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Production of a novel recombinant *Drosophila melanogaster* acetylcholinesterase for detection of organophosphate and carbamate insecticide residues

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Abstract

A novel recombinant *Drosophila melanogaster* acetylcholinesterase (R-*Dm*AChE) produced in *Pichia pastoris* was first reported in this study. We cloned the *Dm*AChE cDNA by reverse transcription PCR with removal of the signal for glycosylphosphatidylinositol (GPI) anchor attachment and the endogenous signal peptide coding sequence, and inserted it into *P. pastoris* vector pPIC9K under control of the alcohol oxidase gene *AOX1* promoter (5′ *AOX1*). The expression cassette of AChE cDNA was then introduced into methylotrophic yeast GS115 and several recombinant strains expressing R-*Dm*AChE were obtained. The secreted R-*Dm*AChE showed high stability in neutral phosphate buffer at 4 °C, and its kinetic parameters were identical to those of the native *Dm*AChE. The bimolecular rate constants of R-*Dm*AChE to dichlorvos, aldicarb and carbaryl were ranging from three to six times higher than of native *Dm*AChE. Within six insecticides, the R-*Dm*AChE was more sensitive than *Ee*AChE, *Nb*AChE and *Hu*AChE. For 10 widely used insecticides, the IC_{50} values to the R-*Dm*AChE were much lower than those to AChEs commonly used in China. With the R-*Dm*AChE-based assay, samples spiked with three concentrations of pesticides caused enzymatic activity inhibition with R.S.D. of 0–13.7%. These results suggest that the R-*Dm*AChE can be useful for detection of organophosphate and carbamate insecticide residues.

Keywords: *Drosophila melanogaster*; Acetylcholinesterase; *Pichia pastoris*; Organophosphate; Carbamate; Insecticide

1. Introduction

Organophosphate and carbamate compounds are broad-spectrum insecticides used for crop protection to control agricultural pests, such as insects, acarids, nematodes, and allied organisms. The primary toxicity of these compounds results from irreversible inhibition of a key nervous system enzyme, acetylcholinesterase (AChE, EC 3.1.1.7) (Yazal et al., 2001). The enzyme has aroused increasing interest in its application for detection of pesticide residues (Villatte et al., 1998; Boublik et al., 2002; Schulze et al., 2005), with the ultimate goal to ensure human, animal and environmental safety.

Routinely, the presence and concentrations of organophosphate and carbamate compounds are being determined by chromatographic methods (Fernández et al., 2001; Viñas et al., 2003; Albero et al., 2003; Sandra et al., 2003), such as gas chromatography or other combinations of methods, which are used as standard detection techniques. However, these techniques involve complicated procedures, which are expensive and time-consuming, and not suitable for rapid, immediate and large-scale sample analysis. AChE-based biosensing analytical methods have been previously

Abbreviations: *Dm*AChE, *Drosophila melanogaster* acetylcholinesterase; R-*Dm*AChE, recombinant *Dm*AChE; *Ee*AChE, *Electrophorus electricus* acetylcholinesterase; *Md*AChE, *Musca domestica* acetylcholinesterase; *Nb*AChE, *Nippostrongylus brasiliensis* acetylcholinesterase; *Hu*AChE, human acetylcholinesterase; R.S.D., relative standard deviation

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developed and play a key role in detection of pesticide residues in food and environmental samples (Bachmann et al., 2000; Bachmann and Schmid, 1999; Devic et al., 2002; Schulze et al., 2002, 2004; Zhang et al., 2005; Sotiropoulou and Chaniotakis, 2005). Rapid assays using AChE-based kits and test strips have been considered as efficient and reliable methods for qualitative detection especially in developing countries. In China, the AChE inhibition assay is legislated as standard method (Wang et al., 2003) and has been extensively used in practice, especially for detection of pesticide residues in vegetables and fruits. Hence, the availability of AChE with high sensitivity to pesticides is crucial for such analysis.

Currently, AChE proteins applied in China are generally produced from some houseflies and the electric eel. The insect-derived material may have high sensitivity with genetic breeding, but each fly produces a tiny amount of the enzyme, which is not appropriate for commercialization. Although electric eel is the most popular source of this protein with sufficient production volume, the enzyme lacks wide sensitivity to the multitude of AChE-inhibiting pesticides (Schulze et al., 2005). Herewith using the current commercially available AChEs for detection of insecticide residues have led to the drawbacks of either low sensitivity or instability, resulting in false negative detection or poor repeatability in practical use. There is a need for greater sensitivity and specificity, and convenient and safe procedures of producing AChEs with lower expense. With the development of genetic engineering techniques, recombinant AChEs have been successfully expressed in baculovirus, yeast, plant, *Xenopus* oocytes and mammalian cells (Duval et al., 1992; Mutero and Fournier, 1992; Morel and Massoulié, 1997; Simon and Massoulié, 1997; Estrada-Mondaca and Fournier, 1998; Mendelson et al., 1998; Hussein et al., 1999, 2000; Mor et al., 2001; Uccelletti et al., 2002). Specifically, *P. pastoris* being methylotrophic yeast, taking advantage of high cell mass yield, high protein content and stable fermentation characteristics were used to produce secreted AChEs from the parasitic nematode *Nippostrongylus brasiliensis* (Juorgen et al., 1998; Hagenson, 1991; Sven et al., 2006) and rat (Gopalakrishna et al., 1980). And wild-type or mutant *Drosophila melanogaster* AChE (*Dm*AChE) has been proved to be more sensitive against a broad range of organophosphate and carbamate insecticides than other ones (Villatte et al., 1998; Boublik et al., 2002; Schulze et al., 2005). However, the source of *Dm*AChE is not suitable for production of sufficient enzyme for application.

Overall objective in this study is to develop a novel recombinant *Dm*AChE for easy and low-cost preparation, and for producing sensitive and stable enzyme for detection of organophosphate and carbamate insecticide residues. The R-*Dm*AChE was heterologously expressed in *P. pastoris*, secreted into the culture medium and extracted. We proved that the R-*Dm*AChE possessed significantly higher sensitivity to the broadly used insecticides in China than other available AChEs. This assay could be an ideal tool for detection of insecticides in agricultural products.

2. Materials and methods

2.1. Materials

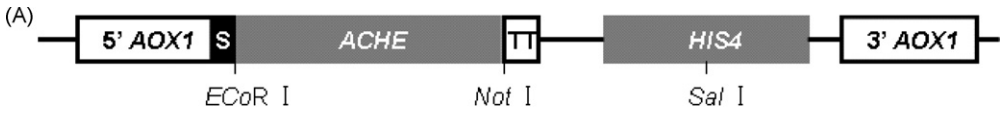
Enzymes and kits for molecular biology were purchased from Invitrogen (CA, USA), Takara (Dalian, China), and Fermentas (MD, USA). Oligonucleotides were synthesized by Invitrogen (Shanghai, China). *Musca domestica* acetylcholinesterase (*Md*AChE) was from Shandong Jingpeng Co., China (Shandong, China). *Electrophorus electricus* acetylcholinesterase (*Ee*AChE) was obtained from Sigma–Aldrich (Product No. C2888, MO, USA). Acetylthiocholine iodide (ATChI), 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), 1,5-Bis (4-allyldimethylammoniumphenyl) pentan-3-one dibromide (BW284c51) and tetraisopropyl pyrophosphoramide (iso-OMPA) were also purchased from Sigma–Aldrich. Insecticides including dichlorvos (CAS No. 62-73-7), omethoate (CAS No. 1113-02-6), paraoxon (CAS No. 311-45-5), paraoxon methyl (CAS No. 950-35-6), aldicarb (CAS No. 116-06-3), carbaryl (CAS No. 63-25-2), carbofuran (CAS No. 1563-66-2), pirimicarb (CAS No. 23103-98-2), monocrotophos (CAS No. 6923-22-4), phorate (CAS No. 298-02-2), propoxur (CAS No. 114-26-1), pirimiphosmethyl (CAS No. 29232-93-7), malathion (CAS No. 121-75-5), triazophos (CAS No. 24017-47-8), acephate (CAS No. 30560-19-1), isoprocarb (CAS No. 2631-40-5), tsumacide (CAS No. 1129-41-5), and methomyl (CAS No. 16752-77-5) were purchased from the China standardization service company (Beijing, China), with a minimum certified purity of 97.7%. Stock solution of each insecticide was prepared at concentration of 100 μg/mL. Other reagents and analytical grade chemicals were obtained from local suppliers.

2.2. Cloning of DmAChE cDNA fragment and the expression vector construction

The experiment was carried out as follows. The total RNA of a strain of *Drosophila melanogaster* provided by the Institute of Genetics, Fudan University (China) was isolated with the TRIZOL® Reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. The first-strand cDNA synthesis was promoted with oligo-dT using RevertAid™ Kits (Fermentas). The primers for amplification were designed according to the *Dm*AChE cDNA sequence (NM_057605). The forward primer (5′-ag*aattc*gtcatcgatcgcctggtcgtgcag-3′) tailored to the 8 N-terminal amino acids (VIDRLVVQ) of *Dm*AChE with removal of the signal peptide, containing a *Eco*R I restriction site italicized, and the reverse primer (5′-a*gcggccgc*ttattacgacgaatcgccatcgcaagtacctgc-3′) based on the complementary sequence of the C-terminal amino acids (AGTCDGDSS) excluding a GPI-anchored segment, linking to a *Not* I restriction site italicized. The amplified AChE cDNA fragment was cloned to T-vector and sequenced for confirmation. The cDNA fragment was cut with *Eco*R I and *Not* I, then the resulting 1752 bp DNA fragment was purified and ligated to the *Eco*R I–*Not* I digested *P. pastoris* expression vector pPIC9K (Invitrogen), constructing the plasmid pPIC9K/*Dm*AChE (Fig. 1). The reading frame was verified by partial sequencing of the recombinant plasmid, which was then linearized with *Sal* I for transformation.

2.3. Expression of R-DmAChE in P. pastoris

The GS115 *P. pastoris* strain (Invitrogen) was used for host strain. The linearized plasmid pPIC9K/*Dm*AChE was integrated into competent yeast cells via homologous recombination, which was performed by electroporation in MicroPulser™ electroporator (Bio-Rad Laboratories Inc., CA, USA) according to the manufacturer's instructions. Each colony on MD plate [1.34% (w/v) yeast nitrogen base, 4×10^{-5}% (w/v) biotin, 2% (w/v) dextrose] was picked and patched on MM plate [1.34% (w/v) yeast nitrogen base, 4×10^{-5}% (w/v) biotin, 0.5% (v/v) methanol]. The plates were incubated at 30 °C for 2 days. Transformants secreting AChE with high activity were obtained according to the method of Ellman et al. with modifications. On MM plate, each colony was covered with 20 μL of screening buffer (10 mM ATChI, 7.8 mM DTNB, 50 mM sodium phosphate, pH 7.6, 0.1% agar). After incubation at 25 °C for 5 min, transformants presenting deeper yellow color on and around their own colonies were selected for fermentation (Morel and Massoulié, 1997).



(B)

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GTCATCGATCGCCTGGTCGTGCAGACATCCTCCGGACCTGTACGCGGTCGCTCCGTGACGGTGCAGGGCAGGGAG   75
 V  I  D  R  L  V  V  Q  T  S  S  G  P  V  R  G  R  S  V  T  V  Q  G  R  E
GTGCATGTCTACACGGGCATCCCCTACGCCAAGCCGCCCGTCGAGGACCTGCGCTTCCGAAAGCCGGTTCCCGCG   150
 V  H  V  Y  T  G  I  P  Y  A  K  P  P  V  E  D  L  R  F  R  K  P  V  P  A
GAGCCATGGCACGGCGTCCTCGACGCCACGCGGTTATCCGCCACCTGCGTCCAAGAGCGTTACGAGTACTTCCCC   225
 E  P  W  H  G  V  L  D  A  T  R  L  S  A  T  C  V  Q  E  R  Y  E  Y  F  P
GGCTTCTCCGGCGAGGAGATCTGGAACCCCAACACCAACGTGTCCGAGGACTGCCTCTACATAAATGTCTGGGCG   300
 G  F  S  G  E  E  I  W  N  P  N  T  N  V  S  E  D  C  L  Y  I  N  V  W  A
CCGGCAAAGGCCCGACTTCGCCATGGTCGGGGTGCCAACGGGGGTGAGCACCCCAATGGCAAACAGGCGGACACT   375
 P  A  K  A  R  L  R  H  G  R  G  A  N  G  G  E  H  P  N  G  K  Q  A  D  T
GACCATCTCATCCACAACGGAAATCCGCAGAACACGACCAACGGACTGCCGATTCTGATCTGGATCTATGGCGGT   450
 D  H  L  I  H  N  G  N  P  Q  N  T  T  N  G  L  P  I  L  I  W  I  Y  G  G
GGCTTCATGACCGGATCGGCCACCCTGGACATCTACAATGCGGATATCATGGCCGCCGTGGGCAATGTAATAGTG   525
 G  F  M  T  G  S  A  T  L  D  I  Y  N  A  D  I  M  A  A  V  G  N  V  I  V
GCCTCCTTCCAGTATCGAGTGGGAGCCTTTGGGTTCTTGCACCTGGCGCCGGAAATGCCGTCGGAGTTTGCGGAA   600
 A  S  F  Q  Y  R  V  G  A  F  G  F  L  H  L  A  P  E  M  P  S  E  F  A  E
GAGGCGCCCGGCAATGTGGGCCTATGGGATCAGGCACTCGCCATTCGCTGGCTGAAGGACAACGCTCATGCCTTC   675
 E  A  P  G  N  V  G  L  W  D  Q  A  L  A  I  R  W  L  K  D  N  A  H  A  F
GGCGGAAATCCGGAGTGGATGACACTGTTCGGAGAGTCGGCTGGATCCAGTTCGGTGAATGCCCAGCTCATGTCG   750
 G  G  N  P  E  W  M  T  L  F  G  E  S  A  G  S  S  S  V  N  A  Q  L  M  S
CCGGTGACGAGGGGTCTGGTCAAGCGCGGAATGATGCAGTCGGGCACTATGAACGCCCCCTGGAGCCACATGACC   825
 P  V  T  R  G  L  V  K  R  G  M  M  Q  S  G  T  M  N  A  P  W  S  H  M  T
TCCGAGAAGGCCGTGGAGATCGGCAAGGCGCTGATCAACGACTGCAACTGCAATGCATCTATGCTGAAGACCAAT   900
 S  E  K  A  V  E  I  G  K  A  L  I  N  D  C  N  C  N  A  S  M  L  K  T  N
CCCGCTCACGTGATGAGCTGCATGCGTTCCGTGGACGCCAAGACCATATCGGTGCAGCAGTGGAACTCCTACTCG   975
 P  A  H  V  M  S  C  M  R  S  V  D  A  K  T  I  S  V  Q  Q  W  N  S  Y  S
GGCATCCTCAGCTTTCCCTCGGCGCCCACCATTGATGGTGCGTTCCTGCCGGCGGATCCCATGACGCTGATGAAG  1050
 G  I  L  S  F  P  S  A  P  T  I  D  G  A  F  L  P  A  D  P  M  T  L  M  K
ACGGCGGATCTGAAGGACTACGACATCCTGATGGGAAATGTCAGGGATGAGGGCACTTACTTCTTGCTGTACGAT  1125
 T  A  D  L  K  D  Y  D  I  L  M  G  N  V  R  D  E  G  T  Y  F  L  L  Y  D
TTCATCGATTACTTCGATAAGGACGATGCCACGGCCCTGCCACGGGACAAATACCTGGAAATTATGAACAATATT  1200
 F  I  D  Y  F  D  K  D  D  A  T  A  L  P  R  D  K  Y  L  E  I  M  N  N  I
TTTGGCAAGGCAACGCAAGCGGAACGCGAGGCCATCATTTTCCAGTATACCAGTTGGGAAGGCAATCCTGGCTAT  1275
 F  G  K  A  T  Q  A  E  R  E  A  I  I  F  Q  Y  T  S  W  E  G  N  P  G  Y
CAGAACCAGCAGCAAATCGGACAGCGCCGTGGGCGATCACTTCTTCACCTGCCCCACCAACGAGTATGCCCAGGCT  1350
 Q  N  Q  Q  Q  I  G  R  A  V  G  D  H  F  F  T  C  P  T  N  E  Y  A  Q  A
CTGGCGGAGCGAGGCGCTTCCGTGCACTACTACTACTTTACACACCGCACAAGCACCTCATTGTGGGGCGAATGG  1425
 L  A  E  R  G  A  S  V  H  Y  Y  Y  F  T  H  R  T  S  T  S  L  W  G  E  W
ATGGGCGTGCTGCACGGCGATGAGATCGAATACTTCTTTGGCCAGCCGCTGAACAACTCCCTGCAGTATCGACCT  1500
 M  G  V  L  H  G  D  E  I  E  Y  F  F  G  Q  P  L  N  N  S  L  Q  Y  R  P
GTGGAGCGTGAGCTGGGCAAGCGTATGCTCAGTGCGGTCATCGAGTTTGCTAAGACGGGAAATCCCGCTCAGGAT  1575
 V  E  R  E  L  G  K  R  M  L  S  A  V  I  E  F  A  K  T  G  N  P  A  Q  D
GGCGAGGAGTGGCCCAACTTCTCCAAGGAGGATCCCGTCTACTATATTTTCAGCACCGACGATAAGATCGAGAAA  1650
 G  E  E  W  P  N  F  S  K  E  D  P  V  Y  Y  I  F  S  T  D  D  K  I  E  K
TTGGCCAGGGGTCCTTTGGCGGCTCGCTGCTCGTTCTGGAATGATTACTTGCCAAAAGTCAGGAGTTGGGCAGGT  1725
 L  A  R  G  P  L  A  A  R  C  S  F  W  N  D  Y  L  P  K  V  R  S  W  A  G
ACTTGCGATGGCGATTCGTCGTAA                                                      1749
 T  C  D  G  D  S  S  STOP
```

Fig. 1. (A) Schematic representation of the construct used for expression of *Dm*AChE in *P. pastoris*. The vector pPIC9K/*Dm*AChE carrying the *AOX1* promoter (5′ *AOX1*), the *AOX1* transcription termination region (TT), the sequence further downstream of *AOX1* (3′ *AOX1*), and the *HIS4* gene. The α-factor was used as a secretion signal (S), upstream of the insert AChE gene. The restriction site (*Sal* I) used for linearization of the vector before transformation is also indicated. (B) Nucleotide and derived amino acid sequence of cDNA encoding the secreted *Dm*AChE (NM_057605).

Recombinant yeast cells were grown in 100 mL of BMGY medium [1% yeast extract, 2% peptone, 0.1 M potassium phosphate, pH 6.0, 1.34% (w/v) yeast nitrogen base, 4×10^{-5}% (w/v) biotin, 1% (v/v) glycerol] by shaking (200 rpm, 28 °C) for approximately for 26 h to OD_{600} of 5.0. Cell pellets were resuspended to an OD_{600} of 1.0 in BMMY medium (same as BMGY, but glycerol was replaced by 0.5% methanol), buffered at pH 7.6 to induce AChE expression on a shaker (200 rpm, 28 °C). Pure methanol (0.5%, v/v) was added once per 24 h to maintain induction, and an aliquot of the culture was removed to monitor the expression level of AChE. The cultures were collected after 72 h fermentation post-induction at the optimal harvest time for further analyses.

2.4. Isolation of R-DmAChE

The yeast culture supernatants were clarified by centrifugation (7000 × *g*, 4 °C). The ammonium sulfate precipitation procedure for concentrating and purifying the recombinant enzyme was to prepare the 40–80% fraction. The fraction was dissolved in 50 mM sodium phosphate, pH 7.6 and dialyzed against the same buffer overnight at 4 °C (Ellman et al., 1961). Native PAGE was carried out on 8% polyacrylamide gels in the absence of sodium dodecyl sulfate (SDS). Enzyme activity was visualized by the method of Karnovsky and Roots (1964) using ATChI as the substrate.

2.5. Characterization of R-DmAChE

AChE activity was determined with 1 mM ATChI as substrate in the presence of 1 mM DTNB in 50 mM sodium phosphate with the optimum pH value, which was investigated using 0.1 M phosphate buffer solutions at pH 5.8–8.0 with the interval of 0.2. The tests were performed by triplicates in 250 μL solution containing 40 U R-*Dm*AChE, 1 mM DTNB and 1 mM ATChI at each pH value. The enzymatic activity was monitored by measuring the absorbance changes at 412 nm in 1 min at 25 °C using an ELx800UV automated microplate reader (Biotek Instruments Inc., VT, USA).

Hydrolysis of ATChI was calculated from the extinction coefficient of DTNB, 13,600 M^{-1} cm^{-1}. One unit was defined as 1 nmol of ATChI hydrolyzed per min at 25 °C.

For determination of the Michaelis constant (K_m), assays were performed at 25 °C in 50 mM phosphate buffer (pH 7.6), containing 0.5 mM DTNB, and with at least 10 concentrations of ATChI ranging from 1 to 50 μM below substrate inhibition. The kinetic parameter was deduced from the non-linear representation of Michaelis–Menten equation with the GraphPad Prism 4 software program (GraphPad Software Inc., CA, USA). The constant of inhibition by excess substrate (K_{ss}), was determined using the Haldane equation (Rosenfeld et al., 2001) with substrate concentrations ranging from 1 μM to 50 mM.

2.6. Sensitivity analysis of R-DmAChE against the inhibitors

The generally accepted inhibition mechanism of AChE by organophosphates and carbamates has been described by Aldridge (1950). The bimolecular rate constants (K_i) for insecticide was determined in the 50 mM phosphate buffer (pH 7.6), using four concentrations of each insecticide in the range of 0.01–100 μg/mL, depending on the insecticide. Insecticides were previously dissolved in acetone and then diluted with water. The maximum acetone concentration in reaction buffer was 1%. AChE was incubated in 50 mM phosphate buffer (pH 7.6) at 25 °C with the inhibitor for various times before addition of 1 mM ATChI. A plot of the natural logarithm of residual activity ($[E]/[E_0]$) versus time was linear for a given inhibitor concentration. K_i values were obtained by dividing the slope of the curve by the concentration. This variation followed a pseudo-first order, In $[E]/[E_0] = -K_i[I]t$ (Villatte et al., 1998).

The value of IC_{50} of each insecticide was determined from the inhibition curve as 50% AChE activity inhibited. The enzyme sample was incubated with the inhibitor for 60 min at 25 °C. The residual AChE activity was assayed according to the method mentioned above and plotted as a function of the concentration of inhibitor. The experiment was repeated three times with duplicates for each concentration.

2.7. R-DmAChE-based assay of practical samples

The R-*Dm*AChE-based assay was developed and applied to detect insecticide residues in vegetable samples. Sample extract (2.5 mL) was incubated with 0.1 mL of R-*Dm*AChE solution for 15 min at room temperature. 1 mM ATChI was used to assay the residual AChE activity (E). The inhibition rate was deduced from this formula, $(E_0 - E)/E_0 \times 100\%$. E_0 was the initial AChE activity obtained with 2.5 mL of 50 mM phosphate buffer (pH 7.6) instead of sample extract. Spiked samples were prepared by adding 10 mL of the pesticide working solution to 5 g of vegetable. The pesticide levels ranged from 0.01 to 2 mg/kg, depending on its inhibitive potency. The solvent was evaporated at room temperature for 4 h. The sample was immerged in 10 mL of 50 mM phosphate buffer (pH 7.6) and extracted for 15 min in an ultrasonic bath. After a brief settling period, the clear extract was removed for AChE-based assay.

2.8. GC-NPD analysis

Capillary gas chromatography (GC) was performed on a 30 m × 0.32 mm i.d., 0.25 μm HP-1 column (Agilent, Waldbronn, Germany), equipped with a nitrogen–phosphorus detector (NPD). The injection port and detector working temperatures were 260 and 270 °C, respectively, with nitrogen as the carrier gas at a flow rate of 1.0 mL/min. The oven working temperature was 240 °C, and the injection volumes were 3 μL. The constant flow rates of hydrogen and air were 100.0 and 50.0 mL/min, respectively. The concentration of each compound in tested samples was determined by comparing the peak areas obtained in the samples with the standards. Chromatographic standards were prepared by spiking blank samples with known amounts of pesticides. The extraction procedure selected was based on a method previously applied for the determination of organophosphorus pesticides in vegetables by GC analysis (Sasaki et al., 1987).

2.9. Statistical analysis

All the experiments on obtaining data were operated by triplicates. The data in parallel were generally statistically analyzed by relative standard deviation (R.S.D.).

3. Results

3.1. Cloning of DmAChE and the expression vector construction

*Dm*AChE is a globular protein of 150 kDa, attached to the membrane via a glycosylphosphatidylinositol (GPI) anchor in the C-terminal hydrophobic peptide (Fournier et al., 1988). *P. pastoris* has the ability to correctly process a C-terminal GPI-anchor signal, and anchor the target protein into the membrane (Morel and Massoulié, 1997). To prevent the addition of the glycolipid anchor characteristic of wild-type enzyme, we amplified the truncated *Dm*AChE cDNA without both the N-terminal signal peptide sequence (amino acids position 1–38) and the GPI anchoring sequence (amino acids position 620–649) using RT-PCR. Sequencing result of the cloned fragment indicated that the truncated *Dm*AChE cDNA nucleotide sequence was identical to the published *Dm*AChE sequence (NM_057605). The plasmid pPIC9K/*Dm*AChE was constructed using yeast α-factor secretion signal under control of the promoter of alcohol oxidase gene (*AOX1*) in order to induce the expression of *Dm*AChE secreted in the culture medium by methanol. The general construct of pPIC9K/*Dm*AChE was shown in Fig. 1.

3.2. Screening of yeast strains with high R-DmAChE expression level

HIS4 gene presented on expression vector allowed selection of transformants by auxotrophic growth without histidine. Linearization with *Sal* I promoted integration into the *his4* locus, generating Mut^+ transformants. Mut^+/His^+ transformants secreting AChE were selected by semi-quantitatively comparing the AChE activity of colonies on MM plates. Colonies failing to change the color of the screening buffer were deemed that could not express recombinant AChE. Several colonies presented much deeper yellow color after incubation with screening buffer for the same time, implying that these transformants possessed high AChE activity. To quantify the expression level of R-*Dm*AChE, we cultured these strains in liquid medium, and induced the expression of recombinant enzyme with addition of methanol. Seven strains were selected among about 1000 transformants with the ability to produce *Dm*AChE highly to 180 units/mg of total protein. These strains were reserved for enzyme production and purification. On native PAGE, a single band of AChE activity was visualized for the culture medium of induced recombinants purified by ammonium sulfate fractionation (Fig. 2A), with the control of *Ee*AChE (Fig. 2B).

3.3. Enzymic characterization of R-DmAChE

AChE is a glycoprotein, and the nature of glycans added by different cells may influence catalytic properties. To understand this point, we analyzed the K_m value of the R-*Dm*AChE expressed by *P. pastoris* (Fig. 3), which was close to those of both the corresponding natural enzyme (17.3 ± 3.1 μM) and

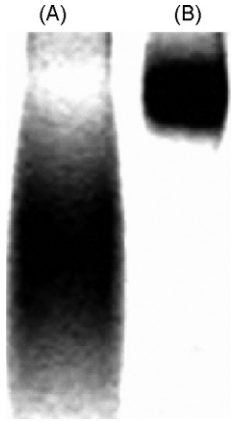

Fig. 2. Analysis of AChE activity by native PAGE. Visualization of enzyme activity was carried out using the Karnovsky–Roots method (1964), with ATChI as the substrate. (A) R-*Dm*AChE and (B) *Ee*AChE.

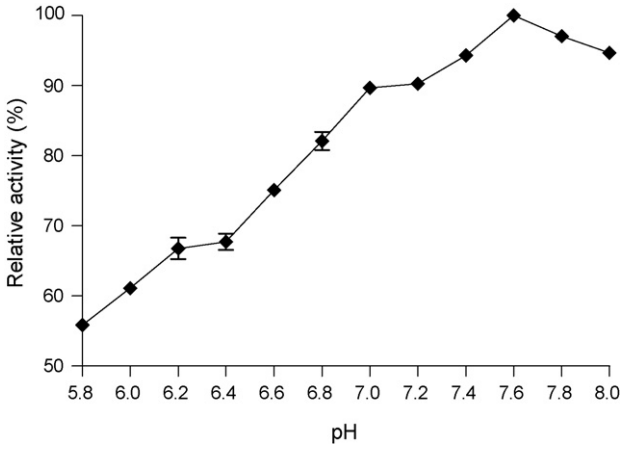

Fig. 4. The influence of various pH values on the enzymatic activity of the R-*Dm*AChE.

the excreted protein expressed in *Xenopus* oocytes (11.4 ± 1.3 μM) (Mutero and Fournier, 1992). Compared with recombinant *Ee*AChE (K_m = 104 ± 10 μM) (Simon and Massoulié, 1997) and *Nippostrongylus brasiliensis* acetylcholinesterase (*Nb*AChE) (K_m = 230 ± 100 μM) (Hussein et al., 1999), the R-*Dm*AChE presented higher affinity to the substrate ATChI. Furthermore, we analyzed the inhibition property of the excessive substrate to R-*Dm*AChE. The K_{ss} value of the R-*Dm*AChE was 50.9 ± 2.8 mM.

Our observation on the enzymatic activity of R-*Dm*AChE in different pH conditions and storage temperatures indicated that the optimum pH value was at 7.6 (Fig. 4) and the enzyme activity remained stable at 4 °C, with only 25% of activity loss after 22 days (Fig. 5). Correspondingly, the activities of the commercially available *Ee*AChE and *Md*AChE were greatly reduced when the enzyme solutions stored at 4 °C after 15 days. For purposes of insecticide residue detection, the recombinant enzymes derived from fermentation should be especially useful with perfect retention of activity and lower costs for partial purification with ammonium sulfate.

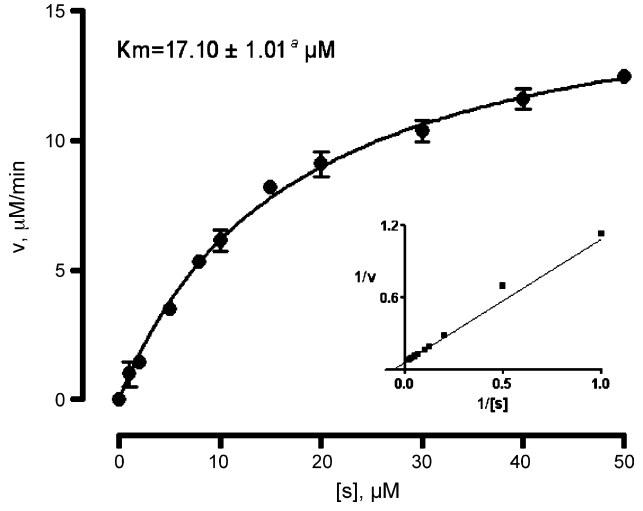

Fig. 3. The substrate-velocity and Lineweaver–Burk plots with calculated K_m value of the R-*Dm*AChE. [a]The value represents the mean from three different experiments.

We tested the K_i values of R-*Dm*AChE to eight insecticides, and our results indicated that the R-*Dm*AChE had similar sensitivity with native *Dm*AChE to omethoate, paraoxon, paraoxon methyl, carbofuran, and pirimicarb (Table 1). Interestingly, the K_i values of R-*Dm*AChE were three to nine times higher than those of native one to dichlorvos, aldicarb and carbaryl, possibly deriving from the conformational change of R-*Dm*AChE post-translationally modified by *P. pastoris*.

The constants of R-*Dm*AChE were higher than of *Ee*AChE, *Nb*AChE and human acetylcholinesterase (*Hu*AChE) to dichlorvos, omethoate, paraoxon methyl, carbaryl, carbofuran and pirimicarb. *Nb*AChE and *Hu*AChE had the similar sensitivity with R-*Dm*AChE to paraoxon. R-*Dm*AChE was less sensitive than *Ee*AChE for aldicarb (Table 1).

The butyrylcholinesterase-specific inhibitor iso-OMPA did not markedly inhibit these three enzymes at concentrations up to 300 μM (Table 2), indicating there was no butyrylcholinesterase activity in the prepared enzyme samples. For the AChE-specific inhibitor BW284c51, the R-*Dm*AChE and *Md*AChE possessed similar IC_{50} values, three- to four-fold compared to that of *Ee*AChE. Two bulky side chains of phenylalanine residues present at the acyl-binding pocket of vertebrate AChEs whereas the invertebrate AChEs possess only a single

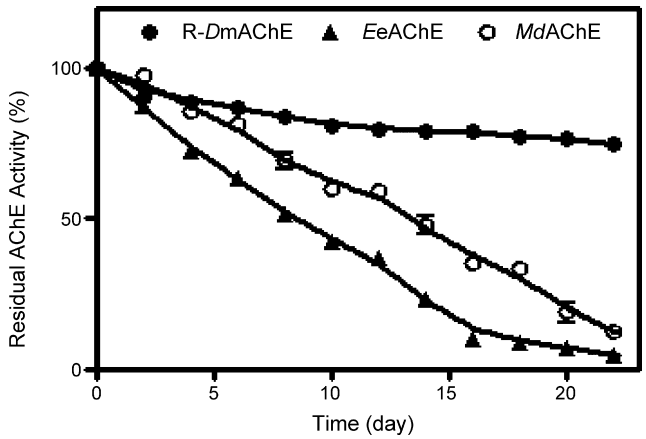

Fig. 5. The stability of R-*Dm*AChE, *Ee*AChE and *Md*AChE preparation buffered at pH 7.6 stored at 4 °C.

Table 1
Bimolecular rate constants (M^{-1} min^{-1}) of the R-*Dm*AChE and other AChEs to various insecticides

Insecticide	R-*Dm*AChE	*Dm*AChE[a]	*Nb*AChE[b]	*Ee*AChE[b]	*Hu*AChE[b]
Dichlorvos	4.4E6	5.0E5	1.3E5	4.3E4	0.6E5
Omethoate	2.2E4	2.2E4	5.2E2	2.3E3	1.1E3
Paraoxon	1.1E6	1.4E6	1.0E6	2.2E5	1.2E6
Paraoxon methyl	4.2E5	4.2E5	1.7E5	0.9E5	3.5E5
Aldicarb	4.8E4	5.5E3	8.0E3	5.0E4	1.6E4
Carbaryl	5.9E5	1.7E5	3.1E4	6.6E4	2.7E4
Carbofuran	5.1E6	5.1E6	4.4E5	1.8E6	1.0E6
Pirimicarb	2.3E5	2.4E5	5.7E4	1.0E3	1.0E4

[a] Refer to Villatte et al. (1998) and Fournier et al. (1988).
[b] Refer to Schulze et al. (2005).

phenylalanine residue at the pocket (Radic et al., 1993), which is likely to be responsible for the BW284c51 specificity. Data in Table 2 show that the recombinant enzyme was particularly inhibited by dichlorvos and carbofuran even at the concentration below 0.01 μM. In addition, for 10 tested insecticides (dichlorvos, omethoate, monocrotophos, phorate, paraoxon, paraoxon methyl, carbofuran, carbaryl, pirimicarb and propoxur), all IC_{50} values for the R-*Dm*AChE were lower than those for other two AChEs. It seemed that the R-*Dm*AChE was better to be used for detection of insecticide residues since it was more sensitive to a wide range of insecticides.

3.4. Establishment of R-DmAChE-based insecticide residues assay for samples

Five different vegetables were fortified at three concentration levels depending on the potency of each insecticide for inhibiting the R-*Dm*AChE. The samples were in parallel analyzed by GC. The recoveries obtained for all pesticides varied from 84 to 120%, indicating efficient handling of the samples. According to the Chinese national standard, entitled ''rapid determination for organophosphate and carbamate pesticide residues in vegetables'' (Wang et al., 2003), the samples are considered to be positive if 50% of the enzyme activity is inhibited after 2.5 mL of extracts incubating with 0.1 mL of AChE solution for 15 min at room temperature. The results of AChE-based assay of the spiked samples (Table 3) indicated that at least 80% of enzymatic activity was inhibited by dichlorvos or carbofuran with a spiked level of 0.01 mg/kg. For pirimiphosmethyl, 0.01 mg/kg caused 50% enzymatic activity loss, which also occurred with vegetable samples spiked with 0.05 mg/kg of triazophos, isoprocarb, or carbaryl. The detection limit of malathion, pirimicarb or methomyl was at the concentration of 0.1 mg/kg in vegetable samples. The repeatability was assessed by R.S.D., which was at the range of 0–13.7%.

Moreover, 12 vegetable samples randomly collected from a local market in Wuxi (China) were analyzed by this kind of assay. Two samples with 77.5 and 67.4% enzymatic activity inhibition of the R-*Dm*AChE were proved to be contaminated with excessive omethoate (Table 4). The applicability of the R-*Dm*AChE-based method was further demonstrated by the data showing that another three positive samples contaminated with pesticides whose concentrations were higher than the MRLs resulted in greater than 50% activity inhibition of the R-*Dm*AChE (Table 4).

4. Discussion

The AChE-based methods are ubiquitously used for rapid detection of pesticide residues, especially in Chinese or other Southeast Asian markets, where still exist the necessity of AChE, carrying more sensitivity, stability at low expense, which could be highly acceptable in practical application. At present, *Dm*AChE is proved to be the most sensitive enzyme adopted as insecticides detecting material in the previously reported AChEs (Villatte et al., 1998; Boublik et al., 2002; Schulze et al., 2005). Heretofore, the production or expression systems including insect, baculovirus infected cells and rat are not appropriate for large-scale AChEs production.

Fortunately *P. pastoris* is a very popular tool for the heterologous expression of recombinant proteins and can produce foreign proteins to high levels (Cregg et al., 1993). We obtained the novel R-*Dm*AChE through recombinant technical procedures of cloning of *Dm*AChE cDNA fragment, expression vector construction and expression of the R-*Dm*AChE in *P. pastori*. The R-*Dm*AChE remains high enzymatic activity

Table 2
IC_{50} (μM) values of various insecticides to three AChEs

Insecticide	IC_{50} value[a]		
	R-*Dm*AChE	*Ee*AChE	*Md*AChE
Dichlorvos	9.77E−04 ± 5.64E−05	8.79E−01 ± 1.85E−01	1.14E−01 ± 1.91E−02
Omethoate	9.35E−01 ± 3.54E−02	>5.00	>5.00
Monocrotophos	7.22E−01 ± 9.54E−03	>5.00	>5.00
Phorate	3.50E−01 ± 6.90E−03	>5.00	>5.00
Paraoxon	1.32E−02 ± 2.67E−03	3.85E−02 ± 3.54E−03	1.97E−01 ± 4.47E−02
Paraoxon methyl	1.05E−01 ± 2.35E−04	6.80E−01 ± 4.24E−02	1.49 ± 1.89E−01
Carbofuran	1.02E−03 ± 9.02E−05	8.95E−03 ± 2.12E−04	3.02E−02 ± 3.60E−03
Carbaryl	6.72E−02 ± 6.43E−03	>5.00	1.15 ± 1.49E−01
Pirimicarb	1.36E−01 ± 1.82E−02	>5.00	>5.00
Propoxur	1.15E−02 ± 1.67E−03	9.44E−02 ± 1.70E−03	2.75E−01 ± 4.92E−02
BW284c51	2.43E−02 ± 4.24E−04	7.30E−03 ± 4.24E−04	2.63E−02 ± 7.07E−04
Iso-OMPA	>300.00	>300.00	>300.00

[a] Mean ± standard deviation ($n = 3$).

Table 3
Mean R-*Dm*AChE inhibition rate and R.S.D. obtained in spiked vegetable samples

Pesticide	Spike level (mg/kg)	R-*Dm*AChE activity inhibition (%), R.S.D. (%) in parentheses[a]				
		Lettuce (*Lactuca sativa*)	Spinach (*Spinacia olenacea*)	Cabbage (*Brassica oleracea* var. *capitata*)	Bell pepper (*Capsicum annuum*)	Eggplant (*Solanum melongena*)
Dichlorvos	0.1	97.44 (1.11)	97.22 (4.04)	99.69 (0.54)	98.79 (1.42)	99.36 (1.12)
	0.05	99.02 (0.99)	97.1 (2.60)	98.74 (1.49)	98.79 (2.13)	99.08 (1.60)
	0.01	80.5 (6.32)	98.49 (1.90)	89.47 (5.75)	95.66 (2.27)	91.52 (5.52)
Pirimiphosmethyl	0.1	99.01 (0.99)	96.9 (3.87)	96.14 (3.34)	99.14 (0.83)	97.58 (3.51)
	0.05	81.71 (1.00)	75.8 (1.62)	74.47 (1.2)	80.03 (1.25)	81 (3.99)
	0.01	50.35 (9.53)	67.05 (4.61)	45.75 (3.02)	58.9 (5.64)	55.56 (4.81)
Malathion	0.5	94.07 (1.12)	99.00 (1.04)	97.18 (0.51)	97.74 (2.05)	98.77 (1.22)
	0.1	62.68 (3.70)	76.28 (3.01)	58.94 (2.03)	60.68 (4.18)	65.55 (1.79)
	0.05	25.25 (4.81)	14.49 (13.66)	11.19 (9.27)	16.58 (5.89)	14.51 (7.26)
Triazophos	0.1	90.07 (1.52)	88.15 (3.55)	79.35 (3.70)	94.3 (3.08)	94.34 (4.73)
	0.05	64.4 (2.18)	54.51 (5.28)	50.19 (6.48)	61.26 (2.07)	62.92 (7.95)
	0.01	22.71 (7.10)	37.46 (9.96)	16.96 (10.52)	19.8 (7.00)	18.94 (7.21)
Acephate	2	70.23 (3.55)	64.00 (2.29)	75.57 (1.74)	80.06 (1.20)	68.87 (2.12)
	1	55.24 (3.94)	51.6 (6.90)	56.08 (4.25)	60.96 (8.19)	47.48 (3.56)
	0.5	36.81 (7.68)	33.04 (9.33)	43.66 (8.53)	46.36 (6.07)	33.61 (9.15)
Isoprocarb	0.5	96.4 (2.55)	95.41 (2.27)	96.05 (1.27)	94.82 (6.20)	100 (0.00)
	0.1	72.44 (1.94)	82.58 (9.22)	77.37 (7.23)	82.83 (4.03)	77.92 (3.67)
	0.05	48.87 (1.49)	49.19 (7.97)	57.43 (7.18)	47.16 (9.64)	49.04 (5.24)
Tsumacide	2	83.95 (7.04)	76.51 (5.60)	75.93 (1.72)	76.15 (4.37)	71.78 (2.34)
	1	58.17 (8.58)	64.22 (6.55)	61.25 (4.99)	51.69 (3.97)	49.68 (11.19)
	0.5	44.74 (1.21)	44.79 (5.74)	44.61 (2.66)	30.38 (7.35)	39.60 (6.21)
Pirimicarb	0.5	93.65 (1.44)	97.25 (1.00)	90.19 (5.84)	91.39 (1.62)	88.9 (8.58)
	0.1	61.49 (7.69)	46.17 (2.43)	50.68 (2.40)	54.76 (3.59)	51.47 (0.05)
	0.05	33.13 (6.19)	29.19 (9.06)	32.84 (8.71)	33.72 (8.60)	28.84 (8.43)
Carbofuran	0.1	97.46 (2.01)	99.66 (0.59)	98.46 (1.44)	95.06 (4.53)	97.61 (2.32)
	0.05	98.68 (1.53)	96.91 (0.55)	98.08 (1.96)	98.89 (1.27)	98.4 (2.82)
	0.01	94.35 (0.59)	91.1 (0.99)	80.32 (9.64)	84.41 (7.28)	90.21 (9.47)
Methomyl	0.5	92.96 (1.83)	88.69 (2.35)	86.97 (0.69)	84.94 (6.25)	87.32 (7.77)
	0.1	59.47 (2.94)	52.59 (1.24)	56.28 (1.89)	49.31 (2.51)	50.75 (3.35)
	0.05	36.05 (1.59)	34.57 (6.74)	31.89 (9.77)	34.95 (2.86)	38.11 (5.81)
Carbaryl	0.5	97.37 (2.54)	96.12 (1.68)	94.82 (2.67)	95.24 (1.64)	97.33 (2.48)
	0.1	76.92 (5.48)	69.79 (2.56)	73.47 (4.74)	85.54 (4.19)	72.74 (4.80)
	0.05	55.03 (6.70)	49.27 (11.13)	52.39 (4.17)	54.58 (11.15)	45.16 (11.34)

[a] $n = 3$ at each spike level for each vegetable sample.

Table 4
R-*Dm*AChE-based assay and GC analysis of marketed vegetables

Code	Vegetable	R-*Dm*AChE-based assay (inhibition rate of AChE (%))	GC analysis (insecticide detected (mg/kg))
1	Eggplant	15.6	nd
2	Cabbage	97.8	Pirimicarb, 1.07
3	Chinese celery (*Apium graveolens* var. *dulce*)	77.5	Omethoate, 0.76
4	Chinese cabbage (*Brassica pekinensis*)	67.4	Omethoate, 0.54
5	Coriander (*Coriandrum sativum*)	18.8	nd
6	Cucumber (*Cucumis sativus*)	13.7	nd
7	Chinese spinach (*Amaranthus tricolor*)	14.3	nd
8	Lettuce	22.0	nd
9	Corn chrysanthemum (*Chrysanthemum segetum*)	91.2	Methamidophos, 1.92
10	Sword bean (Canavalia ensiformis)	62.6	Monocrotophos, 0.83
11	Tomato (*Lycopersicon esculentum*)	22.3	nd
12	Cauliflower (*Brassica oleracea* var. *botrytis*)	15.6	nd

nd, Not detected.

when buffered at pH 7.6 with phosphate, a subtle shift from the native *Dm*AChE of pH 7.0, possibly resulting from the difference in glycosylation of the R-*Dm*AChE in *P. pastoris*. Moreover, the stability of the R-*Dm*AChE is good for practical use, since it remains effective enzyme activity for 22 days when dissolved in phosphate buffer at 4 °C at the optimum pH value mentioned above. However, two main kinds of *Ee*AChE and *Md*AChE in Chinese markets can only remain the activity for 4–7-day storage time under the same condition. Thus this R-*Dm*AChE breaks through the bottleneck of insect-derived AChE in the markets in China because the stability of an enzyme is crucial for practical use.

More importantly, the sensitivity of the R-*Dm*AChE to the tested organophosphate and carbamate insecticides was higher than other AChEs from various sources, represented by its higher K_i values indicating higher detection sensitivity. Compared with two commercially available AChEs from electric eel and housefly commonly used in China, the susceptibility of the R-*Dm*AChE to cholinesterase inhibitors was also substantially higher. Thus the R-*Dm*AChE-based assay was more sensitive and accurate to the spiked and practical samples, which was also confirmed by the chromatographic method. All these indicated that the R-*Dm*AChE-based test could definitely meet the needs of the current legislated standards in China.

5. Conclusion

The procedure for producing R-*Dm*AChE in expression system of *P. pastoris* with higher sensitivity, stability than the main available AChEs, at the same time obtaining larger amount of enzyme, which is more convenient for manufacturing at lower costs.

Although the R-*Dm*AChE-based assay for detection of insecticide residues failed to give quantitative and insecticide-specific information, this method could be used for rapid screening for large numbers of agricultural products containing excessive AChE-inhibiting insecticides. Furthermore, it would help to enforce the national regulation to limit insecticide contamination in agricultural products on spot.

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