Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology) 2021 22(3):204-213
www.jzus.zju.edu.cn; www.springer.com/journal/11585
E-mail: jzus_b@zju.edu.cn

Research Article

https://doi.org/10.1631/jzus.B2000525

Expression of *Drosophila melanogaster* acetylcholinesterase (*DmAChE*) gene splice variants in *Pichia pastoris* and evaluation of its sensitivity to organophosphorus pesticides

Liujia SHI[1,2], Fangfang YANG[1,2], Yanyan XU[1,2], Shoufeng WANG[1,2]✉

[1]*Institute of Pharmaceutical Biotechnology, School of Medicine, Zhejiang University, Hangzhou 310058, China*
[2]*Zhejiang Provincial Key Laboratory for Microbial Biochemistry and Metabolic Engineering, Hangzhou 310058, China*

Abstract: Acetylcholinesterase (AChE) is a key enzyme used to detect organophosphorus pesticide residues by the enzyme inhibition method. An accidental discovery of a mutant strain with AChE activity was made in our laboratory during the process of AChE expression by *Pichia pastoris*. The pPIC9K-*Drosophila melanogaster* acetylcholinesterase (*DmAChE*)-*like* expression vector was constructed by codon optimization of this mutant strain, which was transformed into *P. pastoris* GS115, and positive clones were selected on yeast peptone dextrose (YPD) plate with G418 at 4.0 mg/mL. The GS115-pPIC9K-*DmAChE-like* strain was subjected to 0.5% methanol induction expression for 120 h, with a protein band at 4.3 kDa found by the tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) pattern of the fermentation supernatant. After preliminary purification by ammonium sulfate precipitation, the enzyme activity was detected to be 76.9 U/(mL·min). In addition, the pesticide sensitivity test proved that DmAChE-like is selective and sensitive to organophosphorus pesticides.

Key words: Acetylcholinesterase (AChE); *Pichia pastoris*; Enzyme activity determination; Pesticide sensitivity

1 Introduction

Organophosphorus compounds are the most widely used pesticides due to their broad insecticidal spectrum and relatively low residue (Chen et al., 2010). They can inhibit acetylcholinesterase (AChE, EC 3.1.1.7) activity and cause excessive nerve excitement, thereby achieving the purpose of killing insects (Jokanović, 2009; Hossain and Sikder, 2015; Liang et al., 2019). With the widespread use of organophosphorus pesticides, chronic poisoning in agricultural products has become an issue of increasing concern (van Dyk and Pletschke, 2011; Koureas et al., 2012). The main hazard of organophosphorus pesticides pose to the human body is acute toxicity, which usually occurs after large doses or repeated exposure, manifesting in a series of neurotoxic symptoms. In severe cases, respiratory paralysis and even death may occur (Sakata, 1993; Yang et al., 2018). Therefore, finding a method for the rapid detection of pesticide residues is of great significance to ensure food safety.

Current conventional methods for determining pesticide residues include gas chromatography (GC) or high-performance liquid chromatography (HPLC) combined with mass spectrometry (MS) (Ponomarev and Shtykov, 2000; Albero et al., 2003; Viñas et al., 2003; Apilux et al., 2015), which are complicated and time-consuming protocols requiring skilled technicians and expensive instruments (Villatte et al., 1998; Duford et al., 2013). Thereby, the enzyme inhibition method is currently the routine technology for detecting organophosphorus pesticide residues. It has the characteristics of simple operation and fast detection, and does not require large instruments or advanced professional knowledge (Amine et al., 2006), and thus it is suitable for rapid on-site detection (Mai et al., 2017). Its detection principle is detailed below. Under normal physiological conditions, AChE can hydrolyze acetylcholine into choline and acetic acid. Once the organophosphorus pesticides have entered the human

✉ Shoufeng WANG, sfwang@zju.edu.cn
Liujia SHI, https://orcid.org/0000-0001-5124-7845
Shoufeng WANG, https://orcid.org/0000-0003-0896-5440

Received Sept. 4, 2020; Revision accepted Nov. 16, 2020;
Crosschecked Jan. 29, 2021

body through food intake, they will combine with the active site of AChE to make it phosphorylate, resulting in phosphorylated AChE that is not easily hydrolyzed, thus losing its catalytic activity (Aldridge and Davison, 1953; Fournier and Mutero, 1994; Pope, 1999; Tougu, 2001; Worek et al., 2012; Li et al., 2013). The enzyme inhibition method employs this reaction characteristic, using filter paper or electrode as a carrier to adsorb AChE, and judging the residual amount of pesticides in the sample by the color change of the developer (Yang et al., 2019).

Enzymes used for the enzyme inhibition method include AChE, butyrylcholinesterase, as well as animal and plant esterases. Among these, AChE is the most sensitive to organophosphorus pesticides while being selective, resulting in its wide application. At present, AChE is mainly extracted from insect heads or animal brain tissues (van Dyk and Pletschke, 2011), which sources are relatively limited, preventing the general use of such enzyme inhibition methods. Choosing a suitable and stable enzyme source is the key to rapid and accurate detection. Compared with other sources of *AChE* genes, insect-derived AChE is more sensitive to organophosphorus pesticides (Schulze et al., 2005). We have previously discovered a naturally spliced gene mutant (*Drosophila melanogaster* acetylcholinesterase (*DmAChE*)*-like*) by chance during the in vitro recombinant expression of *DmAChE*. That was the first time *DmAChE-like* was reported. The DmAChE-like enzyme has a smaller molecular weight compared with DmAChE, and is easy to work with. This study planned to demonstrate the AChE activity of DmAChE-like by expressing *DmAChE-like* in *Pichia pastoris*, and compare its differences from DmAChE. We performed pesticide susceptibility experiments to compare the sensitivity and selectivity of DmAChE-like and DmAChE to pesticides, so as to provide a new source of enzymes for routine monitoring of organophosphorus pesticides.

2 Materials and methods

2.1 Strains and media

The pUC57-*DmAChE-like* plasmid was synthesized by Shanghai Sangon Biotechnology (Shanghai, China). The *Escherichia coli* BL21 and *P. pastoris* GS115 strains, as well as the GS115-pPIC9K-*DmAChE* and the integrative expression vector (pPIC9K-top10) were all stored in our laboratory. Colonies of *E. coli* were grown in Luria-Bertani (LB) medium (0.01 g/mL peptone, 5 g/L yeast extract, and 0.01 g/mL sodium chloride), while *P. pastoris* was cultured in yeast peptone dextrose (YPD) medium (0.01 g/mL yeast extract, 0.02 g/mL peptone, and 0.02 g/mL glucose). Transformants of *P. pastoris* were selected on minimal dextrose (MD) medium plates (0.02 g/mL glucose, 0.4 mg/L biotin, 0.013 g/mL yeast nitrogen base (YNB), and 0.015 g/mL agarose), whereas *P. pastoris* positive transformants were fermented in buffered glycerol-complex medium (BMGY) or buffered methanol-complex medium (BMMY) (0.01 g/mL yeast extract, 0.02 g/mL peptone, 100 mmol/L phosphate-buffered saline (PBS, pH 6.0), 0.4 mg/L biotin, 0.013 g/mL YNB, and 0.015 g/mL agarose).

2.2 Reagents

High-fidelity DNA polymerase (PrimeSTAR HS DNA polymerase), restriction endonucleases, and DNA polymerases were acquired from TaKaRa Biotechnology Co., Ltd. (Dalian, China). Kits for polymerase chain reaction (PCR) product purification were purchased from Shanghai Sangon Biotechnology (Shanghai, China). Plasmid small extraction kits, agarose gel DNA purification kit, and column purification kits were obtained from Axygen (Hangzhou, China). Organophosphate insecticides (purity of ⩾98.4%) were purchased from Beijing Century Aoke Biotechnology Co., Ltd. (Beijing, China), and AChE standard products were obtained from Shanghai Yuanye Biological Technology Co., Ltd. (Shanghai, China).

2.3 Plasmid construction

After codon optimization of the original sequence of *DmAChE-like*, the pUC57-*DmAChE-like* plasmid containing the *DmAChE-like* gene was constructed by Shanghai Sangon Biotechnology (Shanghai, China). Using pUC57-*DmAChE-like* as a template, the *DmAChE-like* fragment was amplified by PCR using *DmAChE-like*-up and *DmAChE-like*-down primers (Table 1), and the PCR amplification products were purified by agarose gel DNA purification kit. The *DmAChE-like* and pPIC9K fragments were digested by the restriction enzymes *Sna*BI and *Not*I, and the digested fragments were ligated using the In-Fusion cloning kit for the construction of pPIC9K-*DmAChE-like*.

Table 1 Primers used for plasmid construction in the current study

Primer by function	Sequence (5'→3')
Amplification	
DmAChE-like-up*	GAGGCTGAAGCTTACGTAATG GCTATCTCTTGTAG
DmAChE-like-down*	GAATTAATTCGCGGCCGCTTAC AACAAAGATTC
Verification	
5'-AOX	GACTGGTTCCAATTGACAAGC
3'-AOX	GCAAATGGCATTCTGACATCC

* Underlining indicates sequences of restriction sites. AOX: alcohol oxidase.

2.4 Bioinformatics analysis

Based on the sequencing results, *DmAChE-like* was analyzed by bioinformatics. The DNAMAN V6 (LynnonBiosoft, USA) was applied to analyze the molecular mass, base composition, and distribution of nucleic acid sequences; ExPASy (https://web.expasy.org/compute_pi) was used to calculate its isoelectric point and relative molecular mass; the ProtParam online tool (https://web.expasy.org/protparam) was utilized to predict the protein properties of the protein to be expressed; SignalP software Version 2.0 (http://www.cbs.dtu.dk/services/SignalP-2.0) was used for signal peptide analysis (Nielsen et al., 1997; Goujon et al., 2010; Petersen et al., 2011).

2.5 *P. pastoris* transformation and selection

The pPIC9K-*DmAChE-like* plasmid linearized by *Sal*I was transformed into the host strain GS115 by electric shock, and His^+ recombinant transformants were selected by MD and G418 plates. Colony PCR was performed using universal primers 5'-alcohol oxidase (5'-AOX) and 3'-AOX (Table 1) to obtain positive GS115-pPIC9K-*DmAChE-like* transformants.

2.6 Induced expression

The recombinant GS115-pPIC9K-*DmAChE-like* strain was shake-cultured in 50 mL BMGY medium at 30 °C to get optical density at 600 nm (OD_{600}) of 2.0−6.0. The bacterial solution was centrifuged at 4000*g* for 5 min at room temperature, and the precipitate was collected. The OD_{600} value was adjusted to 1.0 with a fresh BMMY medium. Strain culturing was continued at 30 °C, with 5 mL of sample taken every 12 h before adding anhydrous methanol to a final volume fraction of 0.5%. After 120 h of induced expression, the fermentation supernatant was collected by centrifugation at 10 000 r/min for 10 min.

2.7 Enzyme activity assay

AChE activity was measured by ultraviolet (UV) spectrophotometry according to Ellman et al. (1961). In brief, the reaction solution contained 100 mmol/L PBS (pH 7.4), 10 mmol/L 5,5'-dithiol-bis-2-nitrobenzoic acid (DTNB), 75 mmol/L acetylthiocholine iodide (ATC), and fermentation supernatant. The mixture was incubated at 37 °C for 10 min, and the change in absorbance of the solution was measured within 1 min at 412 nm. Based on the results for the commercially pure AChE enzyme, the relative enzyme activity was obtained from the standard curve (Li et al., 2013). The GS115-pPIC9K-*DmAChE* strain expression product was used as a control.

2.8 Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The process of discontinuous electrophoresis included a protein gel, which consists of 16.5% separation gel, 10.0% sandwich gel, and 4.0% concentrated gel (all volume fraction). During electrophoresis, cathode buffer was added to the inner tank and anode buffer was added to the outer tank. The fermented protein supernatant was concentrated with trichloroacetic acid (TCA). Firstly, electrophoresis was performed at 30 V for 1−2 h. When the leading edge reached the upper edge of the separation gel, the voltage was adjusted to 130 V until the end of electrophoresis (Pardo and Natalucci, 2002; Schägger, 2006). Following the electrophoresis, the gel was stained for 4 h and then decolorized for another hour for imaging.

2.9 Effect of pH and temperature

AChE enzyme activity was measured in PBS with pH values of 5.0, 6.0, 7.0, 7.4, 8.0, and 9.0, respectively. Changes in absorbance were recorded at 412 nm, and the relative enzyme activity was calculated to get the optimal pH. Under different temperature conditions ranging from 25 to 75 °C, the best reaction temperature was obtained by measuring the enzyme activity of DmAChE-like. The GS115-pPIC9K-*DmAChE* strain expression product was used as a control.

2.10 Detection of organophosphorus and carbamate pesticides using the *DmAChE-like* expression product

The fermentation broth was centrifuged to remove the cells, and the supernatant was subjected to ammonium sulfate fractionation. The target product was packed in a dialysis bag and desalted in a low-salt buffer solution, and then concentrated with polyethylene glycol to obtain the crude enzyme solution. Five kinds of pesticide standard products (methamidophos, trichlorfon, carbaryl, chlorpyrifos, and carbofuran) were prepared in dilutions of $10^{-4}-10^{-1}$ mg/mL with distilled water. Volumes of 140 μL of the crude enzyme solutions were mixed with equal volumes of pesticide and left for the reaction to take place at 37 °C for 15 min. At the same time, PBS was used as blank control instead of pesticide. Finally, 560 μL Ellman reagent was added to each reaction mixture to measure DmAChE-like activity for the calculation of the activity inhibition rate (IR):

$$IR=(E_1-E_2)/E_1\times 100\%,$$

where E_1 indicates the enzyme activity of the reaction system without pesticide control and E_2 is the enzyme activity of the reaction system with pesticide inhibition.

Taking the inhibition rate of enzyme activity as the ordinate and the logarithmic value of the pesticide concentration as the abscissa, the pesticide sensitivity curves were drawn.

3 Results

3.1 Identification of clones

The mutant strain was a result of spontaneous mutation introduced during the construction of the expression vector pPIC9K-*DmAChE*, and the complete sequence was obtained by sequencing, with the sequencing results shown in Table 2. The original gene sequence is *DmAChE-like* (original), and *DmAChE-like* is the gene sequence after codon optimization (Fig. 1). A 237-bp *DmAChE-like* gene fragment was amplified from the plasmid pUC57-*DmAChE-like* (Fig. 2a). The *DmAChE-like* was cloned into the expression vector pPIC9K, with the colony PCR results shown in Fig. 2b. Sequencing results showed that the recombinant plasmid pPIC9K-*DmAChE-like* was successfully constructed. Results of electric shock conversion are shown in Fig. 3. These results demonstrated that the number of transformants gradually decreased with the gradual rise of G418 concentration.

A single colony on a G418 plate was picked for PCR using the universal primer (5'-AOX and 3'-AOX) to obtain GS115-pPIC9K-*DmAChE-like* positive transformants. As shown in Fig. 2c, a band of 718 bp appeared (the expected size of the band obtained from the universal primer PCR was 718 bp, showing that the constructed vector has been integrated into the genome of *P. pastoris* GS115).

Table 2 Sequences of genes according to this article

Gene	Sequence (5'→3')
DmAChE-like (original)	ATGGCCATCTCCTGTCGGCAGAGCAGAGTCCTGCCCATGTCCTTGCCCCTGCCTCTGACCA TCCCGCTGCCCCTGGTGCTGGTGCTATCACTGCACCTGTCCGGCGTCTGCGGCGTCATCG ATCGCCTGGTCGTGCAGACATCCTCCGGACCTGTACGCGGTCGCTCCGTGACGGTGCAG GGCAGGGAGGTGCATCCATATCCCCGAGGCTCCAGCTCCTTGGAATCGCTGCTCTGA
DmAChE-like (after codon optimization)	ATGGCTATCTCTTGTAGACAATCTAGAGTTTTGCCAATGTCTTTGCCATTGCCATTGACTATC CCATTGCCATTGGTTTTGGTTTTGTCTTTGCACTTGTCTGGTGTTTGTGGTGTTATCGACA GATTGGTTGTTCAAACTTCTTCTGGTCCAGTTAGAGGTAGATCTGTTACTGTTCAAGGTA GAGAAGTTCACCCATACCCAAGAGGTTCTTCTTCTTTGGAATCTTTGTTGTAA

```
DmAChE-like original  ATGGCCATCTCCTGTCGGCAGAGCAGAGTCCTGCCCATGTCCTTGCCCCTGCCTCTGACC  60
DmAChE-like           ATGGCTATCTCTTGTAGACAATCTAGAGTTTTGCCAATGTCTTTGCCATTGCCATTGACT  60
                      ***** ***** *** * **     *****  **** ***** *****  ****  ****

DmAChE-like original  ATCCCGCTGCCCCTGGTGCTGGTGCTATCACTGCACCTGTCCGGCGTCTGCGGCGTCATC  120
DmAChE-like           ATCCCATTGCCATTGGTTTTGGTTTTGTCTTTGCACTTGTCTGGTGTTTGTGGTGTTATC  120
                      *****  ****  ****  ****  * **  ***** **** ** ** ** ** ** ***

DmAChE-like original  GATCGCCTGGTCGTGCAGACATCCTCCGGACCTGTACGCGGTCGCTCCGTGACGGTGCAG  180
DmAChE-like           GACAGATTGGTTGTTCAAACTTCTTCTGGTCCAGTTAGAGGTAGATCTGTTACTGTTCAA  180
                      **  *  **** ** ** ** ** ** ** ** **  * *** * ** ** ** ** **

DmAChE-like original  GGCAGGGAGGTGCATCCATATCCCCGAGGCTCCAGCTCCTTGGAATCGCTGCTCTGA      237
DmAChE-like           GGTAGAGAAGTTCACCCATACCCAAGAGGTTCTTCTTCTTTGGAATCTTTGTTGTAA      237
                      ** ** ** ** ** ***** **  **** **    ** ********  ** * * *
```

Fig. 1 Alignments of *DmAChE-like* original gene and codon-optimized gene.

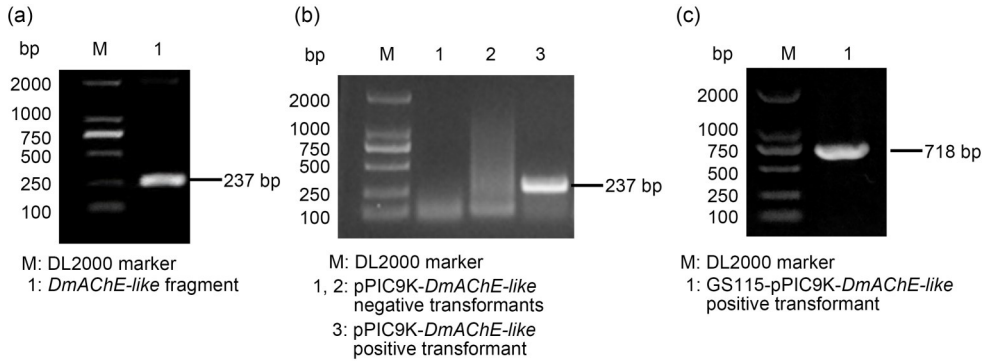

Fig. 2 Identification of PCR constructs. (a) *DmAChE-like* fragment; (b) pPIC9K-*DmAChE-like* positive transformant; (c) GS115-pPIC9K-*DmAChE-like* positive transformant.

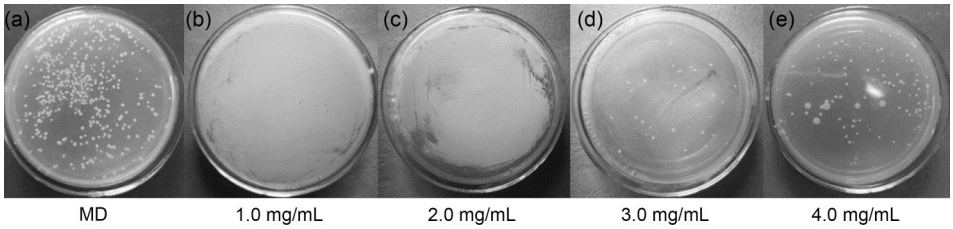

Fig. 3 Screening results for G418 resistant plates of recombinant strain GS115-pPIC9K-*DmAChE-like*. (a) MD medium; (b–e) 1.0 (b), 2.0 (c), 3.0 (d), and 4.0 mg/mL (e) GS115-pPIC9K-*DmAChE-like*. MD: minimal dextrose.

3.2 Bioinformatics analysis of *DmAChE-like*

Due to splicing mutations in the gene, the intermediate sequence of *DmAChE* is missing, resulting in the connection of base pairs 1–194 of the gene sequence with the 1867–1909 bp gene fragment, thus forming *DmAChE-like*. Among them, the open reading frame of *DmAChE-like* is 237 bp long. Through the bioinformatics analysis of *DmAChE-like*, the protein sequence identity between DmAChE-like and DmAChE was found to be 79.5%. The result of protein sequence alignment is shown in Fig. 4. The first 38 amino acids of DmAChE-like are signal peptides, and the last 40 amino acids are mature peptides. The relative molecular weight of DmAChE-like is 4.3 kDa and the theoretical isoelectric point (pI) is 10.6 according to the analysis, with its main physical and chemical properties as shown in Table 3. The specific mechanism of the reaction between DmAChE-like and organophosphorus pesticides is yet unclear and needs to be further investigated in subsequent work.

3.3 Expression and enzyme activity of recombinant protein in the GS115-pPIC9K-*DmAChE-like* strain

A single GS115-pPIC9K-*DmAChE-like* colony was selected to induce expression with methanol at 30 °C for 120 h. A volume of 1 mL fermentation supernatant was taken at each time point, and after desalting and concentrating by TCA, this was used for tricine-SDS-PAGE gel electrophoresis for qualitative analysis. The target band can be observed at 4.3 kDa, as shown in Fig. 5. The enzyme activity of the sampled fermentation supernatant was detected according to the Ellman method. Based on the time curve as shown in Fig. 6, the enzyme activity of DmAChE-like gradually increased with fermentation time, and began to stabilize at 96 h. Its value for DmAChE was 14.7 U/(mL·min) at 120 h, while that of DmAChE-like also reached 12.3 U/(mL·min), with little difference observed. This demonstrates that DmAChE-like does have AChE activity, the level of which is not much different from that of DmAChE enzyme activity.

3.4 Characteristics of DmAChE-like

Under the above conditions, pH=8.0 and temperature (T)=37 °C were optimal for DmAChE-like. As shown in Fig. 7, DmAChE-like reached the highest enzyme activity at an optimum of pH=8.0. Between pH 5.0–8.0, this activity for DmAChE-like gradually increased with the rise of pH, but declined significantly when pH>8.0. Compared with DmAChE, DmAChE-like is less tolerant to acidic environments, while its

```
DmAChE       1 MAISCRQSRVLPMSLPLPLTIPLPLVLVLSLHLSGVCGVIDRLVVQTSSGPVRGRSVTVQGREVHVYTGIPYAKPPVEDL  80
DmAChE-like  1 MAISCRQSRVLPMSLPLPLTIPLPLVLVLSLHLSGVCGVIDRLVVQTSSGPVRGRSVTVQGREVHPY---PRGSSSLESL  77

DmAChE      81 RFRKPVPAEPWHGVLDATRLSATCVQERYEYFPGFSGEEIWNPNTNVSEDCLYINVWAPAKARLRHGRGANGGEHPNGKQ 160
DmAChE-like 78 L---------------------------------------------------------------------------- 78

DmAChE     161 ADTDHLIHNGNPQNTTNGLPILIWIYGGGFMTGSATLDIYNADIMAAVGNVIVASFQYRVGAFGFLHLAPEMPSEFAEEA 240
DmAChE-like    ----------------------------------------------------------------------------

DmAChE     241 PGNVGLWDQALAIRWLKDNAHAFGGNPEWMTLFGESAGSSSVNAQLMSPVTRGLVKRGMMQSGTMNAPWSHMTSEKAVEI 320
DmAChE-like    ----------------------------------------------------------------------------

DmAChE     321 GKALINDCNCNASMLKTNPAHVMSCMRSVDAKTISVQQWNSYSGILSFPSAPTIDGAFLPADPMTLMKTADLKDYDILMG 400
DmAChE-like    ----------------------------------------------------------------------------

DmAChE     401 NVDEGTYFLLYDFIDYFDKDDATALPRDKYLEIMNNIFGKATQAEREAIIFQYTSWEGNPGYQNQQQIGRAVGDHFFTCP 480
DmAChE-like    ----------------------------------------------------------------------------

DmAChE     481 TNEYAQALAERGASVHYYYFTHRTSTSLWGEWMGVLHGDEIEYFFGQPLNNSLQYRPVERELGKRMLSAVIEFAKTGNPA 560
DmAChE-like    ----------------------------------------------------------------------------

DmAChE     561 QDGEEWPNFSKEDPVYYIFSTDDKIEKLARGPLAARCSFWNDYLPKVRSWAGTCDGDSGSASISPRLQLLGIAALIYICA 640
DmAChE-like    ----------------------------------------------------------------------------

DmAChE     641 ALRTKRVF 648
DmAChE-like    --------
```

Fig. 4 Protein sequence alignment results of DmAChE and DmAChE-like.

Table 3 Physical and chemical properties of DmAChE-like

Physical and chemical properties	Predicted result
Formula	$C_{186}H_{313}N_{59}O_{59}$
Molecular weight	4319.9
Number of amino acids	40.0
Theoretical isoelectric points (pI)	10.6
Absorbance 0.1% (1 g/L)	0.3
Total number of negatively charged residues (Asp+Glu)	3.0
Total number of positively charged residues (Arg+Lys)	5.0
Grand average of hydropathicity (GRAVY)	−0.2
Instability index	37.0
Aliphatic index	99.5

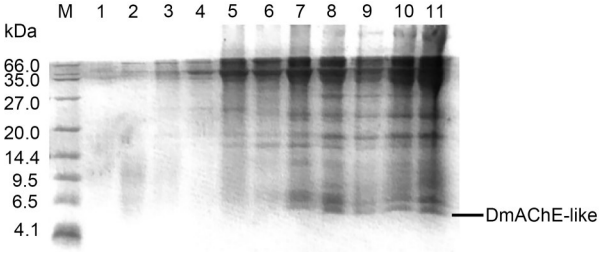

Fig. 5 Tricine-SDS-PAGE of GS115-pPIC9K-DmAChE-like. M: small molecular weight standard protein marker; Lanes 1–11: the activity of GS115-pPIC9K-DmAChE-like protein in fermentation supernatant samples numbered 1–11 was measured at 0, 12, 24, 36, 48, 60, 72, 84, 96, 108, and 120 h, respectively; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

ability to tolerate alkaline environments is improved, and has an increased optimum pH of 8.0 instead of 7.4. When the temperature was changed from 25 to 75 °C, the activity of DmAChE-like showed a trend of rising first and then decreasing (Fig. 8). It reached its peak value when the temperature was 37 °C. At T>50 °C, the rate of decline of enzyme activity became immediately clear. Similarly to DmAChE, DmAChE-like was basically inactive at temperatures above 75 °C.

3.5 Detection of organophosphorus and carbamate pesticides using DmAChE-like

With the aim to study the sensitivity of DmAChE-like to organophosphorus pesticides, we compared the sensitivity of DmAChE with that of DmAChE-like to five organophosphorus pesticides. The enzyme activity determination and inhibition analyses were performed as described in the experimental part. As shown in Fig. 9, DmAChE-like was more easily inhibited than DmAChE under the action of 1.0 mg/mL pesticide. Most pesticides inhibited DmAChE-like by over 50% at

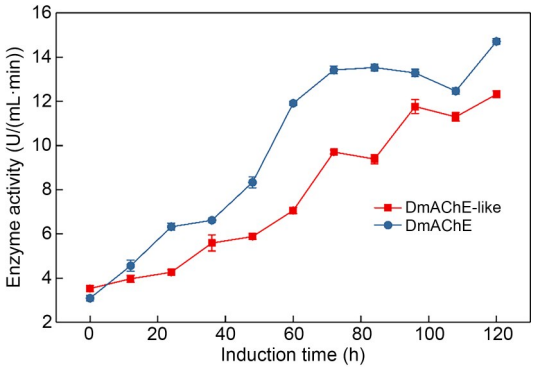

Fig. 6 Enzyme activity–time curve of fermentation supernatant of GS115-pPIC9K-*DmAChE-like*. Data are expressed as mean±standard deviation (*n*=3).

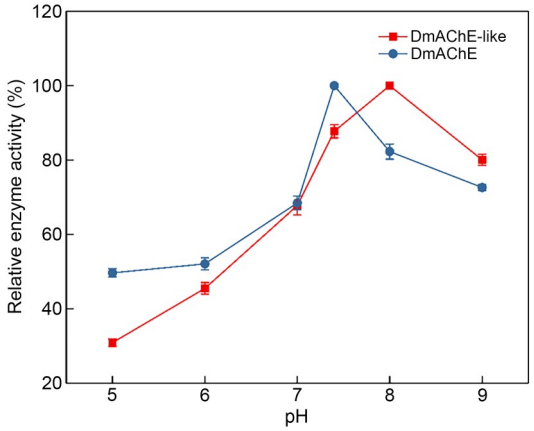

Fig. 7 Test of optimal pH for DmAChE-like. Data are expressed as mean±standard deviation (*n*=3).

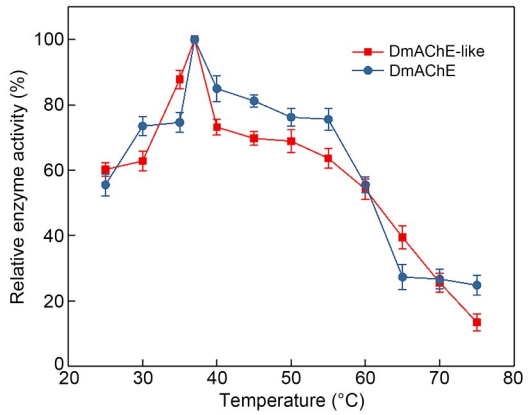

Fig. 8 Test of optimal temperature for DmAChE-like. Data are expressed as mean±standard deviation (*n*=3).

0.1 mg/mL. In addition, DmAChE and DmAChE-like showed different sensitivities to organophosphorus pesticides. As the concentration of carbofuran decreased, the sensitivities of both became more consistent. For the remaining four organophosphorus pesticides,

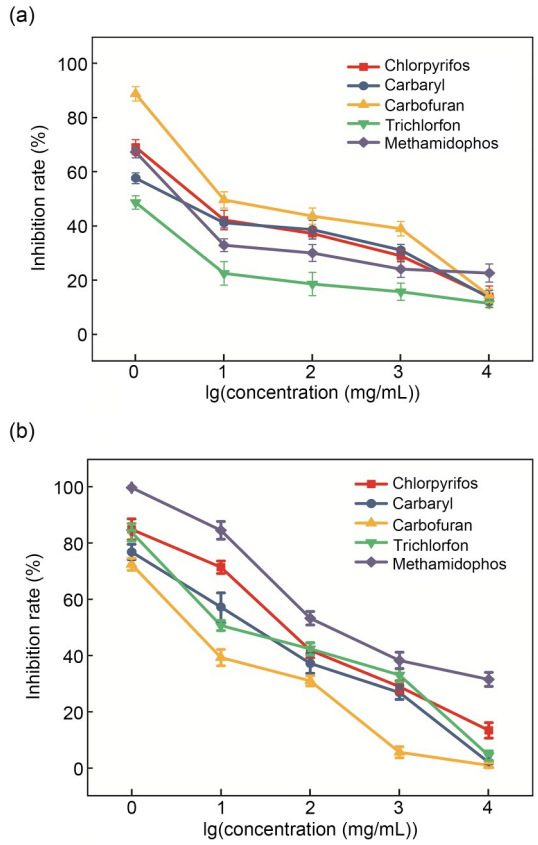

Fig. 9 Curves of DmAChE (a) and DmAChE-like (b) inhibition by organophosphorus pesticides. Data are expressed as mean±standard deviation (*n*=3).

the new splice variant was more sensitive than DmAChE along a decreasing concentration gradient. At the same time, the selectivity between the two enzymes to different organophosphorus pesticides was also different. The order of DmAChE sensitivity to five pesticides was: carbofuran>chlorpyrifos>methamidophos>carbaryl>trichlorfon. The order of DmAChE-like sensitivity to the five pesticides was: methamidophos>chlorpyrifos>carbaryl>trichlorfon>carbofuran. This difference may be attributed to the altered structure of DmAChE-like, leading to changes in the mechanism of its interaction with organophosphorus pesticides.

In summary, DmAChE-like showed good sensitivity for the determination of most selected organophosphorus pesticides. This also showed that a further experimental optimization of DmAChE-like expressed by *P. pastoris* is expected to meet the requirements of pesticide detection and provide a new source of enzyme for routine organophosphorus pesticide monitoring. Currently, only the activity of DmAChE-like has

been verified by the present work; however, subsequent studies are planned to focus on the molecular structure of this splice variant and the interaction mechanism between DmAChE-like and organophosphorus pesticides, thereby laying the foundations of methods to improve enzyme activity.

4 Discussion

Detection methods used for organophosphorus pesticides currently include chromatography, biosensing, and enzyme inhibition. Among these, chromatography and biosensing have higher sensitivity. As shown in Table 4, the use of biosensors in the detection of low concentration organophosphorus pesticides features a good linear relationship, can achieve lower detection limits, and has high sensitivity and selectivity. The enzyme inhibition method mainly uses spectrophotometry to quantify organophosphorus pesticides. This approach has a wider detection range and can meet the organophosphorus pesticide detection requirements (Table 4). In addition, it also has the advantages of low detection cost and fast detection speed, providing the advantage of practicality for on-site pesticide testing.

Furthermore, we used the *P. pastoris* expression system to induce GS115-pPIC9K-*DmAChE-like* expression. After detecting the enzyme activity of the expressed product, we confirmed that DmAChE-like indeed presents AChE activity. In the follow-up pesticide susceptibility experiments, we selected five commonly used pesticides for pesticide susceptibility testing, which proved the sensitivity and selectivity of DmAChE-like to these pesticides. Although its measured sensitivity was not very high, DmAChE-like can be tentatively used as a new enzyme source for pesticide residue detection upon further improving its activity and optimizing detection methods.

The yeast expression system selected for this study is an eukaryotic expression system, which has the advantages of fast reproduction, simple nutritional requirements, and easy industrial large-scale fermentation production (Zhou et al., 2015). In addition, it is capable of a variety of post-translational modifications to proteins in order to help maintain the activity and stability of biological products. In this paper, the original sequence was optimized by codons to make it more suitable for expression in *P. pastoris*. Nevertheless, the expression level of *DmAChE-like* is still low, and ways to effectively increase this level remain a challenge. The expression level of *DmAChE-like* obtained in *P. pastoris* can be improved by increasing the number of gene copies and optimizing fermentation conditions in subsequent work.

In addition, we only tested and verified the enzyme activity of DmAChE-like in this experiment. Further work will focus on the molecular structure of this splice variant and study the interaction mechanism between DmAChE-like and organophosphorus pesticides, to lay the foundations of molecular transformation for the improvement of enzyme activity.

5 Conclusions

The genetically engineered GS115-pPIC9K-*DmAChE-like* strain was constructed in the present study with gene expression induced at the laboratory level. The enzyme activity of the respective fermentation supernatant was tested, which proved that DmAChE-like has AChE activity. A specific protein band was found to be 4.3 kDa by tricine-SDS-PAGE,

Table 4 Performance of various biosensors in the detection of organophosphorus pesticides (OPs)

Detection method	Substrate	Linear range	Limit of detection	Reference
Spectrophotometric	Paraoxon	0.5–150.0 μmol/L	0.2 μmol/L	Tang et al., 2014a
	Parathion	1.0–200.0 μmol/L	0.4 μmol/L	
	Methyl parathion	2.5–200.0 μmol/L	1.0 μmol/L	
Electrochemical microbial biosensor	Paraoxon	0.05–25.00 μmol/L	9.0 nmol/L	Tang et al., 2014b
	Parathion	0.05–25.00 μmol/L	10.0 nmol/L	
	Methyl parathion	0.08–30.00 μmol/L	15.0 nmol/L	
Amperometric acetylcholinesterase biosensor	Paraoxon	0.001–5.000 μmol/L	0.7 nmol/L	Lang et al., 2016
	Dimethoate	0.005–1.000 μmol/L	3.9 nmol/L	
Spectrophotometric	Paraoxon	0.5–10.0 μg/mL	0.136 ng/mL	Liang et al., 2019
	Parathion	5.0–10.0 μg/mL	3.720 ng/mL	

which was consistent with the expected result. The pesticide susceptibility test found that DmAChE-like was sensitive to most selected organophosphorus pesticides, indicating that it can be used as a new enzyme source for the enzyme inhibition method for organophosphorus detection.

Acknowledgments

This work was supported by the National Development and Reform Commission of China (No. 20111158). We thank Wentao JING Engineer (Biological Experiment Teaching Center, College of Life Sciences, Zhejiang University, Hangzhou, China) for providing the RNA samples from the head of *Drosophila melanogaster*.

Author contributions

Liujia SHI performed the experimental research and data analysis, wrote and edited the manuscript. Fangfang YANG discovered the *DmAChE-like* gene and verified its function. Yanyan XU provided assistance for the experiment. Shoufeng WANG contributed to the study design, data analysis, and writing and editing of the manuscript. All authors have read and approved the final manuscript and, and therefore have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Liujia SHI, Fangfang YANG, Yanyan XU, and Shoufeng WANG declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

References

Albero B, Sánchez-Brunete C, Tadeo JL, 2003. Determination of organophosphorus pesticides in fruit juices by matrix solid-phase dispersion and gas chromatography. *J Agric Food Chem*, 51(24):6915-6921. https://doi.org/10.1021/jf030414m

Aldridge WN, Davison AN, 1953. The mechanism of inhibition of cholinesterases by organophosphorus compounds. *Biochem J*, 55(5):763-766. https://doi.org/10.1042/bj0550763

Amine A, Mohammadi H, Bourais I, et al., 2006. Enzyme inhibition-based biosensors for food safety and environmental monitoring. *Biosens Bioelectron*, 21(8):1405-1423. https://doi.org/10.1016/j.bios.2005.07.012

Apilux A, Isarankura-Na-Ayudhya C, Tantimongcolwat T, et al., 2015. Paper-based acetylcholinesterase inhibition assay combining a wet system for organophosphate and carbamate pesticides detection. *EXCLI J*, 14:307-319. https://doi.org/10.17179/excli2014-684

Chen DQ, Chen CHZ, Du D, 2010. Detection of organophosphate pesticide using polyaniline and carbon nanotubes composite based on acetylcholinesterase inhibition. *J Nanosci Nanotechnol*, 10(9):5662-5666. https://doi.org/10.1166/jnn.2010.2477

Duford DA, Xi YQ, Salin ED, 2013. Enzyme inhibition-based determination of pesticide residues in vegetable and soil in centrifugal microfluidic devices. *Anal Chem*, 85(16):7834-7841. https://doi.org/10.1021/ac401416w

Ellman GL, Courtney KD, Andres V, et al., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol*, 7(2):88-95. https://doi.org/10.1016/0006-2952(61)90145-9

Fournier D, Mutero A, 1994. Modification of acetylcholinesterase as a mechanism of resistance to insecticides. *Comp Biochem Physiol C Pharmacol Toxicol Pharmacol Endocrinol*, 108(1):19-31. https://doi.org/10.1016/1367-8280(94)90084-1

Goujon M, McWilliam H, Li WZ, et al., 2010. A new bioinformatics analysis tools framework at EMBL–EBI. *Nucleic Acids Res*, 38(suppl_2):W695-W699. https://doi.org/10.1093/nar/gkq313

Hossain MS, Sikder MT, 2015. Potential human health impacts and medical treatment of acute poisoning with organophosphorus pesticides (OPs): a review. *Int J Environ Prot Policy*, 3(2):6-13. https://doi.org/10.11648/j.ijepp.s.2015030201.12

Jokanović M, 2009. Medical treatment of acute poisoning with organophosphorus and carbamate pesticides. *Toxicol Lett*, 190(2):107-115. https://doi.org/10.1016/j.toxlet.2009.07.025

Koureas M, Tsakalof A, Tsatsakis A, et al., 2012. Systematic review of biomonitoring studies to determine the association between exposure to organophosphorus and pyrethroid insecticides and human health outcomes. *Toxicol Lett*, 210(2):155-168. https://doi.org/10.1016/j.toxlet.2011.10.007

Lang QL, Han L, Hou CT, et al., 2016. A sensitive acetylcholinesterase biosensor based on gold nanorods modified electrode for detection of organophosphate pesticide. *Talanta*, 156-157:34-41. https://doi.org/10.1016/j.talanta.2016.05.002

Li JQ, Ba Q, Yin J, et al., 2013. Surface display of recombinant *Drosophila melanogaster* acetylcholinesterase for detection of organic phosphorus and carbamate pesticides. *PLoS ONE*, 8(9):e72986. https://doi. org/10.1371/annotation/5c68b3d3-240f-4e87-b394-ca5301479cef

Liang B, Wang G, Yan L, 2019. Functional cell surface displaying of acetylcholinesterase for spectrophotometric sensing organophosphate pesticide. *Sens Actuators B Chem*, 279:483-489. https://doi.org/10.1016/j.snb.2018.09.119

Mai CQ, Chen S, Chen Y, 2017. Enzyme inhibition rate method for rapid detection of organophosphorus and carbamate pesticides in cowpea. *Plant Dis Pests*, 8(4):30-32. https://doi.org/10.19579/j.cnki.plant-d.p.2017.04.008

Nielsen H, Engelbrecht J, Brunak S, et al., 1997. Identification of prokaryotic and eukaryotic signal peptides and

prediction of their cleavage sites. *Protein Eng*, 10(1):1-6. https://doi.org/10.1093/protein/10.1.1

Pardo MF, Natalucci CL, 2002. Electrophoretic analysis (tricine-SDS-PAGE) of bovine caseins. *Acta Farm Bonaerense*, 21(1):57-60.

Petersen TN, Brunak S, von Heijne G, et al., 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat Methods*, 8(10):785-786. https://doi.org/10.1038/nmeth.1701

Ponomarev AS, Shtykov SN, 2000. Determination of pesticides and other physiologically active compounds by capillary gas chromatography with an atomic-emission detector without using standard reference materials. *J Anal Chem*, 55(1):47-51. https://doi.org/10.1007/BF02757632

Pope CN, 1999. Organophosphorus pesticides: do they all have the same mechanism of toxicity? *J Toxicol Environ Health B Crit Rev*, 2(2):161-181. https://doi.org/10.1080/109374099281205

Sakata M, 1993. Organophosphorus pesticides. *In*: Suzuki O, Watanabe K (Eds.), Drugs and Poisons in Humans. Springer, Berlin, Heidelberg. https://doi.org/10.1007/3-540-27579-7_60

Schägger H, 2006. Tricine-SDS-PAGE. *Nat Protoc*, 1(1):16-22. https://doi.org/10.1038/nprot.2006.4

Schulze H, Muench SB, Villatte F, et al., 2005. Insecticide detection through protein engineering of *Nippostrongylus brasiliensis* acetylcholinesterase B. *Anal Chem*, 77(18):5823-5830. https://doi.org/10.1021/ac050383p

Tang XJ, Liang B, Yi TY, et al., 2014a. Cell surface display of organophosphorus hydrolase for sensitive spectrophotometric detection of *p*-nitrophenol substituted organophosphates. *Enzyme Microb Technol*, 55:107-112. https://doi.org/10.1016/j.enzmictec.2013.10.006

Tang XJ, Zhang TT, Liang B, et al., 2014b. Sensitive electrochemical microbial biosensor for *p*-nitrophenylorganophosphates based on electrode modified with cell surface-displayed organophosphorus hydrolase and ordered mesopore carbons. *Biosens Bioelectron*, 60:137-142. https://doi.org/10.1016/j.bios.2014.04.001

Tougu V, 2001. Acetylcholinesterase: mechanism of catalysis and inhibition. *Curr Med Chem Cent Nerv Syst Agents*, 1(2):155-170. https://doi.org/10.2174/1568015013358536

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(3):291-307. https://doi.org/10.1016/j.chemosphere.2010.10.033

Villatte F, Marcel V, Estrada-Mondaca S, et al., 1998. Engineering sensitive acetylcholinesterase for detection of organophosphate and carbamate insecticides. *Biosens Bioelectron*, 13(2):157-164. https://doi.org/10.1016/S0956-5663(97)00108-5

Viñas P, Campillo N, López-García I, et al., 2003. Capillary gas chromatography with atomic emission detection for pesticide analysis in soil samples. *J Agric Food Chem*, 51(13):3704-3708. https://doi.org/10.1021/jf021106b

Worek F, Eyer P, Thiermann H, 2012. Determination of acetylcholinesterase activity by the ellman assay: a versatile tool for in vitro research on medical countermeasures against organophosphate poisoning. *Drug Test Anal*, 4(3-4):282-291. https://doi.org/10.1002/dta.337

Yang X, Dai J, Yang L, et al., 2018. Oxidation pretreatment by calcium hypochlorite to improve the sensitivity of enzyme inhibition-based detection of organophosphorus pesticides. *J Sci Food Agric*, 98(7):2624-2631. https://doi.org/10.1002/jsfa.8755

Yang XM, Gu YP, Wu SJ, et al., 2019. Research on a rapid detection method of pesticide residues in milk by enzyme inhibition. *E3S Web Conf*, 79:03013. https://doi.org/10.1051/e3sconf/20197903013

Zhou WJ, Yang JK, Mao L, et al., 2015. Codon optimization, promoter and expression system selection that achieved high-level production of *Yarrowia lipolytica* lipase in *Pichia pastoris*. *Enzyme Microb Technol*, 71:66-72. https://doi.org/10.1016/j.enzmictec.2014.10.007