Video Article

# Functional Characterization of Carboxylesterases in Insecticide Resistant House Flies, *Musca Domestica*

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## Abstract

Carboxylesterase-mediated metabolism is thought to play a major role in insecticide resistance in various insects. Several carboxylesterase genes were found up-regulated in the resistant house fly strain, whereas their roles in conferring insecticide resistance remained to be explored. Here, we designed a protocol for the functional characterization of carboxylesterases. Three example experiments are presented: (1) expression and isolation of carboxylesterase proteins through a baculovirus-mediated insect *Spodoptera frugiperda* (Sf9) cell expression system; (2) a cell-based MTT (3-[4, 5-dimethykthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) cytotoxicity assay to measure the tolerance of insect cells to different permethrin treatments; and (3) *in vitro* metabolic studies to explore the metabolic capabilities of carboxylesterases toward permethrin. The carboxylesterase gene MdαE7 was cloned from a resistant house fly strain ALHF and used to construct a recombinant baculovirus for Sf9 cells infection. The cell viabilities against different permethrin treatments were measured with the MTT assay. The enhanced cell tolerance of the experimental group (MdαE7-recombinant baculovirus infected cells) compared with those of the control groups (CAT-recombinant baculovirus infected cells and GFP-recombinant baculovirus infected cells) to permethrin treatments suggested the capabilities of MdαE7 in metabolizing insecticides, thereby protecting cells from chemical damages. Besides that, carboxylesterase proteins were expressed in insect Sf9 cells and isolated to conduct an *in vitro* metabolic study. Our results indicated a significant *in vitro* metabolic efficiency of MdαE7 toward permethrin, directly indicating the involvement of carboxylesterases in metabolizing insecticides and thus conferring insecticide resistance in house flies.

## Video Link

The video component of this article can be found at https://www.jove.com/video/58106/

## Introduction

Insecticide resistance is currently a major issue of house fly control worldwide[1,2]. Efforts to determine the mechanism of insecticide resistance facilitates better understanding of this issue and thus provide novel strategies to effectively prevent or minimize the spread of resistance development[3]. Carboxylesterases, as one of the major detoxification enzymes, have attracted a lot of attention for their roles in sequestering and metabolizing insecticides in various insects[4,5,6]. Our previous study has identified multiple carboxylesterases in house flies and their expression levels were not only constitutively up-regulated in the resistant ALHF strain but also can be induced to higher levels in response to permethrin treatments[7]. However, the functional characterizations of these carboxylesterase genes in metabolizing insecticides remain to be explored.

Since the first report in early 1980s[8], a baculovirus-mediated foreign gene expression system has been widely employed due to its high protein production efficiency and eukaryotic protein processing capabilities[9]. This binary system is composed of two essential elements: the constructed recombinant baculovirus delivering foreign genes into the host cells, and the large-scale expression of interested proteins by cells infected by recombinant baculovirus. Over the past decades, the baculovirus mediated cell expression system has been widely used to produce thousands of recombinant proteins, ranging from cytosolic enzymes to membrane-bound proteins in insect and mammal cells[10]. Our previous study has successfully expressed multiple CYP450 enzymes in insect Sf9 cells with this system[11]. In this study, we constructed a carboxylesterase-recombinant baculovirus to infect insect Sf9 cells, explored the cell tolerance to different permethrin treatments, and large-scale expressed carboxylesterase proteins *in vitro* for functional exploration. Instead of investigating multiple carboxylesterase isozyme mixtures from insect homogenates as adopted by previous studies[12,13], this baculovirus-mediated insect cell expression system allows the specific expression and isolation of targeted proteins for better characterization of their biochemical and structural properties.

The tetrazolium salt-based assay (MTT) is a high-throughput colorimetric method developed and optimized to measure cell viability. This assay is based on the mechanism that only living cells are capable of metabolizing the yellow-colored MTT reagent to a dark purple colored formazan precipitate, which can be colorimetrically analyzed after dissolved in organic solvents[14,15]. Several more accurate but time-consuming methods, such as Trypan blue exclusion and the thymidine titration assay[16,17], have been developed in recent years. However, the cell-based MTT assay is still currently recognized as the most rapid and easily-operated method to quickly detect cell viability. Here, we use the MTT assay to explore

 

the cell tolerance against insecticide treatments. The enhanced tolerance of cells when infected with carboxylesterase recombinant baculovirus strongly supports the metabolic roles of carboxylesterases to insecticides, which in turn suggests their involvement in insecticide resistance.

Additionally, an *in vitro* metabolic assay was also conducted in this study. Compared with general carboxylesterase assays that use common substrates such as α-napthyl acetate (α-NA) and β-naphthyl acetate (β-NA) to reflect hydrolytic activities of carboxylesterases, the *in vitro* metabolic study is regarded as an accurate way to directly measure activities of carboxylesterases toward insecticides[18]. This method has been successfully employed in various insects to characterize multiple cytochrome P450s in association with insecticide resistance[11,19,20]. However, this method has not yet been applied in carboxylesterase studies. With the availability of carboxylesterase proteins produced by baculovirus-mediated expression system, we can perform an *in vitro* metabolic study of carboxylesterases toward permethrin, which can further provide strong evidence of the involvement of carboxylesterases in conferring pyrethroid resistance in house flies.

## Protocol

# 1. Expression and Isolation of Target Proteins with a Baculovirus-mediated Insect Cell Expression System

1. Directionally clone blunt-ended PCR products of target proteins from house flies.
    1. Design PCR primers of green fluorescent protein (GFP) and the house fly MdαE7 gene based on their sequences and the special requirements of the chosen vector (**Table 1**).
    2. Use a thermostable, proofreading DNA polymerase and primers from step 1.1.1 to perform a 150 µL PCR reaction (30 µL of reaction buffer, 3 µL of 10 mM dNTPs, 1.5 µL of DNA polymerase, 6 µL of house fly template DNA, 7.5 µL of forward primer, 7.5 µL of reverse primer, with water to a final volume 150 µL). Heat the PCR reaction to 98 °C for 30 s, followed by 35 cycles of 98 °C for 10 s, 53 °C for 30 s, and 72 °C for 60 s, and then a final extension at 72 °C for 2 min).
        1. Run a 1% agarose gel with 150 µL of PCR product.
    3. Excise the target DNA fragments from the agarose gel with a sharp, clean scalpel: 1317 bp for MdαE7 and 858 bp for GFP. Purify the DNA using commercially available gel extraction kit following manufacturer's protocol (**Table of Materials**). Dissolve the purified DNA in 15 µL of distilled water.
    4. Run a 1% agarose gel with 1 µL of purified DNA to check integrity. Use another 1 µL of purified DNA to measure concentrations with the spectrophotometer.

2. Construct an entry plasmid for target proteins
    1. Set up the cloning reaction. Mix freshly purified DNA products from step 1.1.3 and commercially available entry vectors containing *att*L-sites (**Table of Materials**) at a molar ratio of 1:1 (0.5-2 µL of fresh PCR product at 70-200 ng/µL concentrations: 0.5 µL vector). Then add 1 µL of commercially available salt solution (1.2 M $NaCl_2$ and 0.06 M $MgCl_2$) and add water to a final volume of 6 µL. Mix gently and incubate at room temperature for 1 h.
    2. Transfer 4 µL of cloning reaction products in step 1.2.1 into 50 µL of chemically competent *E. coli* cells. Incubate on ice for 30 min. Heat-shock the cells for 30 s in a 42 °C water bath without shaking.
    3. Put the tube back in ice for another 2 min. Add 250 µL of room temperature S.O.C. medium (2% tryptone; 0.5% yeast extract; 10 mM NaCl; 2.5 mM KCl; 10 mM $MgCl_2$; 10 mM $MgSO_4$; 20 mM glucose). Incubate at 37 °C for 1 h with gently shaking.
    4. Spread 50-200 µL of the bacterial culture in step 1.2.3 on the selective LB plates (1 g of tryptone; 1 g of NaCl; 0.5 g of yeast extract; 1.5 g of agar dissolved in 100 mL of distilled water. Autoclave and add 0.1% of 50 mg/mL kanamycin). Incubate LB plates for 16 h at 37 °C for the colony growth.
    5. Pick 5-10 colonies and re-suspend them individually into 5 µL of distilled water.
    6. Perform PCR by adding 5 µL of reaction buffer, 0.5 µL of 10 mM dNTPs, 0.25 µL of DNA polymerase, 1 µL of suspended colonies, 1.25 µL of 10 µM M13 forward primer, 1.25 µL of 10 µM reverse primer of the target gene, and water to final volume of 25 µL. Heat the PCR reaction to 98 °C for 30 s, followed by 35 cycles of 98° C for 10 s, 53 °C for 30 s, and 72 °C for 60 s, and then a final extension at 72 °C for 2 min (**Table 1**).
    7. Re-culture 3 µL of suspended colonies from step 1.2.5 in TB media (100 mL of phosphate buffer containing 0.17 M $KH_2PO_4$ and 0.72 M $K_2HPO_4$ with 450 mL of base broth medium containing 6 g of tryptone, 12 g of yeast and 2 mL of glycerol, sterilized, containing 0.1% 50 mg/mL kanamycin) for 16 h. Extract ultra-pure plasmid DNA following manufacturer's protocol.
    8. Use 200 ng/µL ultra-pure plasmid from step 1.2.7 for commercial Sanger sequencing with M13 forward and reverse primers. Verify the correct insertion of the target gene in vector based on the sequence map provided by manufacturer.
    9. Store the sequence-verified ultra-pure plasmid DNA samples of MdαE7 and GFP in -20 °C.
    Note: These are the entry plasmid DNA of MdαE7 and GFP.

3. Construct a recombinant baculovirus by performing Lambda recombination (LR) reaction.
    1. Respectively, mix 1 µL of 300 ng/µL entry plasmid DNA of GFP, MdαE7 from step 1.2.7 or entry plasmid DNA of chloramphenicol acetyltransferase gene (CAT) provided by the cell transfection kit with 5 µL of commercially available (containing *att*L sites) C-term linear DNA (containing *att*R sites) in 250 µL PCR tubes. Add TE buffer to make a total volume of 8 µL.
    Note: The reactions of GFP and CAT were used as control groups.
    2. Add 2 µL of commercially available Lambda recombination (LR) enzyme mix (**Table of Materials**) into each mixture of step 1.3.1 to catalyze the LR reaction. Gently mix and incubate at 25 °C overnight (≈16 h).
    Note: The LR reaction facilitates the recombination of an *att*L-containing entry plasmid DNA with an *att*R-containing C-term linear DNA to generate an *att*B-containing expressing virus. This step produces the recombinant baculovirus for each target protein.

3. Dilute 1 µL of LR reaction products from step 1.3.2 20X. Perform PCR using a Polyhedrin forward primer and V5 reverse primer (**Table 1**). Use 5 µL of PCR products to run a 1% agarose gel to check the quality. **Figure 1** shows an example of the LR reaction product from the MdαE7 gene.

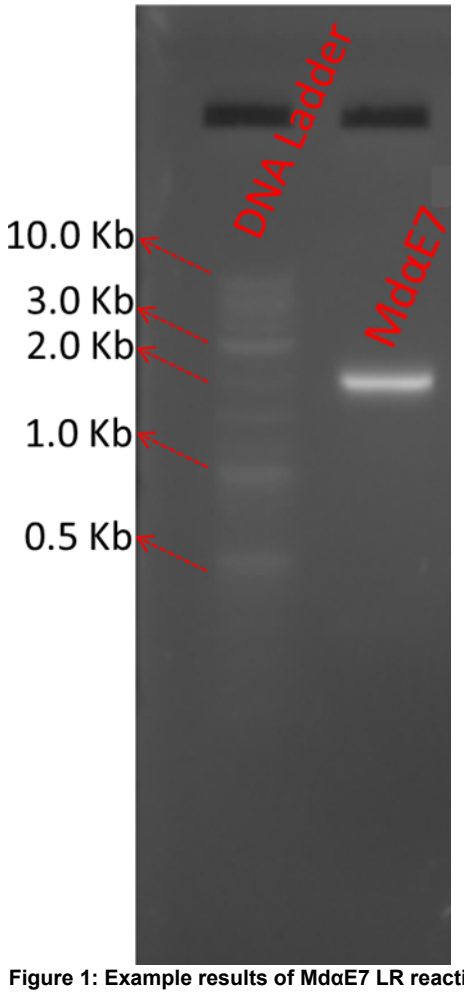


**Figure 1: Example results of MdαE7 LR reaction by PCR analysis.** Dilute 2 µL of LR reaction 200-fold and use 2 µL of dilution together with Polyhedrin forward primer and V5 reverse primer to perform a 25 µL PCR. Use 5 µL of PCR products to run 1% agarose gel to check the quality of LR reaction. a

4. Transfect insect Sf9 cells
    1. Culture insect Sf9 cells with 5 mL of complete cell growth medium (serum-free medium with 10% fetal bovine serum (FBS)) in T25 treated flasks at 27 ˚C.
    2. Remove the supernatant and flush bottom-attached cells with 3 mL of fresh complete cell growth medium. Transfer 0.5 mL of re-suspended cells into a new T25 treated flask. Add 4.5 mL of complete growth medium. Incubate at 27 °C for 3-4 days until the next transfer.
    3. Seed 2 mL of log-phase growth insect Sf9 cells culture (≈3.0-5.0×$10^6$ cells) evenly on the cell culture well. Allow cells to attach for at least 3 h at room temperature in the hood.
    4. Check the cell attachment by observing with an inverted phase microscope at 250X. Remove the cell culture medium and replace with 2 mL of Grace's insect medium.
    5. Prepare transfection mixture A solution (8 µL of commercially available cell infection reagent (**Table of Materials**) with 100 µL of Grace's insect medium) and transfection mixture B solution of each target gene (9 µL of LR reaction products from step 1.3 with 100 µL of unsupplemented Grace's insect medium) in 1.5 mL centrifuge tubes, respectively.
    6. Gently mix transfection mixture A and B together by tapping the tubes. Incubate at room temperature for 35 min in the hood.
    7. Evenly add the mixture from step 1.4.6 dropwise onto the seeded cells from step 1.4.4. Seal wells with tapes and incubate at 27 °C overnight.
    8. Replace 2 mL of Grace's insect medium with 2 mL of complete growth medium. Add 100 µM ganciclovir into each well to negatively select against non-recombinant baculovirus. Seal wells with tape and incubate at 27 °C for 72 h.
    9. Collect 72 h post infection cell culture medium from each well and transfer to 1.5 mL centrifuge tubes. Centrifuge at 1,500 x g for 5 min at 4 °C to remove cells or large debris.
    10. Transfer the supernatant into new 1.5 mL centrifuge tubes. Store them at 4 °C with protection from light.
    Note: These are the P1 virus stock solutions for each target gene.
    11. Amplify the low-titer P1 viral stock (1×$10^5$-1×$10^6$ pfu/mL) to a high titer P2 viral stock (5×$10^7$-1×$10^8$ pfu/mL).

12. Seed 2 mL of log-phase growth insect Sf9 cells culture (≈3.0-5.0×$10^6$ cells) evenly on the cell culture well. Allow cells to attach for at least 3 h at room temperature in the hood.
13. Inoculate 5 µL of P1 virus stock obtained in step 1.4.10 in the cell seeded well of step 1.4.12, respectively. Then, add 100 µM ganciclovir to each well. Seal wells and incubate at 27 °C for 72 h.
14. Collect P2 virus stock solutions of 72 h post infection. Store at 4 °C with protection from light.
    Note: These are the P2 virus stock solutions for each target gene.
15. (Optional) Repeat steps 1.4.12-1.4.14 with 5 µL of P2 virus stock to collect P3 virus stock solutions.
    Note: These are the P3 virus stock solutions for each target gene.
16. Store all the constructed baculovirus at 4 °C with protection from light.
    Note: The GFP and CAT gene were served as control group. **Figure 2** showed the signs of cells when infected by GFP-recombinant baculovirus at different amplification stages.

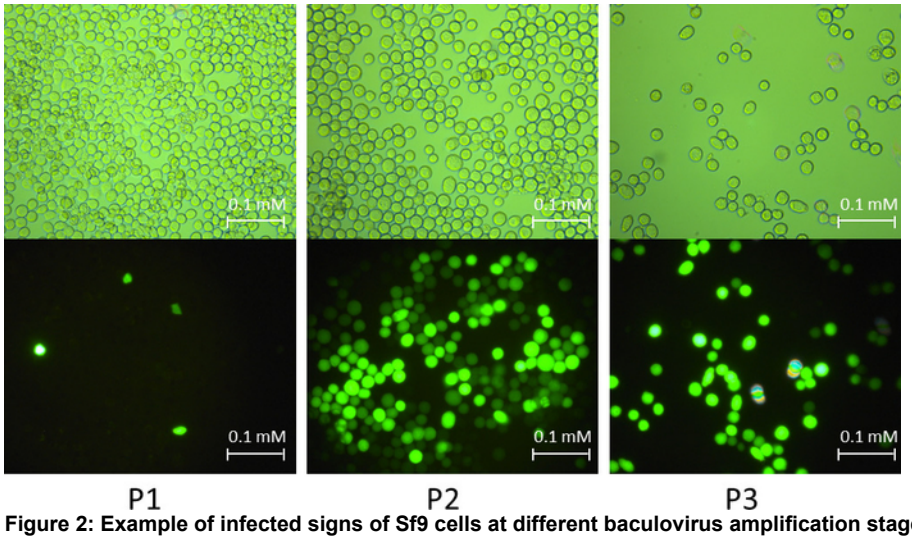


**Figure 2: Example of infected signs of Sf9 cells at different baculovirus amplification stages.** The figure shows GFP-recombinant baculovirus cells under natural light and fluorescent light. At the P1 infection stage, the infection ratio is low. At the P2 infection stage, the infection ratio was significantly enhanced. At the P3 infection stage, almost all the cells showed symptoms of detachment from cell culture plate, increases of cell diameter, as well as the cessation of cell growth. Please click here to view a larger version of this figure.

5. Large-scale expression of target proteins in insect Sf9 cells
    1. Culture 10 mL of log phase growth insect Sf9 cells with complete growth medium in T25 non-treated flasks.
    2. Add 200 µL of P2 virus stock solution (obtained in step 1.4.14) to infect cell cultures in step 1.5.1 for 72 h.
    3. Collect 72 h post infection cell culture medium. Centrifuge at 1,500 x g for 5 min at 4 °C.
    4. Discard supernatant and wash pellets twice with 1 mL of 0.1 M PBS buffer (pH 7.5).
    5. Fully dissolve cell pellets with 1 mL of insect cell protein extraction & lysis buffer (**Table of Materials**). Add 10% glycerol in cell lysis. Immediately store at -80 °C.
    6. Repeat steps 1.5.1-1.5.5 with P2 virus stock solution of CAT proteins to serve as the control group.
       Note: Repeat step 1.5 in triplicate for different protein preparations.

## 2. A Cell-based MTT Cytotoxicity Assay

1. Culture 5 mL of log phase growth (1.5-2.5×$10^6$ cells/mL) insect Sf9 cells with complete growth medium in T25 non-treated flasks.
2. Add 25 µL of P1 virus stock solution (obtained in step 1.4.14) to infect cell cultures in step 2.1 for 48 h with gentle shaking at 27 ˚C.
3. Prepare 100 mM permethrin standard stock solutions in acetonitrile. Use acetonitrile to dilute to 50 mM, 25 mM, 12.5 mM and 6.25 mM by adding 500 µL, 250 µL, 125 µL and 62.5 µL permethrin stock solution in a total volume of 1,000 µL, respectively.
4. Evenly seed 200 µL of infected cell cultures from step 2.2 supplemented with 300 µL of complete growth medium into 24-well plate at a density of 2×$10^5$ cells/mL.
5. Add 4 µL of permethrin standard solutions (6.25 mM, 12.5 mM, 25 mM and 50 mM) into each well to make a final concentration 50 µM, 100 µM, 200 µM and 400 µM, respectively. Only add 4 µL of acetonitrile into wells for cell viability calculations (**Figure 3**).
6. Seal plates with tapes and incubate at 27 °C for 48 h with protection from light.
7. Prepare 5 mg/mL MTT (Thiazolyl Blue Tetrazolium Bromide) reagent dissolved in buffer (2.0 g of NaCl, 0.05 g of KCl, 0.36 g of $NaH_2PO_4 \cdot 2H_2O$, 0.28 g of $NaH_2PO_4$, 0.05 g of $KH_2PO4$ dissolved in 200 mL of distilled water, pH 7.5).
8. Remove cell culture medium from step 2.6 after 48 h incubation without touching the bottom-attached cell layer. Add 200 µL of MTT reagent in step 2.7 into each well. Incubate at 37 °C for 4 h until dark purple formazan precipitates formed in each well (**Figure 3**).
9. Incubate at 37 °C for 4 h until dark purple formazan precipitates form in each well. Add 500 µL of DMSO to fully dissolve precipitates (**Figure 3**).

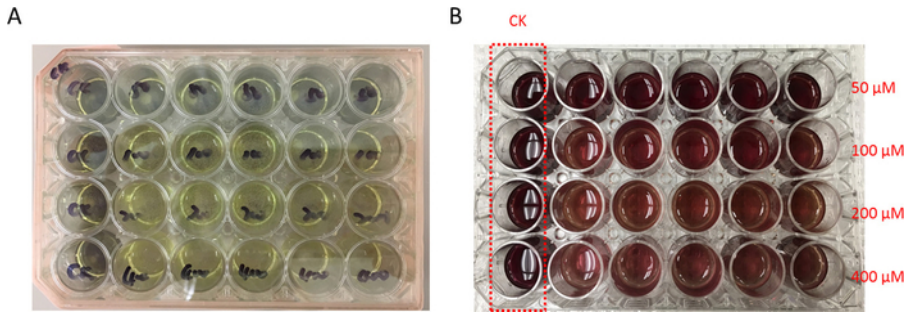


**Figure 3: Example of MTT results.** After 48 h post infection with P1 virus stock solution of CAT recombinant baculovirus solution, evenly seed 500 μL cells expressing CAT gene in a 24-well cell culture plate. Respectively, add 4 μL permethrin at a different dose (6.25 mM, 12.5 mM, 25 mM and 50 mM) in each row to make the final concentration to 50 μM, 100 μM, 200 μM and 400 μM. The control row was treated with acetonitrile only and marked as CK in the plate. (A) After 48 h of incubation at 27 °C, discard the cell culture medium on the upper layer and replace with 200 μL of yellow colored MTT reagents. Then incubate at 37 °C for 48 h. Dark purple colored reduction precipitates form in each well indicating that the survival cells are capable of metabolizing the yellow color MTT reagents into the dark purple colored precipitate. (B) 500 μL of DMSO solvent was added into each well to dissolve the formed precipitate. The absorbance value of each well was detected by spectrophotometer at 540 nm. As permethrin concentrations increase , the color will gradually change from dark red to light red, suggesting that the cell viabilities were gradually decreased. Please click here to view a larger version of this figure.

10. Transfer 200 μL of dissolved solution into 96-well plate. Measure absorbance values at 540 nm using a microplate reader (**Table of Materials**).
11. Calculate cell viability by comparing the absorbance values of permethrin treated cells with those of only acetonitrile treated cells.
12. For the control group, repeat all steps with P2 virus stock solution of chloramphenicol acetyltransferase (CAT) recombinant baculovirus obtained in step 1.4.
13. Repeat 4 times for different virus preparations.

## 3. *In Vitro* Metabolic Assay

1. Prepare 100 mM permethrin standard stock solutions in acetonitrile. Use acetonitrile to dilute to 50 mM, 25 mM, 12.5 mM and 6.25 mM. Detect the corresponding peak area under each concentration using HPLC. Create and calculate the standard curve of permethrin based on the peak area with different permethrin concentrations.
2. Measure the protein concentrations in step 1.5 with Bradford methods[21].
3. Prepare 700 μL of metabolic reaction with 40 μM permethrin standard and 1 mg of proteins obtained in step 1.5 dissolved in 0.2 M Tris-HCl buffer (pH 7.4). Incubate at 30 °C for 2 h with gentle shaking. Protect from light.
4. Quench the reaction by adding 700 μL of ice-cold acetonitrile. Incubate at 30 ˚C for another 30 min with gentle shaking. Protect from light.
5. Centrifuge the reaction mixture at 16,000 x g for 2 min at room temperature. Collect the supernatant and filter through a 0.45 μm membrane. Transfer the filtration into ultraclean brown glass vials for HPLC analysis.
6. Run the HPLC under the optimal chromatographic conditions (Mobile phase A: 90% acetonitrile and 10% water; Mobile phase B: 5% acetonitrile adjusted to pH 2.3 with 85% phosphoric acid). Gradient elute with a flow rate of 1 mL/min and measure at a wavelength of 232 nm.
7. Calculate the depletion percentage of permethrin by comparing with reactions without protein sample added.
8. Use CAT protein obtained in step 1.5.6 to serve as the control.
9. Repeat all the steps with different protein preparations in step 1.5.7.

## Representative Results

**The cell viability toward different permethrin treatments (MTT assay)**

The cytotoxicity of permethrin was examined in MdαE7-recombinant baculovirus infected Sf9 cells (experimental group) and CAT-recombinant baculovirus (provided by baculovirus infected kit) infected cells (control groups). The enhanced cell tolerances to permethrin in MdαE7 expressing cells strongly support the metabolic roles of this carboxylesterase against insecticides and thus protecting cells from chemical damages. In our study, the cell viability against different permethrin concentrations (50, 100, 200 and 400 μM) was calculated in comparison with cells treated with acetonitrile alone. Our results showed that the viability of MdαE7 expressing cells was significantly higher (ranging from 86.00% to 101.92%) (**Figure 4B**) than that of control cells (ranging from 67.59% to 81.43%) (**Figure 4A**) when exposed to permethrin at different concentrations, indicating the important roles of MdαE7 in metabolizing permethrin in insect cells.

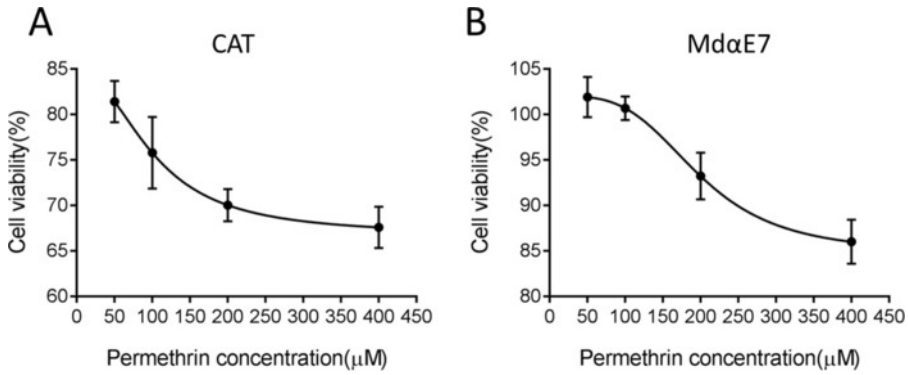


**Figure 4: The viabilities of insect Sf9 cells under different permethrin treatments.** (A) The viability of control cells infected by CAT recombinant baculovirus (B) The viability of experimental cells infected by MdαE7 recombinant baculovirus. Four replications were conducted for each permethrin concentration. Data was shown as mean ± SEM. Please click here to view a larger version of this figure.

***In vitro* metabolism of permethrin by MdαE7**

Permethrin metabolism was assayed by incubating a 40 μM permethrin standard solution together with MdαE7 proteins extracted from infected Sf9 cells. The reactions with CAT proteins served as controls. The depletion percentage of permethrin was calculated in comparison with reactions without enzyme added. Reactions were monitored by reverse-phase HPLC after a 120 min incubation period. Since the permethrin standard is actually a mixture of cis- and trans- isomers, two peaks were observed in the HPLC chromatographic profiles, with elution times of 10.67 min and 10.87 min for trans-permethrin and cis-permethrin, respectively (**Figure 5**). The depletion percentage of permethrin by MdαE7 proteins (39.18±3.78%) was significantly higher than that of CAT proteins (7.29±0.81%) (**Figure 6**), which is not only be consistent with MTT results but also directly demonstrates the capabilities of MdαE7 in metabolizing permethrin *in vitro*.

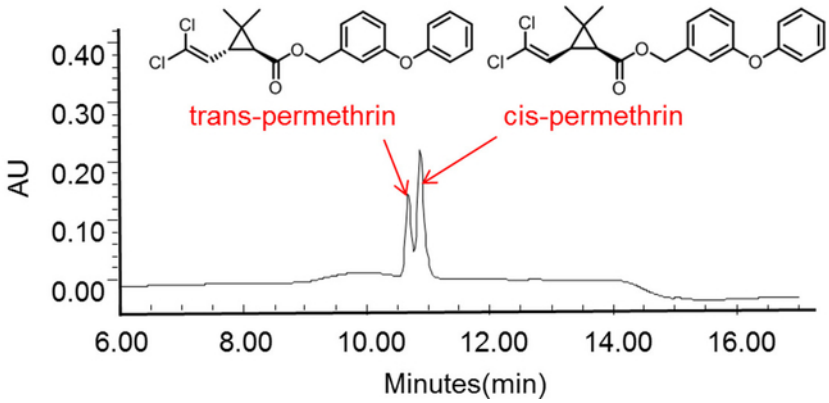


**Figure 5: The HPLC profile of permethrin standard.** The permethrin standard used in this study is a mixture of trans-permethrin and cis-permethrin isomers. The red arrows indicate the peaks for the trans-permethrin isomer and cis-permethrin isomer, respectively. Please click here to view a larger version of this figure.

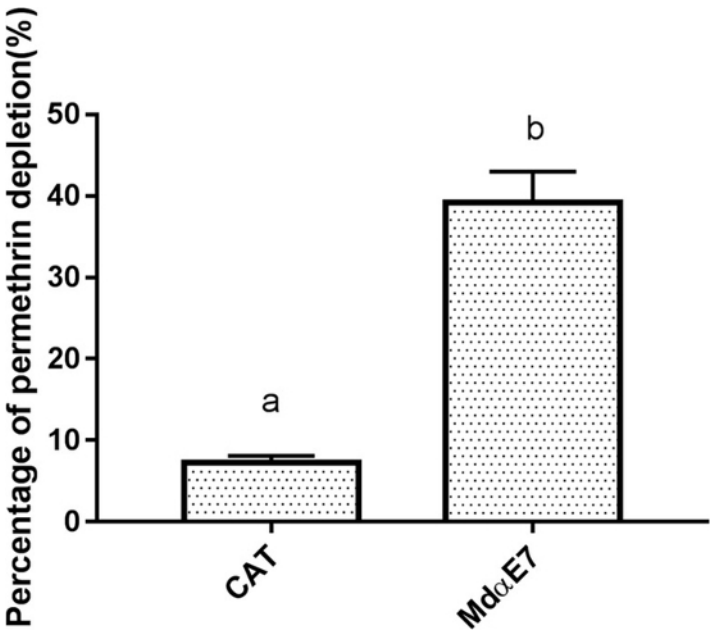


**Figure 6: *In vitro* metabolism of permethrin by MdαE7 and CAT recombinant proteins.** The depletion percentages of permethrin by MdαE7 and CAT proteins were calculated. Three replications were conducted for each permethrin concentration. Data was shown as mean ± SEM.
Please click here to view a larger version of this figure.

## Discussion

In recent decades, heterologous expression systems have been widely used to express and isolate large amounts of proteins, thus allowing biochemical and functional determination and characterization of enzymes *in vitro*. To date, several different model systems including *Escherichia coli*, *Pichia pastoris*, *Saccharomyces cerevisiae*, and *Spodoptera frugiperda* have been adapted for recombinant protein expression, and the choice of the *in vitro* system is crucial for large scale generation of interested proteins[22,23,24,25]. In our study, the baculovirus-mediated insect cell expression system to generate house fly carboxylesterases was chosen because it provides a similar intracellular environment to insect cells and maintains some important eukaryotic processing capabilities[9]. Despite the advantages of the baculovirus-mediated expressing system, its limitations should also be addressed. The baculovirus-mediated insect cell expression system can produce target proteins only if the insect cells were infected with constructed recombinant baculovirus. The current development of creating stably transformed insect cell lines has made it possible not only to constitutively produce interested proteins in the absence of baculovirus infection, but also to improve cell capabilities of glycosylating and folding generated proteins through engineering modifications[9].

To better investigate the roles of carboxylesterases in metabolizing insecticides in cells and thus protecting cells from chemical damages, the MTT assay was conducted to measure the cell cytotoxicity against different permethrin treatments. During this process, a lot of experimental parameters associated with cell metabolism, such as cell medium compositions, cell growth rate, concentration and consumption of energy supply metabolites, can influence the MTT reduction and thereby lead to inaccurate results. To minimize the over/underestimation of the MTT assay, keep the cell culture medium, cell growth state, and the incubation conditions consistent among different experimental treatments[26].
In order to determine the cell viability effects caused by baculovirus infection rather than permethrin, we showed that the infection process of GFP-recombinant baculovirus to insect Sf9 cells, and found that at the P2 infection stage, the cells have the highest infection ratio with lowest cell death ratio compared with to other infection stages (**Figure 2**). This can better explain the reason of choosing cells at P2 infection stage to conduct the MTT assay.

By comparing the viabilities of cells when infected by MdαE7- and CAT-recombinant baculovirus in an MTT study, we found an enhanced viability of cells when expressing MdαE7 proteins, which can better support the metabolic roles of carboxylesterases to permethrin in insect cells and protect cells from chemical damages (**Figure 4**). The *in vitro* metabolic study, which is recognized as a direct method to reflect the metabolic capabilities of carboxylesterases toward insecticides, should also been explored to compensate the limitations of MTT assay in better characterizing protein metabolic functions.

In this study, the heterologous expression of carboxylesterases in insect Sf9 cells with a baculovirus-mediated expression system, the measurement of cell viabilities toward different permethrin treatments by the MTT assay, and the characterization of carboxylesterases through an *in vitro* metabolism assay all work together to provide a systematic, scientific and accurate strategy to investigate the roles of detoxification enzymes in insects, thus facilitating a better understanding of insecticide resistance mechanisms and thereby developing innovative strategies for pest management.

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