Surface Display and Bioactivity of *Bombyx mori* Acetylcholinesterase on *Pichia pastoris*

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**Abstract**

*A Pichia pastoris* (P. pastoris) cell surface display system of *Bombyx mori* acetylcholinesterase (8mAChE) was constructed and its bioactivity was studied. The modified *Bombyx mori* acetylcholinesterase gene (*bmace*) was fused with the anchor protein (*AGz1*) from *Saccharomyces cerevisiae* and transformed into *P. pastoris* strain GS115. The recombinant strain harboring the fusion gene *bmace-AGz1* was induced to display 8mAChE on the *P. pastoris* cell surface. Fluorescence microscopy and flow cytometry assays revealed that the 8mAChE was successfully displayed on the cell surface of *P. pastoris* GS115. The enzyme activity of the displayed 8mAChE was detected by the Ellman method at 787.7 U/g (wet cell weight). In addition, bioactivity of the displayed 8mAChE was verified by inhibition tests conducted with eserine, and with carbamate and organophosphorus pesticides. The displayed 8mAChE had an IC\(_{50}\) of 4.17 \(\times\) 10\(^{-6}\) M and was highly sensitive to eserine and five carbamate pesticides, as well as seven organophosphorus pesticides. Results suggest that the displayed 8mAChE had good bioactivity.

**Introduction**

The intensive use of carbamate (CB) and organophosphorus (OP) pesticides in recent years has led to potentially dangerous effects on human and animal health. The control of pesticide residues in food and the environment is of great importance to minimize the risk to consumers and environmental animal species. Routinely, CB and OP pesticide residues are measured by instrumental methods, such as gas chromatography, liquid chromatography and gas chromatography–tandem mass spectrometry [1,2,3]. There is a growing interest in more rapid and low-cost field-portable detection systems. A promising approach involves the use of screening enzyme-linked immunoassays [4]. However, these assays require broad-specificity antibodies that are difficult to develop. Nevertheless, an enzyme-based method was demonstrated to be an efficient and rapid method for the detection of pesticides because it was inexpensive, allowed high sample throughput, and was easily adapted for use in Asian markets [5].

Previously, acetylcholinesterase (AChE), aldehyde dehydrogenase, alkaline and acid phosphatase, butyrylcholinesterase, organophosphorus hydrolase and tyrosinase have been investigated for their ability to detect pesticides in water and other matrices such as soil, food and beverages [6]. However, AChE has been most often used for enzymatic detection of pesticides because of its broad substrate specificity and good sensitivity [6].

AChE is a key enzyme in the cholinergic system that regulates the level of acetylcholine and terminates nerve impulses by catalyzing the hydrolysis of the neurotransmitter acetylcholine in the synaptic cleft [7,8]. The enzyme activity of AChE can be inhibited by CB and OP pesticides. Therefore, it is feasible to use AChE for the detection of CB and OP pesticides based on the degree of AChE activity inhibition [9]. AChE has been isolated by traditional extraction methods from natural tissues [10,11,12] or from secretions of engineered cells [8,13]. Isolation from these areas requires an enzyme purification step, which leads to higher preparation costs. However, natively displayed molecules on the surface of cells presents another option, which is currently of great interest. Many heterologous proteins and polypeptides have been displayed on the surface of cells, and these displays have been widely used [14,15,16,17,18]. The use of displayed molecules on the cell surface can save tedious purification steps required for enzymes used in traditional immobilization methods. Further, protein engineering can help generate a surface display of enzymes that can be used in efficient high-throughput screening methods for residue detection. In cell surface display development, the anchor protein is a necessary component. The most frequently used anchor is the N-terminal fusion display of \(\alpha\)-agglutinin from *Saccharomyces cerevisiae* (S. cerevisiae), which is composed of a secretion-signal region, an active region, a serine- and threonine-
rich support region, and a putative glycosylphosphatidylinositol anchor-attachment protein. AGz1 protein is one of the agglutinins in N-terminal fusion displays [19]. Different enzymes used for the detection of pesticides, like organophosphorus hydrolase [20] and mouse AChE [21], have been expressed on the surface of microorganisms. In this study we took advantage of the specificity of the displayed AChE for pesticides. Domesticated silkworms have not suffered from pesticide selection, as the sensitivity of the displayed AChE for pesticides may lay the foundation for further sensitivity improvement by developing a displayed AChE system through recombinant molecular methods and application of whole-cell biosensors for the detection of CB and OP pesticides. Here we constructed a cell surface display system for Bombyx mori AChE (BmAChE). The surface display system may serve as the host for recombinant DNA manipulation. The P. pastoris GS115 strains and the integrative expression vector pPIC9K were obtained from Invitrogen Biotechnology Co. (Shanghai, China). E. coli was grown in Luria-Bertani medium (1% yeast extract, 0.5% peptone, 1% sodium chloride). Pichia pastoris was cultivated in yeast peptone dextrose medium (1% yeast extract, 2% peptone, and 2% glucose), and P. pastoris transformants were cultivated on minimal dextrose medium (MD plates (2% glucose, 0.0004% biotin, 1.34% yeast nitrogen base (YNB) and 1.8% agarose). 

Reagents

Gel extraction kits were obtained from Tiangen (Beijing, China). Yeast genomic DNA extraction kits were obtained from Beijing ComWin Biotech Co., Ltd (Beijing, China). PrimerSTAR DNA polymerase, restriction enzymes, and dNTPs were obtained from Takara Biotechnology Co. Ltd (Dalian, China). Primers were synthesized by Shanghai Sangon Biotechnology (Shanghai, China). The mouse anti-FLAG monoclonal antibody, Alex Fluor 488 labeled goat anti-mouse IgG, acetylthiocholine iodide (ATC) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were obtained from Sigma-Aldrich (St. Louis, MO, USA). CB and OP standards were obtained from the National Center of Standard Material (Beijing, China).

Cloning and Assembly of the bmace-AGz1 Gene

The construction scheme for the plasmid containing the bmace-AGz1 gene is shown in Fig. 1; DNA fragments encoding for BmAChE were amplified with the constructed vector pPIC9K-bmace [23] as a template without the signal peptides and the hydrophobic amino acid tail gene. The PCR process was performed using PrimerSTAR DNA Polymerase and the amplification experiment was run at a melting temperature of 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 2 min, with a 30 cycle repeat. Primers used for PCR amplification contained the FLAG tag at 5′ and partial linker at 3′ were the two oligonucleotides F1 and R1, respectively (Table 1). The genome of S. cerevisiae was extracted using the yeast genome extraction kit and the AGz1 gene was amplified using the genome as template and the F2 and R2 primers listed in Table 1. The purified bmace and AGz1 DNA segments (50 ng each) were spliced using overlap extension PCR to assemble the bmace-AGz1 gene with the (Gly, Ser) linker. Then the bmace-AGz1 gene was amplified using the F1 and R2 primers (Table 1). The PCR amplification products were purified by agarose gel DNA purification kit and stored at −20°C.

Construction of the Plasmid for Cell Surface Display

The resulting PCR products from the above step were digested with the restriction enzymes Mlu I and Not I, and then ligated into the expression vector pPIC9K. The ligated products were transformed into competent E. coli DH5α cells for propagation of the recombinant plasmid. The recombinant plasmid pPIC9K-bmace-AGz1 was confirmed by restriction enzyme digestion and DNA sequencing.

P. pastoris Transformation and Selection

Linearized vectors were transformed into P. pastoris as previously described [24]. Transformed cells were spread on MD plates and incubated at 30°C for 3 d to select His+ transformants. Genomic integration was confirmed by performing PCR on genomic DNA with the AOX-F and AOX-R primers (Table 1).

Expression of the bmace-AGz1 Gene

The recombinant P. pastoris clone was grown in 20 mL BMGY medium (1% yeast extract, 2% peptone, 1.34% YNB, 0.0004% biotin, 1% glycerol and 100 mM potassium phosphate (pH 6.0)) in shake culture at 30°C for 24 h until the OD600 reached a value of more than 4. The culture (5 mL) was centrifuged at 3000 g for 5 min. The cells were induced by re-suspension with 20 mL BMGY medium (1% yeast extract, 2% peptone, 1.34% YNB, 0.0004% biotin, 0.5% methanol and 100 mM potassium phosphate (pH 6.0)) and the resulting OD600 was approximately 1. The induction was continued at 20°C for 4 d by adding 200 μL of 100% methanol to the cultures daily.

Characterization of the Displayed BmAChE by Fluorescence Microscopy

The immunofluorescent labeling of yeast cells was carried out as follows: A cell suspension was centrifuged at 8000 x g for 1 min, and the collected cells were washed three times with 0.1 M phosphate buffered saline (PBS) (8.0 g/L NaCl, 1.44 g/L Na2HPO4•7H2O, 0.2 g/L KCl, 0.24 g/L KH2PO4 (pH 7.4)). The cells were suspended and blocked with PBS containing 1% bovine serum albumin (BSA) for 0.5 h (OD600 = 1.0). Anti-FLAG IgG (1 μg) was added to the 200 μL cell suspension and incubated at room temperature for 1.5 h. The cells were then washed with PBS, centrifuged at 6000 x g for 10 min at room temperature, suspended in 200 μL of PBS with 1 μL Alexa Fluor TM 488-conjugated goat anti-mouse IgG (1:200), and then incubated at room temperature for 1.5 h. The PBS washed cells were observed.
under a fluorescence microscope (Nikon Eclipse 80i, Tokyo, Japan). The excitation and emission wavelengths used were 488 nm and 510–535 nm, respectively.

Flow Cytometry Detection

The number of yeast cells displaying $Bm$AChE was determined using a flow cytometer (BD FAcscalibur, CA, USA) with a 488 nm excitation wavelength and a 525 nm emission wavelength to estimate the percentage of $Bm$AChE molecules displayed.

Enzyme Activity Determination of the Displayed $Bm$AChE

The activity of the displayed $Bm$AChE was evaluated spectrophotometrically at 405 nm according to Ellman et al [25], using the substrate ATC and the chromogenic reagent DTNB. A cell suspension (100 μL) of transformed *P. pastoris* was centrifuged at 8000 × g for 2 min and the cells were weighed. The collected cells (approximately 1.03 × 10⁷ cells as determined by a hemocytometer) were washed three times with potassium phosphate buffer (3.075 mL of a 1 M K$_2$HPO$_4$ solution combined with 1.925 mL of a 1 M KH$_2$PO$_4$ solution (pH 7.0)) and re-suspended in 780 μL potassium phosphate buffer (pH 7.0). The enzymatic reaction was activated by consecutively adding 100 μL of 1 mM ATC and 7.8 mM DTNB. The reaction mixture was incubated at room temperature for 5 min and stopped with 20 μL of 1 × 10⁻⁷ M eserine. After centrifugation of the reaction mixture, the supernatant was used to measure the OD at 405 nm with an ELISA reader (Multiscan MK3, Labsystem Co., Finland). One unit of AChE activity was defined as the amount of enzyme hydrolyzing 1 mmol of ATC in 1 min with 1 g of wet cells.

Figure 1. The chemical structures of the different compounds tested. (A) structure of the natural inhibitor of AChE, (B) structures of the CB pesticides, (C) structures of the OP pesticides.

doi:10.1371/journal.pone.0070451.g001

Table 1. Primers used for cloning *bmace* and *AGz1* genes and generating the synthetic gene encoding *bmace-AGz1* fusion protein.

|   |   |
|---|---|
| F1: | 5'-GGCAGCGGTGACCAAGCAGATGATAGAAGGGATGCTTGGGCCAATC-3' |
| R1: | 5'-CCAGGCGCCCTCCCGCTGAAGGGCCTTACACTGCTGAAAGACCAGGT-3' |
| F2: | 5'-GGCCGGCGCAGATAGGATCGTTAGAAGCTCTAGC-3' |
| R2: | 5'-GCCCGCGCCGCGAAGAAGGCTTGGACCAGAT-3' |
| AOX-F | 5'-GCGAAATGGCTCTGGCTG-3' |
| AOX-R | 5'-GCCAAATGGCTCTGGCTG-3'. |

doi:10.1371/journal.pone.0070451.t001
Inhibition of Displayed BmAChE

Inhibition of the displayed BmAChE was carried out in the presence of eserine [26], a well-known AChE inhibitor previously employed to study the enzyme. Transformants were inoculated on MM-B agar plates (1.34% YNB (v/v), 0.00004% biotin, 1% methanol, 100 mM potassium phosphate buffer (pH 7.0) and 1.8% agarose) at 28°C for 3 d. Then 10 μL of 50 mM potassium phosphate buffer (pH 7.0) was placed on one GS115 colony (negative control) and on one GS115/pPIC9K-bmace-AGa1 colony (positive control), and 5 μL of 50 mM potassium phosphate buffer (pH 7.0) and 5 μL of different concentrations of eserine (10⁻²–10⁻⁹ M) were placed on 8 other positive colonies. Following a 10 min reaction period, 3 μL of 10 mM ATC and 3 μL of 7.8 mM DTNB were added to each colony, and the colony color was observed after 10 min at 37°C. Also, an inhibition study of the displayed BmAChE was performed according to Ellman’s method [25] using the yeast cell suspension and different concentrations of eserine.

Detection of CB and OP Pesticides Using the Displayed BmAChE

Five CB pesticides (carbofuran, carbosulfan, isoprocarb, methiocarb and methomyl) and seven OP pesticides (dichlorphos, dimethoate, isocarbophos, malathion, methamidophos, parathion and trichlorphon) were tested (Figure 1). The induced cell suspension was centrifuged at 8000 × g for 2 min, re-suspended and adjusted to an OD₆₀₀ of 2 using 50 mM potassium phosphate buffer (pH 7.0). A volume of 120 μL transformed P. pastoris cell suspension (approximately 0.25 × 10⁷) was mixed with the same volume but different concentrations of CB and OP pesticides. After a 5 min incubation at room temperature, 30 μL of 10 mM ATC and 7.8 mM DTNB were consecutively added, and 20 μL of 1 × 10⁻⁷ M eserine was added to stop the reaction. The reaction mixture was centrifuged at 8000 × g for 2 min and the suspension was removed to microlon plates. The activity of AChE was measured using the multilabel counter at 405 nm. The median inhibition concentration (IC₅₀) for each compound was calculated based on the Log-dose versus probit regression [27]. The lowest concentration that could be detected was measured according to the Inhibition Rate (B/B₀).

Results and Discussion

Construction of the BmAChE Yeast Surface Display System Using P. pastoris

The plasmid for surface display of BmAChE was constructed as shown in Fig. 2. The amplification of bmace generated an approximate 1900 bp DNA fragment, while the AGa1 gene generated an expected 1000 bp fragment. PCR amplification of the assembled bmace-AGa1 gene produced an expected 2900 bp fragment (Figure S1, see supplementary data for the nucleic acid sequence in the Supporting Information). The bmace-AGa1 gene with a FLAG tag (eight amino acids) at the N-termius of AChE was subcloned into the expression vector pPIC9K. The results from sequencing indicated the recombinant plasmid pPIC9K-bmace-AGa1 had been successfully constructed. PCR amplification using AOX-F and AOX-R primers (Table 1) with the genome of the selected transformants as template produced the 2900 bp amplified fragment, indicating that the constructed vectors were integrated into the genome of P. pastoris GS115.

Characterization of the Displayed BmAChE by Fluorescence Microscopy and Flow Cytometry

The display of BmAChE on the yeast cell surface was evaluated by immunofluorescence microscopy. Fluorescence was observed on the cell surface of the pPIC9K-bmace-AGa1 transformant strains.

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**Figure 2. Construction of the BmAChE-display with the P. pastoris expression system based on α-agglutinin.** The bmace and AGa1 DNA segments were spliced using overlap extension PCR to assemble the bmace-AGa1 gene and inserted into pPIC9K for the pPIC9K-bmace-AGa1 construction.

doi:10.1371/journal.pone.0070451.g002
using a fluorescence microscope, and fluorescence was not observed from the control cells (Fig. 3). The images demonstrated that the bmace-AGa1 fusion protein was anchored on the P. pastoris surface.

The expression of the BmAChE fusion protein on the surface of P. pastoris was further analyzed by indirect immunofluorescence labeling using flow cytometry (Fig. 4). A difference was detected in the amounts of BmAChE-α-agglutinin fusion protein expression obtained from P. pastoris pPIC9K-bmace-AGa1 transformants. Fluorescence was detected in about 25% of the constructed cells. These studies confirmed that BmAChE was displayed on the cell surface of P. pastoris.

Enzyme Activity Determination Using Displayed BmAChE

AChE activity was measured based on the Ellman method [25] using ATC and DTNB. The hydrolytic activity of the BmAChE enzymes displayed on the surface of the cells was 787.7 U/g (wet cell weight) after being induced with methanol for 4 days at 28°C.

The inhibition analysis of displayed BmAChE

The inhibition characteristics of eserine, the AChE specific inhibitor, were performed on MM-B plates. As shown in Fig. 5, the color of the original P. pastoris GS115 colonies was white (Fig. 5, colony 1), while the color of the transformed P. pastoris GS115/pPIC9K-bmace-AGa1 colonies was yellow (Fig. 5, colony 2). When the concentration of eserine was between 10⁻⁷-10⁻⁸ M, the color of the pPIC9K-bmace-AGa1 colonies gradually turned yellow (Fig. 5, colony 8–10). When the concentration of eserine was 10⁻⁹ M (Fig. 5, colony 10), the color was close to the positive control (Fig. 5, colony 2). Based on colony color, the inhibition characteristics of eserine for displayed BmAChE were estimated for a concentration series of eserine solutions (1×10⁻⁵, 2×10⁻⁶, 5×10⁻⁶, 4×10⁻⁷, 5×10⁻⁷, 6×10⁻⁷ and 7×10⁻⁸ M). The B/B₀ decreased with increasing eserine concentrations (Fig. 6 (A)). The IC₅₀ of BmAChE was 4.17×10⁻⁸ M. The results showed that the recombinant BmAChE that was displayed on the yeast surface exhibited high-sensitivity to eserine. Compared with the BmAChE expressed in Trichoplusia ni (BTI-Tn-5B1-4) cells [28], the sensitivity for eserine in our report was at about the same level, which indicated that BmAChE retained its natural activity after being displayed on the cell surface.

Detection of CB and OP Pesticides Using Displayed BmAChE

The displayed BmAChE enzyme was used to detect five CB and seven OP pesticides. Measurement of enzyme activity and inhibition studies were performed as described in the experimental section. The inhibition of BmAChE with CB pesticides is shown in Fig. 6 (B). An IC₅₀ of 1.92×10⁻⁸ M was obtained with carbofuran, while an IC₅₀ of 1.13×10⁻⁷ M was obtained with carbosulfan, 1.11×10⁻⁷ M with isoprocarb, 6.58×10⁻⁸ M with methiocarb and 6.41×10⁻⁸ M with methomyl. Inhibition of BmAChE with seven OP pesticides is shown in Fig. 6 (C) and Table 2. Among them, trichlorphon showed the highest inhibitory effect on BmAChE activity with an IC₅₀ of 2.40×10⁻⁷ M and a limit of detection of 3.89×10⁻⁸ M. The maximum European Union (EU) residue limit was recently set at 0.01 mg/kg (approximately 4.0×10⁻⁸ M) for pesticide residues in all agricultural products for

![Figure 3. Fluorescence microscopy assay of recombinant P. pastoris cells displaying BmAChE: The fluorescence at 519 nm emitted with excitation at 495 nm was observed by fluorescence microscopy. (a) and (c), phase micrographs of recombinant yeast cells; (b) and (d), fluorescent micrographs of recombinant yeast cells. GS115/pPIC9K-bmace-AGa1 (a, b); GS115 as a control (c, d). doi:10.1371/journal.pone.0070451.g003](image)

![Figure 4. Flow cytometry detection of BmAChE displayed on the recombinant yeast surface. doi:10.1371/journal.pone.0070451.g004](image)

![Figure 5. P. pastoris display of BmAChE on an MM-B plate: 1, GS115; 2, GS115/pPIC9K-bmace-AGa1; 3–10, GS115/pPIC9K-bmace-AGa1 inhibited by different concentrations of eserine (clone 3: 10⁻² M, clone 4: 10⁻¹ M, clone 5: 10⁻⁸ M, clone 6: 10⁻⁵ M, clone 7: 10⁻⁶ M, clone 8: 10⁻⁸ M, clone 9: 10⁻⁶ M, clone 10: 10⁻⁶ M). doi:10.1371/journal.pone.0070451.g005](image)
food or animal feed [29]. Therefore, the activity of the displayed
BmAChE has sufficient sensitivity for the determination of most of
the selected CB and OP pesticides. As seen in Table 2, for all five
tested CB pesticides (carbofuran, carbosulfan, isoprocarb, methio-
carb and methomyl), the sensitivity values of the displayed
BmAChE were better than those of the common housefly (Musca
domestica) and those of the common fruit fly (Drosophila melanogaster
AChEs. In addition, the sensitivity of our displayed BmAChE for
the representative OP pesticides (dimethoate, isocarbophos and
trichlorphon) is much better than the housefly AChE [30]. For
dichlorphos, the sensitivity of the displayed BmAChE was at the
same level as with the Drosophila melanogaster AChE [31], but a little
less than that of the Bombyx mandarina AChE [32]. Further
experimental optimization of the P. pastoris displayed BmAChE
enzyme is expected to meet or exceed the pesticide detection
requirements and the displayed BmAChE enzyme will be used for
routine monitoring of CB and OP pesticides.

Analytical equipment-based methods typically used for the
analysis of pesticides are not practicable enough to be used for
simple, fast detection of large numbers of samples. Rapid assays
using AChE-based methods have been proposed as an efficient
and rapid method for the detection of pesticides, especially in
many Asian markets [5]. Until now, most of the AChE enzymes
used for the detection of pesticides have been extracted from fish
and insect heads [33,34], requiring much preparation time,
resulting in high costs for enzyme purification. However, the yeast-
display technology has provided an alternative means for
engineering a low-cost AChE enzyme with desirable activity and
the developed cells can be immobilized by chemical methods or
with physical methods for development of whole-cell biosensors
[35]. Also, the yeast expression system is capable of folding and
glycosylating heterologous eukaryotic proteins [36,37]. In partic-
ular, P. pastoris also has the advantage of high-density cultivation in
inexpensive medium compared with other yeasts [38]. Therefore,
the displayed AChE on the cell surface of P. pastoris potentially has
many benefits and practical applications for pesticide detection.

AChE has been most often used for the detection of pesticides
because of its broad-substrate specificity. In this study the AChE
gene from Bombyx mori was cloned from a constructed vector and a
P. pastoris cell surface display system was developed for the first
time. The surface-displayed BmAChE was evaluated with eserine,
and with CB and OP pesticides. The results demonstrated that the

![Figure 6. Inhibition curve of displayed BmAChE. (A), inhibition curve of displayed BmAChE for eserine; (B), inhibition curve of displayed BmAChE for CB pesticide (n = 3); and (C), inhibition curve of displayed BmAChE for OP pesticides (n = 3). B, the average absorbance at the indicated concentrations; B0, the average absorbance at zero concentration. The data were fitted with a four-parameter-logistic equation to calculate the IC50 using OriginPro 7.5 software. The data points are mean values and the errors observed from triplicate determinations.

doi:10.1371/journal.pone.0070451.g006](http://www.plosone.org/doi/10.1371/journal.pone.0070451.g006)
Table 2. The median inhibition concentration (IC₅₀) of displayed BmAChE for five CB and seven OP pesticides.

| Analytes       | Displayed BmAChE | Expressed housefly AChE [30] | Expressed Drosophila melanogaster AChE [31] | Expressed Bombyx mandarina AChE [32] |
|----------------|------------------|------------------------------|---------------------------------------------|-------------------------------------|
|                | IC₅₀ (M)         | IC₅₀ (M)                     | IC₅₀ (M)                                    | IC₅₀ (M)                            |
| Carbofuran     | 1.92 × 10⁻⁴      | 1.85 × 10⁻⁴                  | 1.02 × 10⁻⁴                                 | –                                   |
| Carbosulfan    | 1.13 × 10⁻⁴      | –                            | –                                           | –                                   |
| Isoprocarb     | 1.11 × 10⁻⁴      | –                            | –                                           | 7.50 × 10⁻⁷                         |
| Methiocarb     | 6.58 × 10⁻⁶      | –                            | –                                           | –                                   |
| Methomyl       | 6.41 × 10⁻⁶      | 3.67 × 10⁻²                  | –                                           | 6.00 × 10⁻⁶                         |
| Trichlorphon   | 2.40 × 10⁻⁷      | 6.05 × 10⁻³                  | –                                           | –                                   |
| Dimethoate     | 1.14 × 10⁻⁴      | 7.74 × 10⁻²                  | –                                           | –                                   |
| Methamidophos  | 1.07 × 10⁻⁶      | –                            | –                                           | –                                   |
| Isocarbophos   | 9.10 × 10⁻⁷      | 7.10 × 10⁻²                  | –                                           | –                                   |
| Malathion      | 1.41 × 10⁻⁶      | –                            | –                                           | –                                   |
| Parathion      | 1.13 × 10⁻⁶      | –                            | –                                           | –                                   |
| Dichlorphos    | 9.03 × 10⁻⁷      | 4.87 × 10⁻⁴                  | –                                           | 2.40 × 10⁻⁷                         |

doi:10.1371/journal.pone.0070451.t002

Supporting Information

Figure S1 Nucleic acid sequence of the 2900 bp fragment.

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