymatic analysis.

Methyl parathion hydrolase (MPH; E.C.3.1.8.1), isolated from the soil-dwelling bacterium *Pseudomonas sp.* WBC-3 in our previous study, is an enzyme that catalyzes the degradation of methyl parathion and, consequently, generates a yellow product with specific absorption at 405 nm [17]. After systematic study of the enzyme, we believe that its potential applications as a labeling enzyme must be explored for the following reasons.

- First, the 3-D crystal structure of the enzyme showed that all its C and N-terminals were exposed on the protein surface (Fig. 1) [18, 19]; hence, adopting a fusion protocol with this enzyme would be feasible.
- Second, although MPH is a dimer in the crystalline state, its active form in solution is a monomer as determined by gel-filtration chromatography [20]; this suggests that the enzyme protein in a solution has less tendency to aggregate.
- Third, MPH can be efficiently expressed in *E. coli* with good solubility; this phenomenon could be attributed to the second point stated above.
- The fourth and the most important feature is that, compared with the other commonly used labeling enzymes, MPH is rarely found in animals, plants, and microorganisms; thus, difficulties due to background noise caused by the presence of an endoenzyme would not be encountered.

We therefore attempted using MPH as a labeling enzyme and demonstrated its application in the detection of WSSV. The results are reported herein.

Materials and methods

Materials

Restriction enzymes, DNA polymerase, and T4 DNA ligase were obtained from Takara (Dalian, China) and Promega (USA). Single-strand oligonucleotides were synthesized by Sangon (Shanghai, China) and Biojet (Wuhan, China).

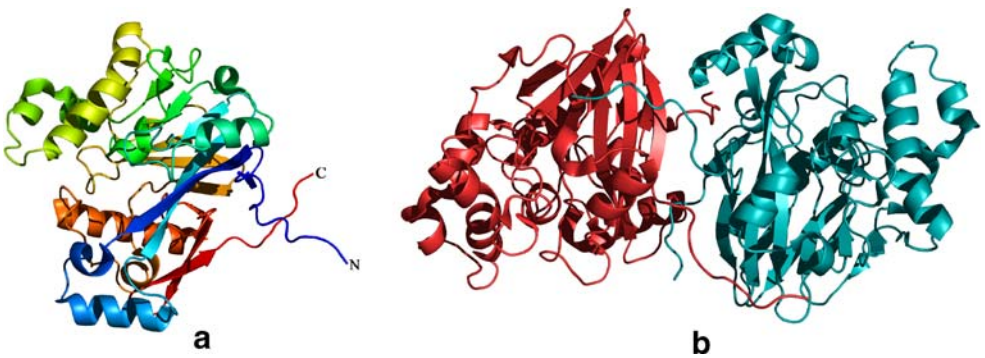

Fig. 1 The 3D structure of MPH. (**a**) monomer showing C and N-terminals; (**b**) the crystal structure of the dimer

DNA-purification columns were purchased from Omega. HRP/Anti-E Tag Conjugate, CM-Sepharose Fast Flow for ion-exchange chromatography, and RPAS Purification Module were provided by GE Healthcare. Ampicillin, bovine serum albumin (BSA), and tetracycline were purchased from Sigma. BCA Protein Assay Kit and 96-well ELISA microplates were obtained from Pierce and Corning (USA), respectively. Purified WSSV and the hemolymph of infected shrimp were obtained by using the procedure described by Dai et al. [21]. The pCANTAB5E-A1E and pET5a-MPH plasmids were constructed in our previous study. *E. coli* DH5α was used for all bacterial transformations and plasmid propagations. *E. coli* SM547 was selected as the host cell for protein expression. All other reagents used were of analytical-reagent grade.

Construction of expression vectors

All oligonucleotides were designed to incorporate proper restriction sites for cloning. The *E. coli* phosphatase expression vector pASK75-EAP [22] was used as a starting plasmid for construction of all the expression vectors used in this study. The plasmid pASK75-EAP was digested by StuI and HindIII to extract the *E. coli* phosphatase gene and yield a linearized plasmid to accommodate new inserts. Because there is a StuI site in the A1E- and MPH-encoding sequences, SnaBI restriction enzyme, which generates a flat terminal similar to that of StuI, was chosen to replace StuI for cloning the genes of interest. Primers UPA1ES (5′-GCCCTACGTACCATGGCCGAGGTGA-3′) and DOWN A1EH (5′-GCACCAAGCTTTTAGCGCTTGATTTCCAAC-3′), spanning the A1E scFv-encoding gene, were synthesized to amplify the A1E scFv gene with pCANTAB5E-A1E as a template. SnaBI and HindIII restriction sites were introduced in the N and C-terminal primers, respectively. The fragment was first cloned into the pGEM-T vector, and the correct orientation was identified by polymerase chain reaction (PCR) and restriction analysis. The resulting plasmid pGEM-TA1E was digested by SnaBI and HindIII, and A1E scFv-encoding fragment was recovered and inserted into the StuI/HindIII site of pASK75, yielding the A1E scFv expression vector pASK75-A1E. The MPH expression vector pASK75-MPH was similarly constructed using primers UPMPHS1 (5′-TATTACGTACAGCCGCACCGCAGGT-3′) and DOWNMPH (5′-GGGCAAGCTTAGTAGTTCACCGGCA-3′) to amplify the MPH-encoding sequence with the pET5a-MPH plasmid as the template.

For the construction of the MPH-A1E fusion gene, the MPH gene with SnaBI and XbaI restriction sites and the A1E scFv fragment with the SpeI and HindIII sequences were amplified and cloned into the pGEM-T vector, using a procedure similar to that described above; this step yielded the plasmids pGEM-TMPH2 and pGEM-TA1E2. MPH and A1E scFv fragments were recovered after separately digesting pGEM-TMPH2 with SnaBI and XbaI, and pGEM-TA1E2 with SpeI and HindIII. The synthesized oligonucleotides containing the [–(Gly–Ser)$_5$–] coding sequence (in bold) 5′-*CTAGA*AGCGGCTCTGGTTCCGGTAGCGGTT CCGGCA-3′ and 3′-TTCGCCGAGACCAAGGCCATCG CCAAGGCCG*TGATC*-5′ were annealed at 65 °C for 5 min to obtain a dsDNA fragment with XbaI and SpeI sticky ends (in italic). The resulting short fragment was then used to link the A1E scFv- and MPH-encoding fragments when cloning them into the StuI/HindIII site of pASK75. The structures of the three expression vectors are shown in Fig. 2. Each expression vector was transformed in the host cell strain SM547 for the expression of recombinant proteins.

Extraction and purification of recombinant proteins

The recombinant proteins SM547/pASK75-A1E, SM547/pASK75-MPH, and SM547/pASK75-MPH-A1E were cultured in Luria–Bertani (LB) broth to the exponential phase, and expression of the recombinant proteins was induced with 200 μg mL^{-1} tetracycline for over 16 h at 28 °C. The cells were harvested by centrifugation at 6,000 rpm for

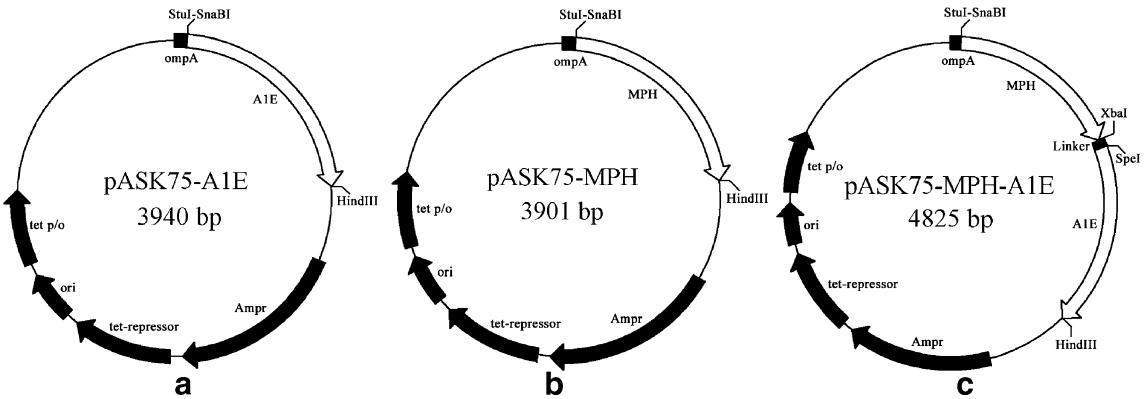

Fig. 2 Vectors constructed for expression of (**a**) A1E, (**b**) MPH, and (**c**) MPH-A1E fusion proteins

10 min, and the periplasmic proteins were extracted using the osmotic shock method described by French et al. [23].

MPH in the periplasmic extract was purified by cation-exchange chromatography (CM Sepharose Fast Flow). Initially, the column was washed with 20% (*v*/*v*) ethanol and then equilibrated with 0.01 mol L^{-1} sodium phosphate buffer (pH 7.0). The sample solution was applied to the column, and the unbound proteins were washed out with an equilibration buffer. The proteins bound to the column were eluted using a salt gradient in the equilibration buffer. The fractions with enzyme activity were pooled, and the concentration was determined by using the BCA Protein Assay Kit.

Soluble A1E scFv and MPH-A1E in the periplasmic extract were purified with the RPAS Purification Module. The soluble A1E scFv and fusion protein MPH-A1E carried a 13-amino-acid peptide tag (E-tag) at their C-terminals, which was recognized by an anti-E tag monoclonal antibody (GE Healthcare). The RPAS Purification Module contains a 5-mL HiTrap anti-E tag affinity column for fast purification of the E-tagged scFvs. The A1E scFv and fusion protein MPH-A1E were bound to the column at neutral pH (pH 7.0) and were easily eluted from the column by reducing the pH (to pH 3.0). The protein concentration in the collected sample solutions was determined by the BCA Protein Assay Kit.

Western blot assay

Expression of A1E scFv and fusion protein MPH-A1E was identified by Western blot assay. The *E. coli* periplasmic extracts were electrophoresed on a 10% sodium dodecylsulfate–polyacrylamide gel (SDS–PAGE) for 20 min at 50 V, followed by 1 h at 150 V. The samples were then electrotransferred to a nitrocellulose membrane for 30 min at 15 V by a Semi-Dry Transfer Cell (Biorad) with a transfer buffer (12 mmol L^{-1} Tris-Cl, pH 8.3, 96 mmol L^{-1} glycine, and 20% *v*/*v* methyl alcohol). Subsequently, the membrane was blocked at 37 °C for 2 h in a blocking buffer (2% (*w*/*v*) skim milk in a wash buffer TBST (20 mmol L^{-1} tris-buffered saline, pH 7.2–7.4, 150 mmol L^{-1} NaCl, and 0.1% (*v*/*v*) Tween-20)). After three washes of 10 min with the wash buffer, the membrane was incubated in HRP-conjugated anti-E tag antibody at 1:10,000 dilution for 2 h at 37 °C. The membrane was washed thoroughly as mentioned above and incubated in the substrate mixture containing 0.1 mg mL^{-1} 3,3′,5,5′-tetramethylbenzidine (TMB) and 0.1% (*v*/*v*) H_2O_2 for color development.

Measurement of enzyme properties

The molecular weight of MPH and its state in solution were determined using an XL-I analytical ultracentrifuge (Beckman Coulter) equipped with a four-cell An-60 Ti rotor. Purified MPH (0.8 mg mL^{-1}) in 100 mmol L^{-1} sodium phosphate buffer (pH 7.5) was centrifuged at 4 °C and 60,000 rpm for 4 h, using sodium phosphate buffer as the control. For determining its molecular weight, the data were analyzed using SEDFIT [24] from http://www.analyticalultracentrifugation.com/download.htm.

The thermostability of MPH and the optimum reaction pH and temperature were investigated using a stopped-flow spectrometer with 0.125 mmol L^{-1} *O*,*O*-dimethyl-*O*-(4-nitrophenylmethyl) phosphorothioate as the substrate and a reaction time of 1 min. Thermostability analysis was performed by incubating the enzyme solution at 40 °C, 50 °C, 60 °C, 70 °C, and 80 °C and taking samples every 10 min at 25°C. The storage stability of the enzyme at room temperature, 4 °C, and −20 °C was also measured periodically using a procedure similar to the thermostability assay. For determining the optimum reaction pH, the pH was set in the range 6.0–12.0. The optimum reaction temperature experiments were carried out at 4 °C–50 °C.

Enzyme kinetic assays

Using the stopped-flow spectrometer, the kinetic properties of purified MPH and MPH-A1E with *O*,*O*-dimethyl-*O*-(4-nitrophenylmethyl) phosphorothioate as the substrate were calculated at pH 7.0 and 25 °C by assaying the initial velocities over a range of *O*,*O*-dimethyl-*O*-(4-nitrophenylmethyl) phosphorothioate concentrations from 0.025–0.15 mmol L^{-1}. K_{cat} was defined as the number of substrate molecules that were converted to products per enzyme molecule per second. K_m is the substrate concentration that provides a reaction velocity that is half of the maximal velocity obtained under saturating substrate conditions. The values of K_{cat} and K_m were obtained from Lineweaver–Burk plots on the assumption that simple Michaelis–Menten kinetics were followed.

Determination of antibody affinity constants

The affinity constants (K_{aff}) of the A1E and MPH-A1E fusion protein were determined by the protocol described in our previous study [21]. In brief, WSSV at concentrations of 5, 2.5, 1.25, or 0.625 μg mL^{-1} was coated on the microplates, and the plates were incubated overnight at 4 °C in 0.01 mol L^{-1} phosphate-buffered saline (PBS) buffer (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4 in 1 L distilled H_2O; pH 7.4). Twofold serial dilutions of the scFv and fusion protein were subsequently added to each concentration of coated WSSV, followed by the application of the secondary HRP/Anti-E Tag Conjugate. Finally, a substrate mixture containing 0.1 mg mL^{-1} TMB and 0.1% (*v*/*v*) H_2O_2 was added to each well of the microplates, and

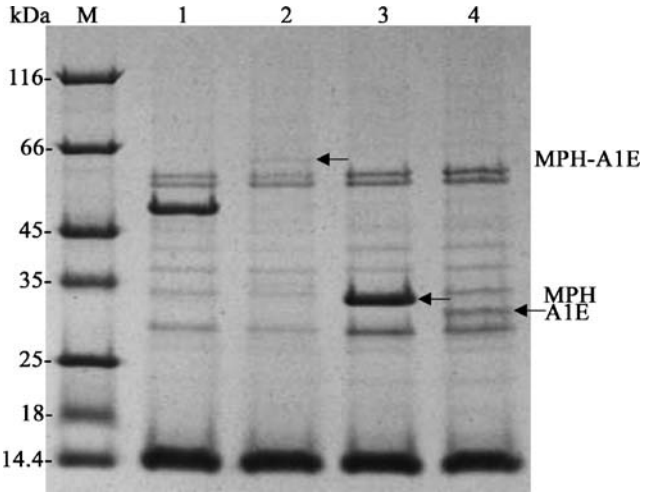

Fig. 3 SDS–PAGE analysis of the periplasm of *E. coli* SM547. *Lane M*, low molecular weight protein marker (kDa); *lane 1*, periplasm of *E. coli* SM547 expressing EAP (the negative control); *lane 2*, periplasm of *E. coli* SM547 expressing the MPH-A1E fusion protein; *lane 3*, periplasm of *E. coli* SM547 expressing MPH; and *lane 4*, periplasm of *E. coli* SM547 expressing A1E scFv

the absorbance was recorded at 450 nm. K_{aff} was calculated according to the mathematic formula:

$$\text{Kaff} = (n-1)/(n[\text{Ab2}] - [\text{Ab1}])$$

where [Ab1] and [Ab2] represent the respective antibody concentrations required to achieve 50% of the maximum absorbance obtained at two different concentrations of coated antigen ($[Ag]_1 = n\,[Ag]_2$), and n is the dilution factor between the antigen concentrations used.

ELISA for WSSV

The purified WSSV and shrimp hemolymph samples were subjected to the MPH-A1E-mediated ELISA assay. For the detection of purified WSSV, the wells of 96-well microplates were coated with the previously indicated quantity of WSSV ranging from 0–100 ng and incubated at 4 °C overnight. The wells were then blocked with 4% skim milk in PBS to cover any unoccupied sites on the plastic surface. After incubating with 100 μL 100 μg mL^{-1} MPH-A1E at 37 °C for 1.5 h, the wells were thoroughly washed with PBST (0.05% Tween-20 in PBS buffer) and PBS buffer. Finally, 100 μL substrate solution containing 0.25 mmol L^{-1} *O*, *O*-dimethyl-*O*-(4-nitrophenylmethyl) phosphorothioate was added to the wells for color development, and the absorbance was recorded at 405 nm. For assay of the shrimp hemolymph sample, a similar procedure was followed with the exception that PBS-diluted samples containing 1 μL of shrimp hemolymph were coated instead of purified WSSV. The infected shrimp hemolymph was analyzed using the purified WSSV as the positive control, and equal volumes of PBS and healthy shrimp hemolymph were used as the negative controls.

Results

Expression of A1E scFv, MPH, and MPH-A1E

As a preliminary experiment, the expression vectors were separately transformed in the host strain *E. coli* SM547. Positive transformants were confirmed by PCR and restriction analysis. Expression of the recombinant proteins was induced by tetracycline. The proteins in the *E. coli* periplasm were extracted and analyzed using SDS–PAGE (Fig. 3). Expression of A1E scFv and MPH-A1E in the SM547 were confirmed by Western blot analysis (Fig. 4). The periplasm extracted from the SM547/pASK75-EAP, which was constructed in our previous study, was used as the negative control. The molecular weights of A1E scFv and MPH were found to be approximately 31 kDa and 34 kDa, respectively; these values are consistent with those in previous reports [21, 20]. The protein band corresponding to the fusion protein was approximately 65 kDa. The molecular weight of the fusion protein matched the sum of the molecular weights of A1E and MPH.

MPH properties

MPH was purified by cation-exchange chromatography at pH 7.0; the protein was eluted using a 0–1.0 mol L^{-1} NaCl concentration gradient and harvested at approximately 200 mmol L^{-1} NaCl. The purified MPH was used for measurement of thermostability, storage stability, optimum pH, and optimum temperature. To obtain a homogeneous

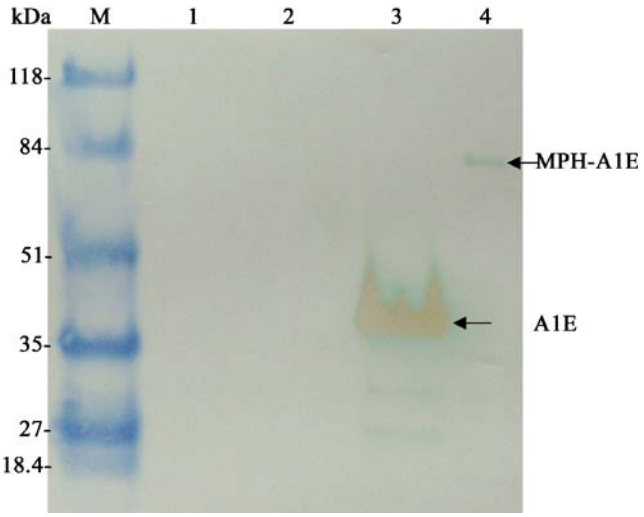

Fig. 4 Western blot analysis of the periplasm of *E. coli* SM547. *Lane M*, pre-dyed protein marker (kDa); *lane 1*, periplasm of *E. coli* SM547 expressing EAP (the negative control); *lane 2*, periplasm of *E. coli* SM547 expressing A1 scFv without the E-tag; *lane 3*, periplasm of *E. coli* SM547 expressing A1E scFv with the E-tag; and *lane 4*, periplasm of *E. coli* SM547 expressing the MPH-A1E fusion protein

protein sample, the enzyme used for analytical ultracentrifugation was further purified by gel-filtration chromatography.

To determine the molecular weight of MPH and its state in solution, the purified MPH was concentrated to 0.8 mg mL^{-1} and centrifuged at 4 °C and 60,000 rpm for 4 h on the XL-I analytical ultracentrifuge equipped with a 4-cell An-60 Ti rotor; sodium phosphate buffer was used as the control. The result showed two types of proteins in the sample whose molecular weights were 22 kDa and 37 kDa. According to the SDS–PAGE analysis, the smaller protein was an interferential protein while the larger protein corresponded to MPH (Fig. 5). Since a protein with a relatively large molecular weight was not observed in the entire study, we concluded that MPH exists as a monomer in solution.

The optimal pH and temperature of MPH were 11.0 and 40 °C, respectively. The enzyme was stable at temperatures ranging from 25 °C to 60 °C. These results were consistent with those of our previous study [20]. The storage stability of MPH at room temperature, 4 °C, and −20 °C was also measured periodically. As expected, the enzyme activity did not decrease when stored at −20 °C for 10 months, at 4 °C for 2 weeks, and at room temperature for 3 days (Fig. 6).

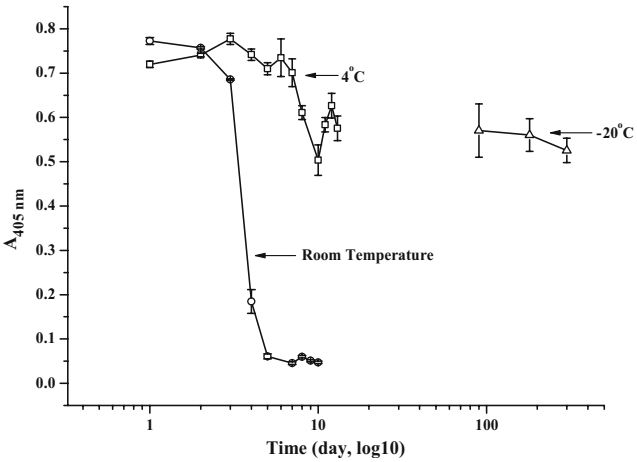

Fig. 6 The storage stability of MPH at room temperature, 4 °C, and −20 °C

Characterization of the properties of MPH-A1E fusion protein

The MPH-A1E fusion protein was constructed by fusing A1E scFv to the C-terminal of MPH by insertion of a [–$(Gly–Ser)_5$–] linker peptide. To examine whether the fusion affected the biological function of the fusion partners, the kinetic properties of MPH and the binding affinity of A1E scFv were determined in the fusion protein and were compared with those of the parent proteins. As shown in Table 1, the fusion protein had approximately 39.67% MPH catalytic activity and 61.80% antibody affinity. The molecular weight of the fusion protein was approximately twice that of MPH and A1E scFv, and this primarily caused the decrease in the catalytic activity and antibody affinity. These results suggest that the fusion protocol did not have any significant influence on the native structure of both the fusion partners. Thus, the MPH-A1E fusion protein was found to be a potential candidate for use in the detection of WSSV.

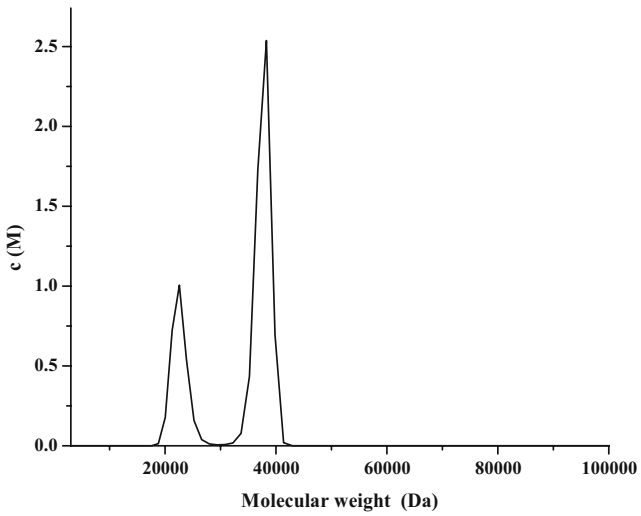

Fig. 5 Analytical ultracentrifugation assay of MPH. Using the XL-I analytical ultracentrifuge equipped with a four-cell An-60 Ti rotor, MPH (concentrated to 0.8 mg mL^{-1}) was centrifuged at 4 °C and 60,000 rpm for 4 h; sodium phosphate buffer (pH 7.5) was used as the control. To determine the molecular weight of MPH, the data were analyzed using SEDFIT

Detection of WSSV using the MPH-A1E fusion protein

To evaluate the practical utility of the MPH-A1E fusion protein, it was used as an ELISA reagent for the detection of WSSV. The indicated quantities of purified WSSV or infected shrimp hemolymph were coated on the wells of the 96-well ELISA plates, and the plates were then incubated overnight at 4 °C; this was followed by addition of MPH-A1E. After the binding reaction, the MPH substrate was added, and the yellow product was measured at 405 nm. The detection limit was set as the time point when the absorption ratio of the positive control (purified WSSV) to the negative control (PBS) was twofold or higher. As shown in the calibration curve (Fig. 7), the MPH-A1E fusion protein specifically recognized as little as 25 ng purified WSSV protein. This detection limit was approximately the same as that in a previous study, which used A1E scFv and the HRP/Anti-E Tag Conjugate protocol [21]. When the hemolymph samples of infected shrimp were applied, WSSV in 1 μL hemolymph of the infected shrimp was directly detected by the MPH-A1E fusion protein (Fig. 8).

Table 1 Characteristics of fusion protein MPH-A1E, A1E scFv and MPH

Protein	K_{aff} (mol^{-1} L)	Enzyme kinetics			
		Specific activity (U mg^{-1})	K_m (μmol L^{-1})	K_{cat} (s^{-1})	K_{cat}/K_m (s^{-1} μmol^{-1} L)
MPH-A1E	(3.51±0.62) ×10^7	10.29±1.54	84.75±56.21	43.48±26.73	0.545±0.187
A1E scFv	(5.68±0.64) ×10^7	–	–	–	–
MPH	–	25.94±3.07	27.79±6.22	114.70±13.19	4.37±1.64

Discussion

Based on the experimental data, by applying the fusion protocol, we could demonstrate the desired properties of the fusion partners, i.e. the high residual activities of enzyme catalysis and antibody affinity. This was possible largely due to the contribution of the linker peptide that acts as a spacer between the fusion partners and reduces the possible interference between them when the proteins fold and function. This study of WSSV assay indicates that the fusion system has a good sensitivity for application in direct ELISA. In summary, MPH meets all the requirements for analytical assays—it is a good catalytic agent, produces easily comprehensible signals, can be easily fused with other proteins, is quantitatively expressed in *E. coli*, and has a lower tendency to aggregate. With a simple purification method as the only requirement, the MPH-scFv fusion protein can serve as a ready-to-use ELISA reagent.

Moreover, MPH is believed to be a recent product of molecular evolution; it has probably appeared after the use of pesticides began about 50 years ago. Thus far, it has been found only in a limited number of bacterial strains [25–27]; its occurrence in animals and plants has not yet been reported. Besides, MPH is not a constructive enzyme but an inductive enzyme; this implies that bacterial cells would not produce MPH unless methyl parathion is introduced in its environment for a certain time period. These two features are particularly important to avoid the interference of background signal in ELISA and enzyme-linked immunohistochemistry.

However, the toxicity of its substrate—methyl parathion—may be a limitation for the use of MPH as a labeling reagent. Methyl parathion is a highly toxic insecticide (Environmental Protection Agency (EPA) toxicity class I) and is classified as a Ia (extremely hazardous) pesticide by the World Health Organization (WHO) [28]. Some or all the formulations of methyl parathion may be classified as restricted use pesticides (RUPs). However, many laboratory reagents are highly poisonous, for example, the commonly used substrates *p*-nitrophenyl phosphate (pNPP) for APs and 5-fluoroindole-3-acetic acid and *o*-dianisidine for HRP. The risk of being poisoned can be carefully controlled and restricted to minimal levels by employing stringent laboratory standards. According to the Pesticide Information Profiles (PIPs), which are documents providing specific pesticide information relating to health and environmental

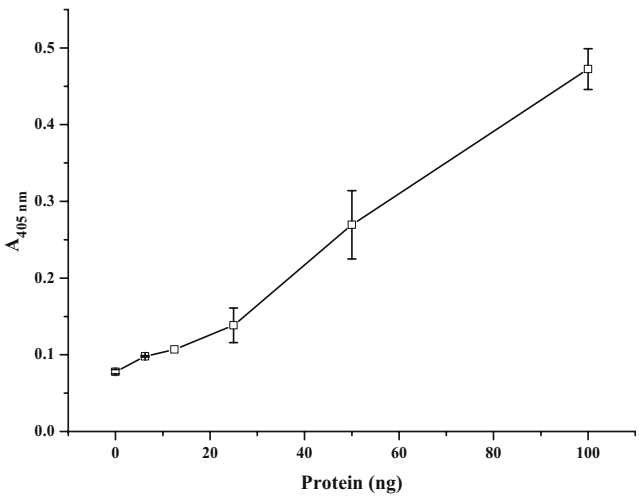

Fig. 7 Calibration plot for detection of WSSV

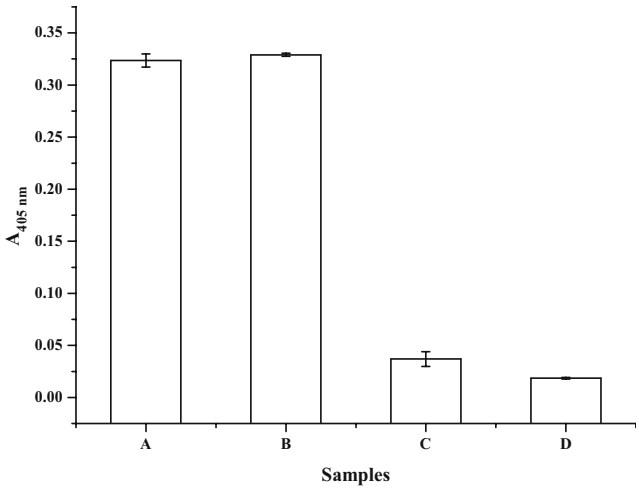

Fig. 8 Detection of WSSV in the hemolymph of an infected shrimp by using the MPH-A1E fusion protein. **a**, 1.02 μg μL^{-1} purified WSSV (positive control); **b**, fresh hemolymph of an infected shrimp; **c**, fresh hemolymph of a healthy shrimp; and **d**, PBS (negative control). The amounts of positive control and the hemolymph sample were 3 μL and 1 μL, respectively

effects, the reported LD50 values for acute toxicity of MPH in rats are 6–50 mg kg^{-1} and 67 mg kg^{-1} when administered via the oral and dermal routes, respectively. With regard to chronic toxicity, studies with human volunteers have found that the minimum toxic dose of MPH that causes the appearance of mild cholinesterase inhibition is 22–30 mg day^{-1} for each individual after at least four weeks of MPH exposure (data from the extension toxicology network (EXTOXNET), http://extoxnet.orst.edu/ghindex.html). The substrate dosage (0.25 mmol L^{-1}, 6.578×10^{-3} mg $well^{-1}$) used for enzyme analysis is much lower than the minimum acute toxicity dose in animals and the chronic toxicity dose in humans. Moreover, this concern could be addressed by developing non-toxic or less-toxic substrate derivatives.

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