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Research Article

Quantitative Analysis of *Philosamia cynthia ricini Ace 2 (Pcrace2)* Gene at Transcriptional Level and Prokaryotic Expression

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Abstract

The present work was carried out to investigate quantification of Ace2 gene expression that was obtained from midgut, fat body, malpighain tubule, head and haemolymph tissues of Philosamia cynthia ricini which was exposed to micro-amounts of pyrethroid (0.002 mg/L fenvalerate) insecticides. Actin3 used as internal control gene. A qPCR was employed and data were analyzed by excel version 16; the target gene was amplified by PCR and inserted into pET28a vector; recombinant plasmid DNA was transformed into E. coli BL21 competent cells; the hypothesized colonies were chosen on LB plate containing Kanamycin and confirmed by PCR, restriction enzyme digestion and sequencing analysis. The verified recombinant pET28a-Pcrace2 plasmid DNA was expressed through inducer (IPTG) for 24 h and protein was subjected for SDS-PAGE analysis. These experiments were done in June 2018 to March, 2019 at Sericultural Research Institute, Zhenjiang, China. Results showed that expression of *Pcrace2* gene can be increased due to fenvalerate exposure in all tissues under investigation, except at 9 hrs in FB and 3 hrs in haemolymph. 1485bp of *Pcrace2* gene CDS with 494 Amino acid sequences was successfully amplified; its phylogeny tree analysis revealed that *Pcrace2* gene has closely related to Ace 2 gene of B. Mori with accession number ABY50089.1; the partial (630bp) CDS of *Pcrace2* sequence was cloned and 23.1kbp protein was induced using 7.5 mM IPTG and 5.0 mMIPTG but control sample did not. Two concentration band sizes of inducer were similar which desires future work and relative expression level in FB tissue and the ORF of the gene requires further work as well.

Keywords: *Ace* 2; Cloning; *Philosamia cynthia ricini*; Prokaryotic Expression; qPCR

Introduction

The quality of silk production primarily depends on quality seeds, uncontaminated forages, disease free eggs and suitable environmental factors [1-7]. Most of the insecticides in common use today are toxic to insects. All pesticides are poisonous [11], although the degree of toxicity depends on the dose of the material and mechanism of action, among other factors [3,10]. Silkworms are highly susceptible to synthetic chemicals that pollute the environment [8], which result in great loss of cocoon yields [9].

Acetylcholinesterase (EC 3.1.1.7, AChE) is not only main target site for coining of insecticides but also used as a core site to develop and invent nerve agents and therapeutic drugs [12,13]. Therefore, AChE, which breaks down acetylcholine into acetic acid and choline, is the main target of nerve agents such as sarin as well as insecticides [14]. In light of these perspectives, it has received great attention by researchers and emerged as one of the significant research areas today [14,15]. Furthermore, because of its impressive catalytic power, the enzyme has been subjected to several pioneering studies in enzymology as well. In general, it is obvious that resolution of atomic structure of AChEs and extensive site-directed mutagenesis studies have also broadened scope of research work. However, evaluation and comparison of the results of different studies on AChE is complicated by the occurrence of different molecular forms and variability of the enzyme from species to species [16].

Indeed, AChE is a key element of cholinergic system which is catalysing hydrolysis of the neurotransmitter acetylcholine, thereby ceasing transmission of nerve impulses at synapses of cholinergic neurons in the central and peripheral nervous systems in both vertebrates and invertebrates [10,12]. Particularly, it is attached to cellular or basement membranes of presynaptic cholinergic neurons and post-synaptic cholinoceptive cells within the neuromuscular junction. Then, the signal transmission at neuromuscular junction involves the release of acylcholine by interaction with the acycholine receptor. Consequently, this feature of the event is characterized by a considerably wide substrate specificity and capable of cleaving neutral substrates with high reaction rates [10,12-16].

In the broader context, AChE can be found in various cells and tissues, for instance, skeleton, smooth and cardiac muscles, nerves, blood and glands of human [17,18]. The ubiquity of the enzyme suggests that it has diverse functions [19] which is not merely restricted to cholinergic system but also plays a number of biological roles in the regulation of cell growth and morphogenesis during embryogenesis and regeneration acting through its non-cholinergic sites [20-23].

In case of mammals, AChE is encoded by a single *Ace* gene while in most insects it is encoded by *Ace* 1 and *Ace* 2 genes even in some invertebrates more than two genes may be involved [24-26]. This gene is inhibited by organophosphate (OP), carbamate (CB) and Pyrethroid, which leads to the accumulation of ACh in postsynaptic membrane. Thus, this phenomenon results in death of the insect from convulsions [27]. The present study aimed at quantification, cloning and expression of the yellow Eri silkworm (*Philosamia cynthia ricini) Ace 2* (*Pcrace2*) gene when a third day of fifth instar larvae of *Philosamia cynthia ricini* were exposed to 0.002 mg/L of the Fenvalerate insecticide in order to understand the effects of this chemical on the silkworm.

Materials and Methods

Chemicals

TRIzol, chloroform, isopropanol, dNTP, SYBR Premix, Ex Taq, Fenvalerate, ddH_2O, IPTG, PBS, expression strain BL21 competent cell and other routine chemical reagents were used and all of them were purchased from TaKaRa Biotechnology (Dalian, China) and Sangon Biotech (Shanghai) Co., Ltd. Primers were designed by primer six software and synthesized by Shanghai Sangon Biological Technology and Services (Shanghai, China).

Material of silkworm, insecticide and leaves

A yellow Eri silkworm (*Philosamia cynthia ricin)* strain was reared at Eri Silkworm Seed Production Center of Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang, China, under normal conditions at 25 °C, 75 ± 5% relative humidity (RH) [1,5] and 12 h light:12 h dark photo-period with fresh *Ailanthus altissima* leaves. Rearing and whole experimental activities were carried out between June 2018 to March 2019. The Fenvalerate insecticide with 0.002mg/L dose was chosen based on the tolerated level from previous investigations in our laboratory. It was purchased from Hanghzou Qingfeng Imp. & Exp. Co., Ltd. (Hanghzou city, Zhejiang Province, China). The 3rd day Larvae of the 5th instar of *Philosamia cynthia ricini* were treated with this insecticide. *Ailanthus altissima* leaves were dipped into solutions of Fenvalerate diluted with distilled water for 20 seconds at the chosen dose of 0.002 mg/L. The organic solvent and water on the leaves were let to evaporate under room temperature. The treatment group of one hundred larvae was fed on the treated leaves while similar number of larvae in the control group were fed on non-treated leaves. In so doing, different tissues (midgut, head, fat body, malpighian tubule and haemolymph) of five larva samples from both treatment and control groups were dissected at an

interval of 0 h, 3 h, 6 h, 9 h, 12 h, and 24 h of the post-treatment and stored at −80 °C refrigerator till required for the next experiment for extraction of total RNA.

Extraction of total RNA

The yellow Eri silkworm midguts (MG), fat body (FB), head (HD), malpighain tubule (MT) and Haemolymph(HM) preserved were ground with liquid nitrogen and total RNAs of each tissue was extracted separately according to protocol of the manufacturer (Takara Biotechnology, Dalian Co. Ltd). Each concentration of RNA was quantified using a Nano-Drop 1,000 spectrophotometer. The purity of RNA sample was also assessed at an absorbance ratio of A260/280 and A260/230 and adjusted with diethylpyrocarbonate (DEPC) H_2O to a concentration of 500ng/µL. The integrity of the RNA was confirmed by 1% agarose gel electrophoresis. It was then kept at −80 °C refrigerator.

Quantitative real-time RT-PCR

Reverse transcription (RT) was carried out using cDNA synthesis kit according to the manufacturer's instructions (TaKaRa Biotechnology) i.e., 1000 ng of each sample of total RNA was reverse-transcribed in 20 µL of reaction system containing:1 µL Oligo dT Primer (50 µM), 1 µL Random 6mers (50 µM), 1 µL dNTP mixture (10 mMeach), 1 µL Total RNA, 10.5 µL RNase Free dH_2O, 4 µL 5 × Prime Script buffer (for Real time), 0.5 µL RNase inhibitor40 µ/µL, and 1µL Primer Script II RTase (200 µ/µL).

According to the manufacturer's instructions for the SYBR Premix Ex TaqTM (TaKaRa Biotechnology) a real time PCR volume reaction was set in a 20 µL system for each tube and was performed using thermal cycle PCR machine (Model7300; ABI, Shanghai, China) with the SYBR Premix Ex Taq Kit (TaKaRa Biotechnology) i.e., 10 µL SYBR, 0.8 µL P1, 0.8 µL P2, 0.4 µL Rox, 6 µL ddH_2O and 2 µL cDNA template. The reaction was carried out under the following conditions: denaturation at 95 °C for 5 minutes, followed by 45 cycles of 95 °C for 15 seconds, 60 °C for 31 seconds, followed by 1 cycle 95 °C for 15 seconds, 60 °C for 1 minute, and the last stage again by 1 cycle 95 °C for 15 seconds, and 60 °C for 15 seconds. The primers used were: Pcractin3 (P1) F: 5'-GGG CCG GAC TCG TCA TATT-3' and Pcractin3 (P2) R: 5'-ATC ACA GCC CTC GCT CCAT-3'with internal reference genes actin3. Then, data of the reaction were processed using sequence detection software (ABI), and analysed was done using the model of threshold cycle for relative quantification that is, allowing the fold change to be calculated using the formula 2.

cDNA synthesis for cloning

The total RNA of µL was subjected to cDNA synthesis with Prime Script TM (Shanghai, China) as in the aforementioned protocol kit. Then it was amplified through PCR using the primer PcrAce2-new-F 5'-ACG ATA CAT CT CGT TAA GCA AAT A-3' and PcrAce2-new-R 5'-GAA AGG AGC ATA AAG TAA AG CAA T-3' primers with PCR temperature conditions standardized as in the proceeding conditions: initial denaturation at 94 °C for 5 minutes, followed by 33 cycles of denaturation at 94 °C for 30 secconds, annealing at 55 °C for 30 second, extension at 72 °C for 2 minutes and a final extension of 72 °C for 7 minutes. The amplicon's length was proved by 1% agarose gel electrophoresis using 2kb DNA molecular weight marker and stored at -20 °C till PCR experiment was begun to obtain double strain DNA for rubber recovery.

Amplification of *Pcrace2* gene cDNA and rubber recovery

From preserved *Pcrace2* gene cDNA, a 2 µL cDNA was taken and amplified using the following gene-specific primers employing PCR with temperature conditions: initial denaturation at 94 °C for 5 minutes, followed by 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 60 °C for 30 seconds, extension at 72 °C for 1 minute and a final extension of 72 °C for 7 minutes. The amplicon's length was proved by 1% agarose gel electrophoresis using 2kb DNA molecular weight marker; and rubber recovery was performed as per manufacturer's instructions (Sangon Biotech (Shanghai) Co., Ltd). Then, DNA quality was determined by assessing absorbance ratio 260/280 nm using Nano Drop (ND-1000) Spectrophotometer with value fall between 40-80 ng/µL was taken as better quality. To clone gene in frame with pET28a vector, restriction sites were added to the primer Pcrace2-630-F (BamHI): 5'-GCG GAT CGC GGA TCC GCT GGT GGA GGA AGC GTA A-3' and Pcrace2-630-R (XhoI): 5'-CGG GCG CCG CTC GAG AAA GTA ATT GGT GGG GC-3'.

Double digestion and cloning of *Pcrace2* gene to pET28a vector

The rubber recovered product of *PcrAce2* gene and pET28a plasmid previously recovered in our laboratory were subjected to restriction endonuclease digestion with BamHI and XhoI in the proportion described in the following Table 1.

Then mixture of the solution was digested for 4 h at 37 °C. After 4 h of digestion, product was exposed to heat shock at 75 °C for 10 minutes to inactivate restriction endonuclease enzyme function. Then, the products were ligated using T_4 DNA ligase enzyme

ace 2 *P.cynthia ricini* gene		pET28a plasmid	
ddH_2O	4	ddH_2O	14
eGFP/template DNA/	12 µL	Plasmid recover	2 µL
10xkbuffer	2 µL	10xkbuffer	2 µL
BamHI	1 µL	BamHI	1 µL
XhoI	1µL	XhoI	1 µL
Total volume	20 µL		20 µL

Table 1: Double digestion of target gene and selected plasmid vector.

and buffer for overnight at 16 °C in the ratio of 4 µL plasmid, 12 µL gene of the digestion products, 2 µL of 10xT_4 buffer and 2 µL of T_4 ligase. Then, 50 µL *E. Coli* BL21 competent cells were mixed with 10 µL of ligated mixture of solution and incubated on ice for 30 minutes. After incubation, the cells were subjected to heat shock at 42 °C for 90 seconds followed by snap cooling on ice for 3 minutes. Further 0.8 mL of LB broth was added and incubated at 37 °C with 250 rpm constantly shaking for 90 minutes. Then it was centrifuged at 12000 rpm for 1 minute, 0.3 mL was discarded and the remaining 0.5 mL sediment was gently mixed; from the mixture 0.035 mL(35µL) was plated on solid LB agar containing 1 µL/mL of Kanamycin and incubated overnight at 37 °C.

Conformation of transformation by PCR and re-digestion

The colonies suspected for recombinant clones of pET28a-Pcrace2 gene were screened and inoculated into LB broth with 1 µL/mL Kanamycin at 37 °C for overnight cultured at 250 rpm. Then, the PCR was carried with pET-28a-F 5'-TAATACGACTCACTATAGGG-3' and pET-28a-R-5'-TGCTAGTTATTGCTCAGCGG-3' primers for confirmation with PCR temperature conditions: initial denaturation at 94 °C for 5 minutes, followed by 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 60 °C for 30 seconds, extension at 72 °C for 1 minute and a final extension of 72^{0}C for 7 minutes. In so doing, 1% agarose gel electrophoresis was run using 2kb DNA molecular weight marker; and rubber recovery was performed as per manufacturer's instructions (Sangon Biotech (Shanghai) Co., Ltd) and some fragment part was sent to Shanghai Biotechnology services co., Ltd for sequencing. In turn, the recombinant plasmid DNA extract was subjected to re-digestion with BamHI and XhoI as in the aforementioned procedures. Then again 1% agarose gel electrophoresis was run with both 2kb and 5kb DNA molecular weight markers in order to cross check the ligation success. And then the successful ligation products were stored at 4-8 °C for the next steps.

Expression of recombinant pET28a-*Pcrace2* gene

From recombinant pET28a-Pcrace2 gene, a positive transformant was selected considering the above stated methods. The positive clones were inoculated into 1mL LB containing 1 µL/mL kanamycin and incubated for overnight at 37 °C at 250 rpm with constant shaking. The incubated culture was reinoculated into three tubes with 4 µL fresh liquid medium with ration of 1:80 with kanamycin and shaking at 37 °C and 250 rpm with constant shaking 2:30 to 3 h until their OD_{600} reached between 0.8–1.0. When the intended OD_{600} values was obtained, the culture was divided into 2 sections with volume of 2 µL per each 10 mL culturing tube. Then, each sample was adjusted for their inducer concentration in the following manner: sample one was 7.5 mM IPTG, sample two was 5.0 mM IPTG and sample three (control) was 0.0 mM IPTG, respectively. In so doing, all tubes were incubated for 24 h at 37 °C with 250 rpm in a constant shaking. And then 1.5 mL amount was poured into separate and marked 1.5 mLEp tubes with 7.5 mM IPTG, 5.0 mM IPTG and 0.0 mM IPTG one by one, respectively; tubes were centrifuged at 12,000 rpm for 2 minutes; supernatant was discarded; 500 µL PBS was used to wash a sediment; 50 µL PBS was added to each 1.5 mL EP tubes and well mixed with pellet; 10 µL 5x SDS PAGE protein dying buffer poured to each tubes mixed and vortex as well; proteins were denatured at boiling point (100°C); after heat shock, mixture was centrifuged at 12,000 rpm for 2 minutes; and 5 µL supernatant was poured into well of 12% SDS-PAGE along with 12% SDS PAGE protein molecular weight markers as standard and analysed.

Results

Quantification of *Pcrace2* gene

Real-time RT-PCR

To investigate expression profile of *Ace* 2, at transcriptional levels of *Pcrace2* gene in different tissues and at various time interval after exposure to insecticide of *P. cynthia ricini* were determined by qRT-PCR. Results showed that *Pcrace2* gene transcripts were found in various tissues with distinct levels. Comparatively, it was expressed highest in the head (HD) and followed by the FB, and progressively lower amounts were expressed in BD, MG, and MT, respectively (Figure 1).

The trends of expression revealed that the level of *Pcrace2* expression has increased in all checked tissues (such as HD, FB

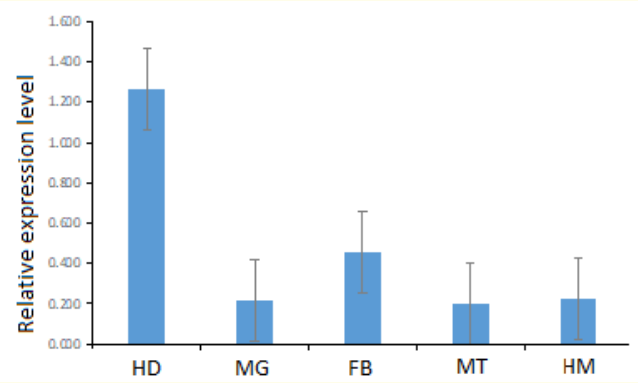

Figure 1: *PcrAce2* gene expression level in different tissues of P. *cynthia ricini*.

and HM) of *P. cynthia ricini* (Figures 2 - 4), except at 3 h and 9 h of HM and FB, respectively. And then, when worms were exposed to Fenvalerate insecticide their level of expression of *Pcrace2* rose up with time in relation to the control. Thus, a prolonged exposure to insecticide causes an over-expression of the gene to a certain level within similar tissues before it calms down to lower points.

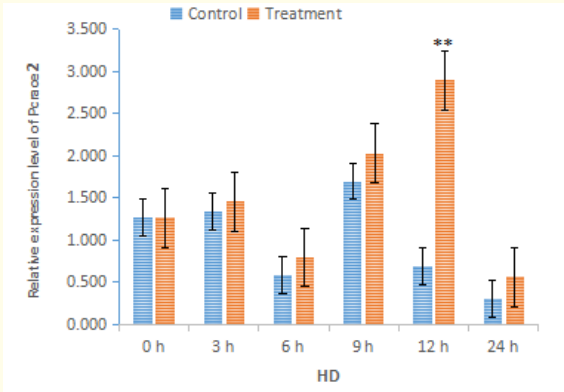

Figure 2: *Pcrace2* gene relative expression level in HD at different time intervals.

** significant at p < 0.001.

In the head tissue, the treatment group has more expression level of *Pcrace2* gene than control in all time of the investigation. The level of expression is statistically significant with p value < 0.001 due to fenvalerate insecticides exposure (Figure 2).

The expression level of *Pcrace2* gene of treatment group in fat body tissue at different time intervals were greater than

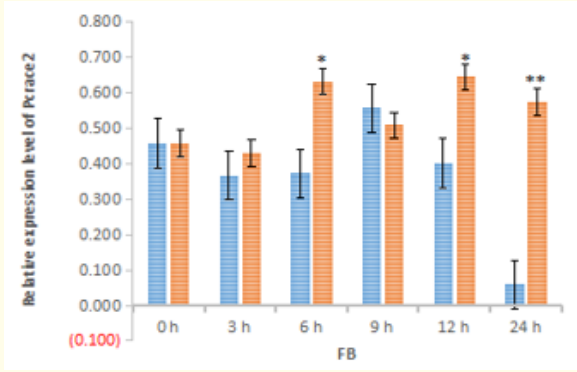

Figure 3: *Pcrace2* expression level in FB at different time intervals.

* significant at P < 0.05, ** significant at p < 0.01.

control, except at 9 h of the exposure. The level of expression was statistically significant with p < 0.05 in the case of 6 h and 12 h and at 24 h, it was significant with p < 0.01 (Figure 3).

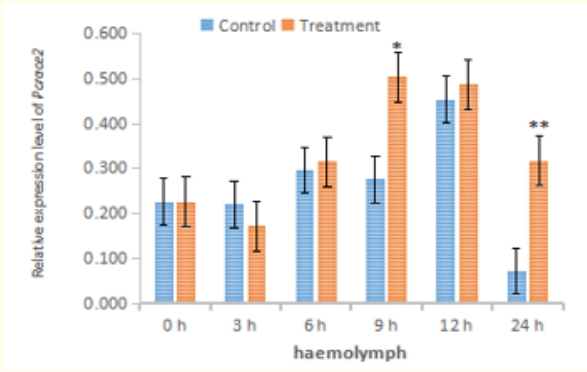

Figure 4: *Pcrace2* gene relative expression level in HM at different time intervals.

* significant at P < 0.05, ** significant at p < 0.01.

Similarly, in haemolymph tissue, treatment group has more expression level than control group except at 3 h of the exposure which control group has more expression than treatment. Statistically, the expression was significant with p < 0.01 at 24 h and p < 0.05 at 9h (Figure 4).

Prokaryotic expression of the target gene

Sequencing of CDS sequence of *Pcrace2* gene

The cDNA obtained from HD tissue at 12 h was amplified. Its PCR product had a band size of 1500bp when viewed under Ultraviolet

(UV) light (Figure 5). To verify the length, 50 µL of PCR product was sent to the company for sequencing. The obtained a result from the company has an ORF of 1485bp length of CDS sequence and 494 amino acid coding sequences (Figure 6).

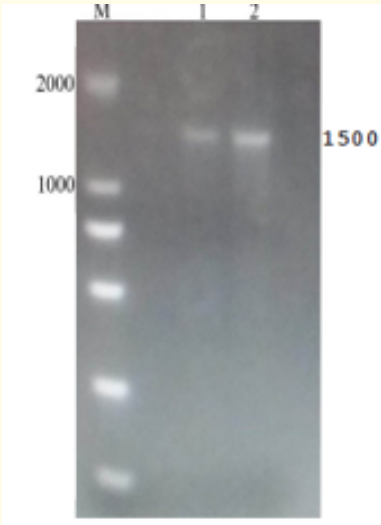

Figure 5: CDS sequencing of *Pcrace2* gene.

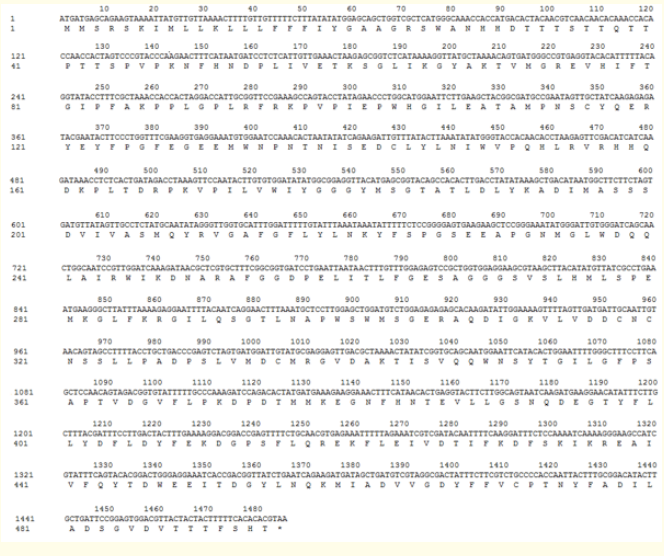

Figure 6: CDS sequence of *Pcrace2* gene.

The full length of *Pcrace2* gene CDS sequence has ATG at the beginning of the sequence (transcription starting site) and TAA which was represented by * is stop codon of the target gene where transcription was wrapped up (figure 6, DNAMAN8 software).

Phylogeny tree analysis of the *Pcrace2*

From the NCBI database sources, 8 sequences were downloaded and aligned them with *Pcrace2* gene CDS sequence using a Mega7 software. The result of the phylogeny tree analysis revealed that *Pcrace2* gene has closely related to *Ace* 2 gene of *B. Mori* (accession number ABY50089.1) while other sequences were more divergent from our results (Figure 7).

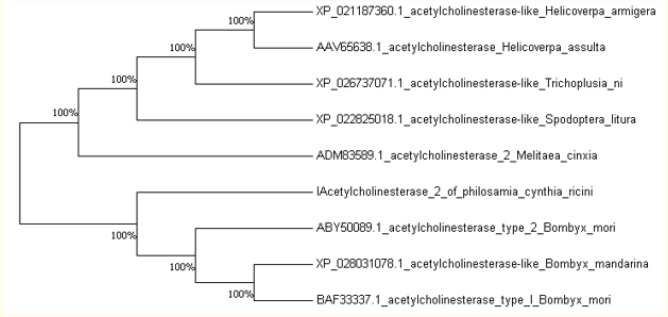

Figure 7: Phylogeny tree of *Pcrace2* gene with other genes in NCBI.

The phylogeny tree was construction using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.13554370 is shown. The evolutionary distances were computed using the p-distance method and are in the units of the number of amino acid differences per site. The proportion of sites where at least 1 unambiguous base is present in at least 1 sequence for each descendent clade is shown next to each internal node in the tree. The analysis involved 9 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 492 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

Cloning

Amplification of the partial (630bp) CDS of the *Pcrace2* gene

The total RNA was extracted using Trizol® reagent and checked for purity by spectrophotometry of Nano Drop-1000 (ND-

1000) and subjected to cDNA synthesis. The result of amplified *Pcrace2* encoding gene fragment was analyzed on 1% agarose gel electrophoresis by pipetting a volume of 5 µL 2kbp DNA molecular weight marker mixed with 1 µL 6*loading buffer of DNA marker to lane M and 50 µL PCR products which was properly mixed with 10 µL of 6*loading buffer to lane 1, and electrophoresis reaction was run at 350 volt and 180mA for 25 minutes. The result of analysis was shown a band size of 0.63kb (Figure 8).

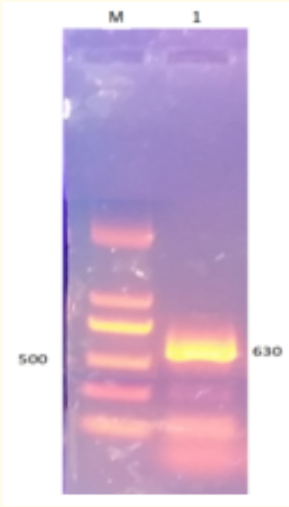

Figure 8: PCR result of *Pcrace2* gene.

Where: lane M is 2kbp DNA molecular weight marker, lane 1 is RT-PCR product of *Pcrace2* gene fragment.

Double digestion by BamHI-XhoI restriction enzymes

A rubber recovered PCR product of *Pcrace2* gene fragment and pET28a plasmid DNA vector were subjected to double-digestion with BamHI-XhoI endonuclease restriction enzymes for 4 h at 37 °C then exposed to heat (75 °C) shock for 10 minutes to inactivate the enzymes. Products were analysed on 1% agarose gel electrophoresis with volume of 5 µL of 2kbp DNA molecular weight marker and 1 µL of 6*DNA loading buffer were mixed and pipetted into lane M while 3 µL of double-digestion product of *Pcrace2*, 2 µL of ddH_2O and 1 µL of 6*loading buffer were mixed properly and poured into lane 1 as well; the electrophoresis reaction was run at 350 volt and 180mA for 25 minutes. Results are shown with a band size of 0.63kb (Figure 7).

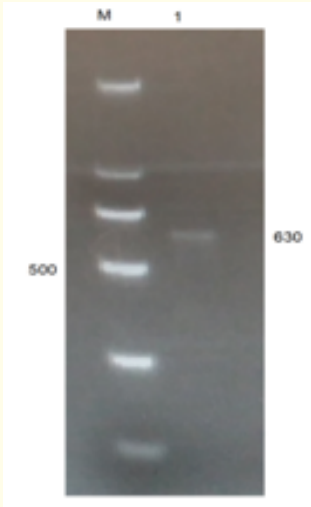

Figure 9: Double digestion of *Pcrace2* gene fragment.

Where: lane M is 2kbp DNA molecular weight marker, lane 1 is double digestion of *Pcrace2* gene fragment.

As aforementioned procedure, similar steps were permitted to run electrophoresis for restricted product of pET28a plasmid DNA vector except using 5kbp DNA molecular weight maker instead of 2kbp, the result shown a band size of 5.369kbp of linearized vector (Figure 8).

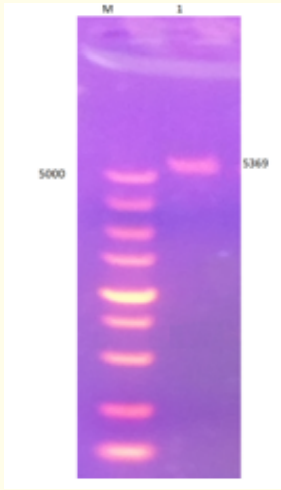

Figure 10: Double digestion of pET28a plasmid DNA vector.

Where: lane M is 2kbp DNA molecular weight marker, lane 1 is double digestion of pET28a plasmid DNA vector.

Pcrace2 gene cloning and conformation of transformation

The double-digested products were ligated and inserted into expression vector pET28a and transformed into BL21 competent cells of *E. Coli* genome. After overnight incubation at 37 °C , 12 good size mono colonies were randomly picked up on LB agar plates containing 1µl/ml kanamycin and cultured overnight. Then they were subjected to PCR analysis. The result of PCR product was evaluated on 1% agarose gel electrophoresis. Out of 12 colonies, 2 colonies have a band at 0.63kbp which portrays the successful insertion of intended fragment into pET28a vector (Figure 9).

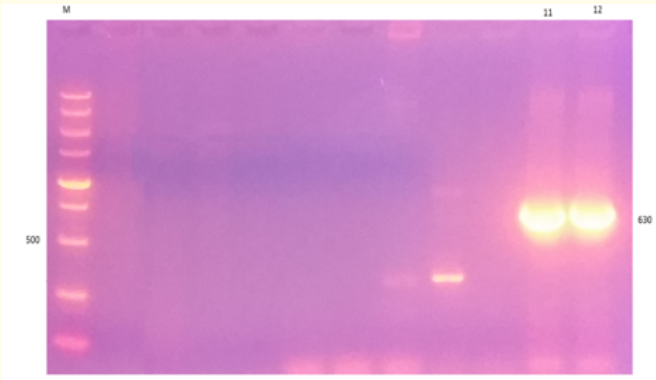

Figure 11: 1% Agarose gel electrophoresis of colony PCR products from positive clones.

Where: lane M is 5kbp DNA molecular weight marker, lanes of 11 and 12 are PCR product of transformants.

As we can seen in the figure 9, Lane M - stands for 5kbp a DNA molecular weight marker of the reference point against which the result was measured, Lane 11 and 12 have a band size at 0.63kbp of PCR amplified *Pcrace2* gene from recombinant vector while remaining Lanes have not shown hypothesized size length of the band which indicates that there were no successful insertions of target fragment of the gene into the vectors which could not longer be used for further experimental purposes.

The positive colonies of PCR result of recombinant plasmid DNA were extracted and subjected to re-double digestion using BamHI-XhoI endonuclease restriction enzymes for pET28a-Pcrace2 recombinant test. And then analysed again on 1% agarose gel electrophoresis. Two bands were observed of which one is specific to linearized pET28a vector at 5.369 kbp and the other *PcrAce2* gene at 0.63kbp (Figure 10).

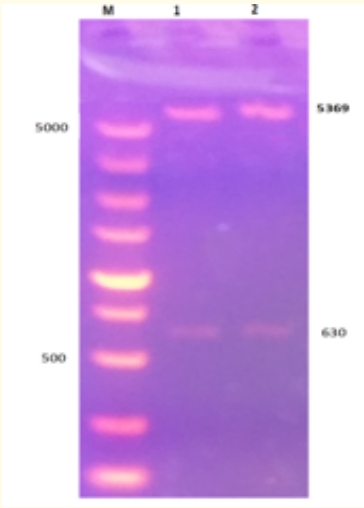

Figure 12: 1% Agarose gel electrophoresis of re-digestion of pET28- *Pcrace2* plasmid.

Where: lane M is 5kbp DNA molecular weight marker, lane 1-2 are re-double digestion of recombinant plasmid DNA.

Based on the result of the PCR and 1% agarose gel electrophoresis evaluation, the positive colonies of the pET28a-Pcrace2 plasmid DNA were also sent to company in order to verify the insertion of the target gene into plasmid which aligned with NCBI and the partial sequences was translated into amino acid sequences using DNAMAN8 (Figure 13).

```
          10        20        30        40        50        60        70
1     GCTGGTGGAGGAAGCGTAAGCTTACATATGTTATCGCCTGAAATGAAGGGCTTATTTAAAAGAGGAATTTTACAA
1      A  G  G  G  S  V  S  L  H  M  L  S  P  E  M  K  G  L  F  K  R  G  I  L  Q
            85        95       105       115       125       135       145
76    TCAGGAACTTTAAATGCTCCTTGGAGCTGGATGTCTGGAGAGAGAGCACAAGATATTGGAAAAGTTTTAGTTGAT
26     S  G  T  L  N  A  P  W  S  W  M  S  G  E  R  A  Q  D  I  G  K  V  L  V  D
            160       170       180       190       200       210       220
151   GATTGCAATTGTAACAGTAGCCTTTTACCTGCTGACCCGAGTCTAGTGATGGATTGTATGCGAGGAGTTGACGCT
51     D  C  N  C  N  S  S  L  L  P  A  D  P  S  L  V  M  D  C  M  R  G  V  D  A
            235       245       255       265       275       285       295
226   AAAACTATATCGGGTGCAGCAATGGGAATTCATACACTGGGAATTTTGGGCTTTCCTTCAGCTCCAACAGTAGACGGT
76     K  T  I  S  V  Q  Q  W  N  S  Y  T  G  I  L  G  F  P  S  A  P  T  V  D  G
            310       320       330       340       350       360       370
301   GTATTTTTGCCCAAAGATCCAGACACTATGATGAAAGAAGGAAACTTTCATAACACTGAGGTACTTCTTGGCAGT
101    V  F  L  P  K  D  P  D  T  M  M  K  E  G  N  F  H  N  T  E  V  L  L  G  S
            385       395       405       415       425       435       445
376   AATCAAGATGAAGGAACATATTTCTTGCTTTACGATTTCCTTGACTACTTTGAAAAGGACGGACCGAGTTTTCTG
126    N  Q  D  E  G  T  Y  F  L  L  Y  D  F  L  D  Y  F  E  K  D  G  P  S  F  L
            460       470       480       490       500       510       520
451   CAACGTGAGAAATTTTTAGAAATCGTCGATACAATTTTCAAGGATTTCTCCAAAATCAAAAGGGAAGCCATCGTA
151    Q  R  E  K  F  L  E  I  V  D  T  I  F  K  D  F  S  K  I  K  R  E  A  I  V
            535       545       555       565       575       585       595
526   TTTCAGTACACGGACTGGGAGGAAATCACCGACGGTTATCTGAATCAGAAGATGATAGCTGATGTCGTAGGCGAC
176    F  Q  Y  T  D  W  E  E  I  T  D  G  Y  L  N  Q  K  M  I  A  D  V  V  G  D
            610       620       630
601   TATTTCTTCGTCTGCCCCACCAATTACTTT
201    Y  F  F  V  C  P  T  N  Y  F
```

Figure 13: Partial CDS sequence of *Pcrace2* gene.

Expression

Expression of recombinant pET28a-*Pcrace2* and SDS PAGE analysis

The positive recombinant plasmids pET28a-Pcrace2 were subjected to transformation in BL21 competent cells. Their positive clones' supernatant were induced with 7.5 mM IPTG, 5.0 mM IPTG and 0.0 mM IPTG (control) for 24 h. After induction, supernatant was collected and subjected to SDS-PAGE analysis. The colonies induced of recombinant pET28a-Pcrace2 plasmid and non IPTG induced (recombinant without IPTG treatment) were run along the side of protein molecular weight marker. A result of 23.4kDa protein band was observed for both samples of IPTG treatments, whereas in case of control the hypothesized protein bands were not detected (Figure 14).

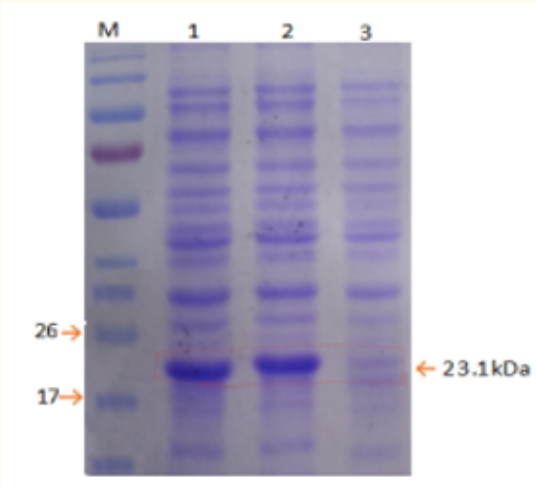

Figure 14: SDS-PAGE analysis of *Pcrace2* gene.

Where: Lane M - protein weight marker, Lane 1 -supernatant of 7.5 mM IPTG induced sample, Lane 2 - supernatant of 5.0 mM IPTG induced sample and Lane 3-supernatant of 0.0 mM IPTG (control sample).

Discussion

Acetylcholinesterase (EC 3.1.1.7, AChE) is a key enzyme in the family of serine hydrolases that possesses active site with esterasic subsite in which serine, histidine, and glutamic acid are expected to be engaged in a charge relay system [28-30]. Its main function is to catalyse hydrolysis of neurotransmitter acetylcholine [27] which results in termination of impulse transmission at cholinergic synapses. This enzyme also plays a crucial role in the nervous system of insects in such a way that understanding each function of elements and signal pathways in cholinergic system is essential to develop inhibitors of AChE enzyme as insecticide [10,12,17,18,29,30]. Obviously, AChE enzyme is highly susceptible to insecticides, and in case of domesticated silkworm susceptibility of the enzyme is more severe [30,31].

Different enzymes are used for detection of pesticide effects on non-targeted organisms. For instance, organophosphorus hydrolase and mouse AChE have been expressed on the surface of micro-organisms [8]. In this study, we took the domesticated yellow strain of *Philosama cynthia ricini* (a member of the Saturniidae family [33]) as *Ace* 2 gene source to investigate sensitivity of the AChE enzyme to Fenvalerate insecticide exposure. Thereafter, we quantified its presence in midguts (MG), fat body (FB), head (HD), malpighain tubule (MT) and haemolymph (HM) tissues of the silkworm. We then cloned and expressed it in its head taking into consideration our quantified value presented in Figure 1 and previous research findings which argued that AChE genes were mainly expressed in the brain [32,34,35].

The qRT-PCR result of the current work also indicated that expression level of *Pcrace2* genes varied within same tissue and peaks its level of expression in the beginning followed by gradual decline after the exposure. For instance, at 12 h post-exposure, *Pcrace2* gene expression in the head reached the highest point with $p < 0.01$ (Figure 2). This finding was similar to the report of Zhu., *et al*. [36] who looked at AChE inhibition effect on medaka (*Oryzias latipes*) due to exposure to three different insecticides. Even though the general trend of expression we had obtained in FB tissues were similar with Zhu and his colleagues, the variation was not as such which needs further intervention.

The qRT-PCR result of *Pcrace2* gene which was quantified at 12 h after exposure from head samples were highly expressed in heads and reduced after 24 h of the exposure (Figure 2), this result was similar with Hui and his colleagues [14] who argued that *Ace* genes are often expressed in heads, based on this circumstance, we were chosen *Pcrace2* gene which was quantified at 12 h to amplify (Figure 8) for cloning into pET28a plasmid and transformed into BL21 competent cells. The employed pET system with *E. coli*

BL21 competent cells had a well noticed expression band in both samples (Figure 14) upon induction of 7.5 mM IPTG and 5.0 mM IPTG concentration for 24 h. Control samples did not bear similar protein band at specified length which depicted that the inducer (IPTG) clearly played its role in inhibiting the lac repressor binding to DNA and allow for T_7 RNA polymerase to assess the promoter site gene to express target gene of fragment. While, there was not any size difference detected between two treatment samples due to change in IPTG concentration. These results indicated that mutant part of *Ace* gene in our target gene was not influenced our experiment (data was not shown) so we infer that the target gene is not mutated in response to insecticides, our argument is similar with Wang., *et al*. [37]. They constructed plasmids containing different mutant sites of the *Ace* gene that mutant AChE showed significantly higher remaining enzyme activity than the wild-type, indicating the close relationship between the mutated sites and AChE's sensitivity to physostigmine and phoxim. Juan., *et al*. [38], reported that the open reading frame of *Pcrace2* gene is 1497 with 497 amino acid sequences, on the contrary we obtained 1485bp ORF with 494 amino acid sequences. But, based on our phylogeny tree analysis, *Pcrace2* shared the closest relation with *Ace* 2 gene of *B. Mori* (accession number ABY50089.1), we had similar results with Juan and his co-workers phylogenetic tree that they stated that *Philosamia cynthia ricini* had close genetic relationship with Bombyx mori and B*ombyx mandarina.* Hence these issues need further investigation.

Conclusion

In conclusion, the level of expression of *Pcrace2* gene was higher in the head (HD) than in other tissues and during early period of feeding on Fenvalerate insecticide with in a similar tissue, the trends of the relative expression level of *Pcrace2* gene in all tissues under investigation were shown increment then gradually declined down in the later hours, except in 3 h of HM and 9 h of FB tissues. The *Pcrace2* gene has 1485bp of open reading frames (ORFs) with 494 amino acid sequences which its partial recombinant protein has been expressed which verifies that pET28a recombinant vector is suitable for expression of target protein which enable us to understand that *Pcrace2* gene has not mutated to cope with adverse effects of insecticides. We concluded that the insecticide fenvalerate has adverse effects on *P.cynthia ricini's Ace 2 gene* expression at transcriptional level. In this study, we applied 0.002 mgL dose of fenvelarate insecticide to treat our samples for 24 h and collected sample tissues at 0 h, 3 h, 6 h, 9 h, 12 h and 24 h post-treatment. Moreover, 7.5 mMIPTG and 5.0 mMIPTG was applied to induce target fragment's protein for 24 h in constant shaking at 250 rpm. Further researches concerning these issues, and length of the ORF and amino acid of the gene need to be done.

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Conflict of Interest

The authors declare that there is no conflict of interest in the conduct of this experiment and publication of the findings.

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