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Regulation of Alkaline Phosphatase Gene in Cotton Bollworm, *Helicoverpa armigera* by Cantharidin Extracted from Dried Meloidae, Blister Beetles

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Abstract The high toxicity of cantharidin was validated by previous researchers, particularly on lepidopteran insects. Nevertheless, toxicity mechanism is not well understood so far. In China, cantharidin has been used directly as an insecticide or along with other insecticides for a synergistic effect in pest management. Evidently, for the effectiveness of a synergist, it must prevent the action of metabolic enzymes, particularly those involved in insecticide degradation, biological and environmental stresses. Alkaline phosphatase, the ubiquitous enzyme is an important enzyme implicated in diverse functions with reports of its involvement in resistance towards insecticides. The current studies were performed to study the influence of cantharidin towards regulation of *Helicoverpa armigera* alkaline phosphatase gene (HaALP) (GenBank: EU729322) using real-time qPCR. Our results confirmed down regulation of alkaline phosphatase within the midgut of *Helicoverpa armigera* in response to a sub-lethal dose at 96 hrs post treatment by1/0.48-fold compared to control. This specific down regulation of *Helicoverpa armigera* alkaline phosphatase gene was also confirmed through an immunoblotting blotting assay.

Keywords Cantharidin; lepidopteran; synergist; alkaline phosphatase; *Helicoverpa armigera*; down-regulation

Introduction

The ubiquitous enzyme, alkaline phosphatase (ALP) is an essential metalloenzyme that splits phosphate groups of organic compounds in biochemical reactions. ALPs are known for their hydrolytic activity which hydrolyzes phosphomonoesters in an alkaline situation in nature. Normally, in animals ALPs are primarily present in the intestinal epithelium. The principal role of these enzymes is to deliver phosphate ions out of mononucleotide and ribonucleo-proteins which play a significant role in a variety of metabolic functions. ALPs involvement in transphosphorylation reaction has also been reported [1]. The ubiquitous occurrence of ALPs indicates their involvement in a variety of molecular functions, though their precise physiological activity and the natural substrate is not well understood [2]. ALPs also take part in numerous physiological processes and react against biotic and abiotic stresses. [3-7]

Cantharidin is a well-recognized natural toxin extracted from the blister beetles [8-9]. In China, this natural toxin has been used as a biological pesticide formulated as an emulsifible concentrate (EC) [10]. In earlier investigations, it has been reported to act as an antifeedant with insecticidal action [11]. However, the way it causes toxicity in insects is not very well understood. The activity of cantharidin *in vivo* as an inhibitor of ALPs was earlier documented in

armyworm, *Mythimna separata* [12]. Moreover, the synergistic activity of cantharidin was studied in diamondback moth, *Plutella xylostella* while using it alongside other commercial chemical insecticides, specifically, abamectin, bisultap, chlorfluazuron, endosulfan, and methomyl in different commercial formulations. Cantharidin mixture with chlorfluazuron demonstrated the maximum synergistic activity and ranked at the top [13].

The present studies were designed to examine the gene regulation of *Helicoverpa armigera* alkaline phosphatase using real-time qPCR. For the investigation an *H. armigera* alkaline phosphatase gene (HaALP) (GenBank accession no. EU729322) was cloned from the insects' midguts. The immunoblotting assay was used to confirm the results of real-time qPCR.

Materials and Methods

Experimental Insect

Experiment insect, *H. armigera* were supplied by Zhongke Baiyun Biotech. Co., Ltd. Beijing, China. The insects were raised with artificial diet under laboratory conditions [14] maintained at 27+1ºC and 45 to 55% RH with a 12 h light period in 24-cell plastic boxes.

Candidate Chemical

Candidate chemical, cantharidin was isolated through a chemical process [15] in the laboratory from beetles commercially procured from the market.

Molecular Cloning

Midguts from larvae were dissected on ice, washed with ice cold 1x phosphate buffer. RNAiso Plus (TaKaRa, Dalian, China) was added to the grounded midgets using liquid nitrogen. Using the suggested protocol total RNA was extracted. RT-PCR using RevertAid™ Reverse Transcriptase (Fermentas, Thermo Fisher Scientific Inc.) was used for the synthesis of complementary DNA (cDNA) in a reaction mixture of 20 µL .

The sequence specific gene primers were used for amplification of a target sequence of alkaline phosphatase in *Helicoverpa armigera* (HaALP) (GenBank: EU729322) from cDNA through PCR (Table 1). Two restriction sites *Bam*HI and *Xho*I were included in a sense and anti-sense primers, respectively for restriction digestion reaction. The PCR program for the amplification was set at: Denaturation for 3 min at 94 ºC (First step) followed by 30 cycles of 94 ºC for 30 s, 60 ºC for 45 s, 72 ºC for 2 min and last extension of 7 min at 72 ºC. The resultant PCR band was visualized on 1 % agarose gel having ethidiun bromide through a BioRad™ imaging system. The specific amplified product of the target gene was isolated by gel purification kit (Biomiga Inc. San Diego, California), and subsequently incorporated to pMD-19 simple Tvector (TaKaRa, Dalian, China). The resultant plasmids were transformed into DH5α, *Escherichia coli*. The ampicillin incorporated (100 µg mL^{-1}) LB agar plates were used to select the positive transformants at 37 ºC after an overnight incubation. The positively selected clones were sent to Shanghai Sunny Biotech Co., Ltd for sequencing. The obtained sequences were examined by DNAman (Lynnon, Quebec, Canada).

Production of Recombinant Plasmid and its Expression

The recombinant plasmid, pMD-19T-HaALP was double digested with *Bam*HI and *Xho*I and the resultant fragments were isolated and subsequently ligated to pET-32a prokaryotic expression vector (Novagen, Merck KGaA, Darmstadt, Germany) for the construction of the recombinant plasmid, rHaALP by TaKaRa quick ligation kit. The resultant plasmids were transformed into competent cells, BL-21 (DE-3) for culturing in LB liquid media having 100 µg mL^{-1} ampicillin at 37ºC at 220 rpm up to the absorbance value of 0.5 nm at OD_{600}, followed by addition of 1 mM isopropyl-*β*-D-thiogalactopyranoside (IPTG) and continued culturing for 8 h at 30 ºC for the protein expression. The resultant protein was run on 12% SDS-PAGE for confirmation and visualized by the BioRad™ imaging system.

Insect Treatment

The sub-lethal concentration of cantharidin, 25 µg g^{-1} was earlier determined through a bioassay experiment. The starved 3rd instar larvae for 8 h were introduced to an artificial diet comprising 25 µg g^{-1} of cantharidin. From the treated diet larvae were picked at different intervals of 24, 48, 72 and 96 h, and quickly frozen by subjecting to liquid nitrogen for total RNA and protein isolation. A batch of ten larvae each for three biological replicates was used.

Gene Expression Analysis

Flash frozen larvae from treatment and control were stored at -80ºC. For the extraction of total RNA, a batch of 10 randomly selected larvae stored at -80ºC, were used. At first, midguts were dissected on ice. After washing midgets with ice cold 1x phosphate buffer, larvae were grinded in liquid nitrogen prior to the RNAiso Plus addition (TaKaRa, Dalian, China). The total RNA from samples were isolated in line with the manufacturer's protocol. The RNA integrity and quality were observed on an agarose gel. DNA contamination (Genomic) was disintegrated by DNase-I (Fermentas). Reverse transcription was performed to synthesized cDNAs from all the samples by RevertAid™ Reverse Transcriptase (Fermentas). The resultant product was stored at -80ºC.

Quantitative real-time PCR was performed in PCR strips by BioRad iQ™ 5 cycler. A fluorescent dye, SYBR Green was used to get realtime signals both from target and reference genes. The qPCR mixture composed of 1 µL diluted cDNA templates by 10x, 0.5 µL of 10 µM sense and antisense primers (Table 1) mixed with Maxima™ SYBR Green/ROX qPCR Master Mix (Fermentas) in a 25 µL reaction mixture. The conditions for real-time PCR were set at: first denaturing at 95ºC for 30 s, 40 cycles of 95ºC for 10 s, 62ºC for 30 s and 72ºC for 30 s. The data for real-time were obtained at 72ºC. A total of three replicates were used for each treatment. A β-actin was kept as an internal control in the process. The signal quantification levels of the relative transcript were obtained by the comparative CT method. The comparative expression of the target gene was estimated that corresponds to $2^{-\Delta\Delta CT}$ [16].

ATGGTGACACTGTTCCCGTACGTAGTGGCGGTGCTGTGCGGCGCGACGAGCGCGCGCGCCTA
CTGGCTGCATCCCGCGGCGCCGGCCGCCGCCAGCCGCGCCGAGACCTCTGCCAACTACTGGG
CGCAAGACGCGCAGGCCGCCATCAACGCTCGCCTGGAACGAGTTGAAAGCGTGAAGAAAGC
GCGTAACGTCATCATGTTCCTGGGCGACGGCATGTCGGTGCCCACGCTCGCCGCCGCGCGCA
CGCTGCTCGGTCAGCGCCAAGGGAAAACGGGAGAGGAGACAAAGTTGCATTTCGAGACTTT
CTCCACAATCGGATTAGTGAAGACGTACTGTGTGGACGCCCAGATTGCAGACTCCGCATGTAC
TGCCACAGCGTATTTGTGTGGTGTAAAAAATAACTATGGCGCCATAGGCGTAGACGGCACGGT
ACGCCGAGGAGACTGTCAAGCCGCTTCAAACACTGCGACACACGTCGAGTCCATCGCGGAG
TGGGCGCTCGCTGACGGACGAGATGTCGGTATTGTGACGACGACTCGTATCACTCACGCGTC
TCCGGCGGGCACGTTCGCGAAGACGGCGAACCGCACCTGGGAGAACGACGGTGAAGTGTCG
CAGATGGGCTTGGACGCCAAGGACTGCCCTGACATCGCGCATCAGTTGGTACACCATCATCC
CGGTAACAAGTTCAAGGTTATTTTTGGTGGTGGCAAGCGTGCCTTTTTGCCAAATACTGAACA
GGACGAAAAAGGATCTTATGGTAGAAGGATAGACAACCGCAATCTCATCAAAGAGTGGGAGG
ATGATAAGGTTTCTCGTAATGTCAGCCATCAATATGTTTGGCACCGCGAGCAGCTAATGCGTCT
AAAGGAGGACCTGCCTGAATACATGTTGGGACTGTTCGAGAGCAGTCATATGACCTATCACTT
GAAATCAGACCCTCAGTCTGAACCCACTCTCGCTGAACTAACAGAGGTGGCAATTCGGTCAT
TAAGACGCAATGAGAAGGGATTCTTCCTGTTCGTGGAGGGGGGGCGTATCGACCACGCGCAC
CACGACAACCTGGTGGAGCTCGCACTCGACGAGACGCTGGAGATGGACAAGGCCGTGGCCA
CCGCCACGAAGATGCTCTCAGAGGACGACTCGCTCATCGTGGTCACTGCCGACCACGCACAC
GTCATGACTTTCAATGGCTACTCTAACCGTGGTCATAACATCCTCGGGCCCTCCAGGGATGTC
GGACTAGACAATGTGCCTTACATGACGCTAACGTATGCCAATGGACCCGGATTCCGTCCACAC
GTTAACGACATTAGACCAGATGTTACCCTTGAGCCAAACTATCGCACCCTGGACTGGGAGTCG
CACGTGGACGTGCCGCTGGTGGACGAGACGCACGGCGGCGACGACGTGGCCGTGTTCGCGC
GCGGGCCGCACCACTCCATGTTCACGGGGCTGTACGAGCAGAGCCAGCTGCCGCACCTCATG
GCCTACGCCGCCTGCATCGGCCCCGGCCGGCACGCCTGCGCCAGTGCCGCGCACTTGCCTAG
CGCGCACTTCTTCGTAGCTTTGCTCGCTCTATTCATTTCCATTTTACTGCGATAA

MVTLFPYVVAVLCGATSARAYWLHPAAPAAASRAETSANYWAQDAQAAINARLERVESVKKAR
NVIMFLGDGMSVPTLAAARTLLGQRQGKTGEETKLHFETFSTIGLVKTYCVDAQIADSACTATAY
LCGVKNNYGAIGVDGTVRRGDCQAASNTATHVESIAEWALADGRDVGIVTTTRITHASPAGTFA
KTANRTWENDGEVSQMGLDAKDCPDIAHQLVHHHPGNKFKVIFGGGKRAFLPNTEQDEKGSYG
RRIDNRNLIKEWEDDKVSRNVSHQYVWHREQLMRLKEDLPEYMLGLFESSHMTYHLKSDPQSE
PTLAELTEVAIRSLRRNEKGFFLFVEGGRIDHAHHDNLVELALDETLEMDKAVATATKMLSEDDS
LIVVTADHAHVMTFNGYSNRGHNILGPSRDVGLDNVPYMTLTYANGPGFRPHVNDIRPDVTLEP
NYRTLDWESHVDVPLVDETHGGDDVAVFARGPHHSMFTGLYEQSQLPHLMAYAACIGPGRHAC
ASAAHLPSAHFFVALLALFISILLR-

Figure 1: Gene and the deduced amino acid sequence of the amplified product of Helicoverpa armigera alkaline phosphatase

Immunoblotting Analysis and Production of HaALP Antibody

Purified recombinant protein (0.5 mg mL^{-1}) was mixed with complete Freund's adjuvant in 1:1 for immunization of New Zealand white rabbit. The rabbit was immunized with antigen every two weeks. A total of six injections were used to immunize rabbit before terminal bleed. ELISA was used to confirm titer of antibody using conjugated secondary antibody Goat anti-rabbit IgG-HRP.

Protein was extracted from midguts for both treatment and control larvae frozen at -80ºC, by the TRIzol method [17] and dissolved in 1% sodium dodecyl sulphate (SDS). Immunoblotting was carried out to see the binding of the polyclonal antibody to the recombinant alkaline phosphatase to confirm results of real-time qPCR.

Results

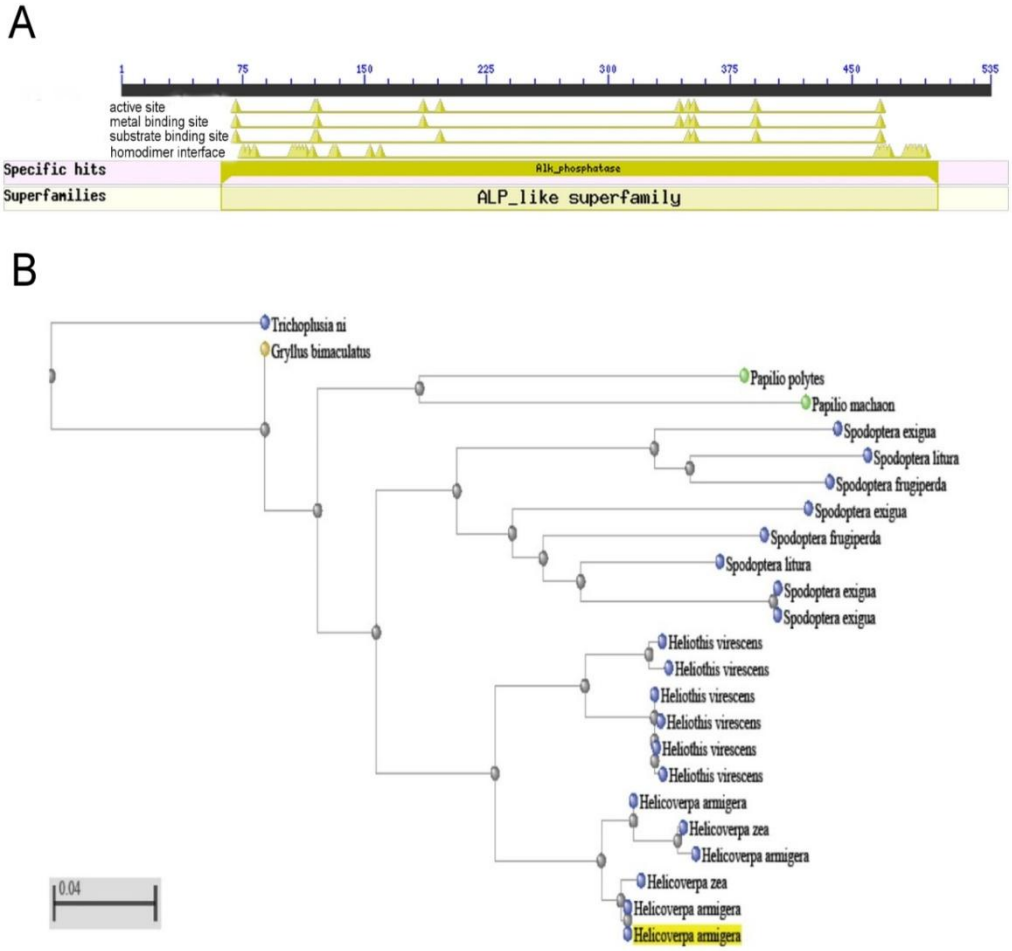

Figure 2: The HaALP amino acid sequence of H. armigera. (A) The small triangles in yellow color show active sites, metal binding sites, substrate binding sites and homodimer interfaces; (B) The neighbour joining method of phylogenetic associations in intra and inter species in relation to H. armigera.

Gene Cloning and Expression

The target gene HaALP was PCR amplified using a pair of reverse and forward gene specific primers. The resultant product having a size of 1608 bp was visualized by the agarose gel with ethidium bromide (EtBr). The product (Fig. 1) was further confirmed through gene sequencing by Shanghai Sunny Biotech Co., Ltd, P.R. China.

The deduced amino acid sequence of *Helicoverpa armigera* alkaline phosphatase was subjected to BLAST analysis at NCBI for comparison with other sequences available on the database. The deduced sequence showed high to moderate homology with other sequences of ALP superfamily in insects (Fig. 2A). The comparison of the deduced sequence showed high homology inside species with *Helicoverap zea*, *Helicoverpa virescens* and outside with *Spodoptera exigua*, respectively (Fig. 2B).

The amplified target fragment ligated into pET-32 expression vector used for the production of the target protein was confirmed with SDS-PAGE. The expressed target protein was further confirmed with specific antibody produced against it through ELISA.

Gene Expression Profile of HaALP

The quantitative real-time PCR was performed to obtain the transcript level of HaALP under the effect of sub-lethal concentration of cantharidin. Normalized gene expression results revealed that the transcription level of HaALP was down regulated by 1/0.97, 1/0.94, 1/0.74 and 1/0.48-fold compared to control at 24, 48, 72 and 96 h post treatment, respectively, in response to cantharidin treatment (Fig. 3). Melting curve confirmed signal specificity from our target amplification (Fig. 4). The real-time qPCR product visualized on agarose gel also confirmed the reduction in the transcript level (Fig. 5).

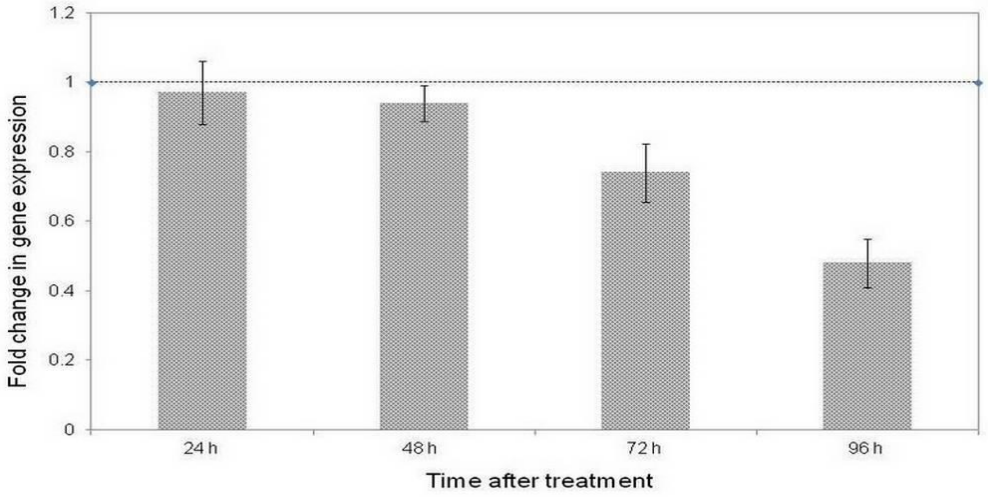

Figure 3: The time course expression of ALP in response to cantharidin treatment by real-time qPCR. Real-time qPCR of ALP mRNA transcript at different time intervals from 24 to 96 h post treatment. Bars in the plot show change in gene expression ratio of target gene compared to untreated control. Error bars show standard deviation among the three replicates.

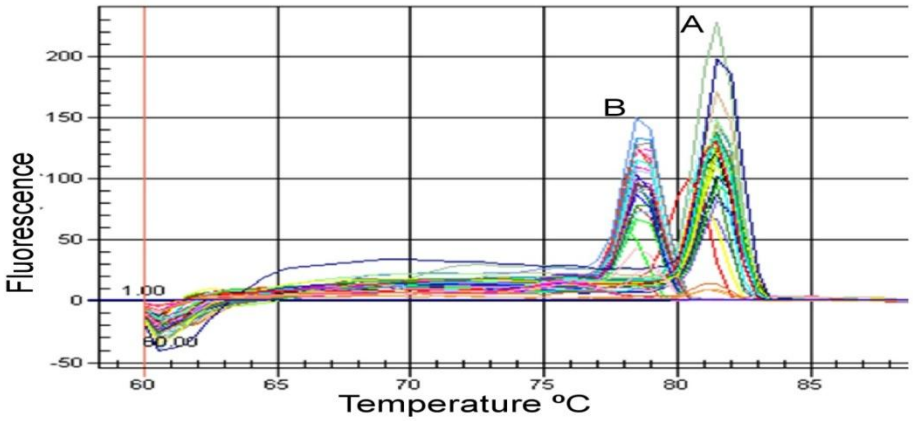

Figure 4: Melting curve analysis. (a) the melting curve of HaALP. (b) the melting curve of internal cortrol, β-actin.

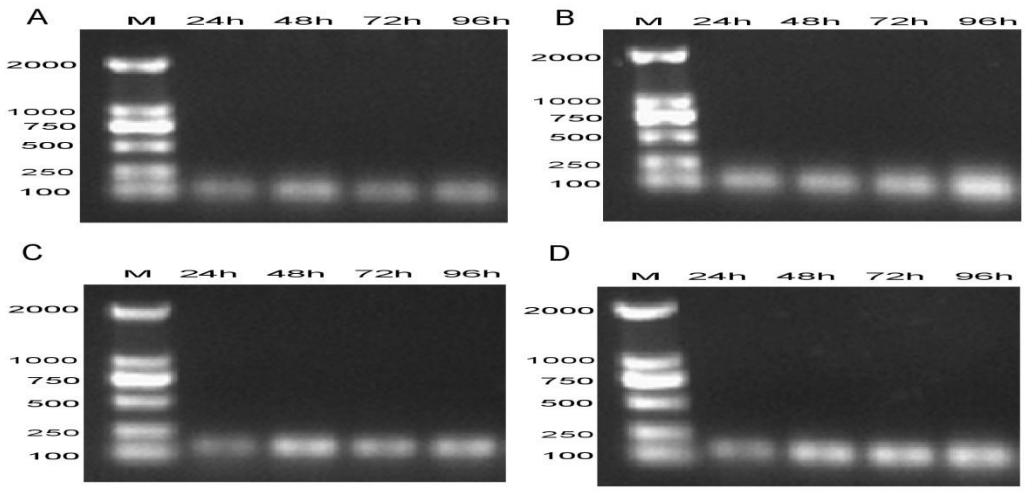

Figure 5: Agarose gel electrophosesis of real-time qPCR products. (a) HaALP expression under treatment. (b) HaALP expression without treatment. (c) β-actin expression under treatment treatment. (d) β-actin expression without treatment

Imunoblotting Assay

Recombinant antibody produced and confirmed against the target gene was used for imunoblotting. The imunoblitting results showed the gradual decline of HaALP level after 24, 48, 72 and 96 hrs post treatment compared to the control. These results conformed results from the real time qPCR of gene transcript (Fig. 6).

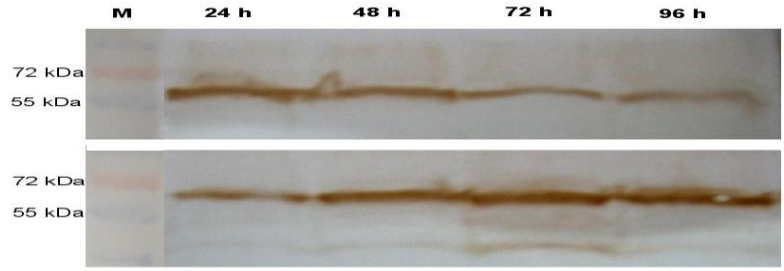

Fig. 6: Western blotting of ALP in cantharidin treatment compared to control at 24, 48, 72 and 96 h. (c) Semi-quantitative RT-PCR results of β-actin transcription level in cantharidin and control samples at 24, 48, 72 and 96 h.

Discussion

Primarily, bioactivity of cantharidin was considered by its high tendency and specificity to cantharidin binding protein (CBP) [18]. Except for cantharidin's high affinity to PP2A-AC nature of its precise biochemical and physiological properties and mechanisms of action is unidentified. [19-23].

Cantharidin was determined to be highly toxic to armyworm, *Mythimna separata* in a laboratory bioassay study [12]. Besides its lethal concentration its sub-lethal concentration was applied to *M. separata* to determine its effects on metabolic enzymes *in vivo,* such as ALP. Based on the experimental data it was established that cantharidin inhibits ALP activity. In a rather different investigation, cantharidin was found to have synergistic properties when used with other commercial insecticides suggesting its involvement in several mechanisms for its toxicity in insect pests [13]. These investigations are in line with our studies of down regulation of ALP and involvement of ALP in toxicity of insecticides towards *H. armigera*.

In earlier investigation ALP in *H. armigera* was found in higher concentration in resistant insects compared to susceptible insects and was considered as an important factor for resistance towards insecticide [24]. Evan though glutathione-S-transferase, cytochrome P450 and esterases are regarded as significant detoxification enzymes against

insecticides, several other investigations also determined induction of ALP by insecticides and also its higher level in resistant insects strains [24-26]. Induction of the ALP was also reported by fenvalerate which was reported at the gene transcription level [27]. Cantharidin as an inhibitor of ALP [12] and further validated in the current studies may help to counter the menace of resistance and act as a synergist to enhance the effect of insecticides against resistant strain of insects

Although, in our experiment we demonstrated the down regulation of ALP gene in response to the sub-lethal dose of cantharidin using real time qPCR and immunoblotting assay, there is a need to understand the molecular mechanism of the down regulation of ALP and to investigate the factors in detail responsible for the down regulation of the gene.

Conclusions

On the basis of our current investigations, it was found that cantharidin down regulated ALPs in *Helicoverpa armigera.* As the ALPs play a vital role in biotic and abiotic stresses, its down regulation by cantharidin may suggest its role as an insecticide synergist. Moreover, it may also be recommended with other chemical insecticides where insecticide resistance due to higher levels of ALPs is suspected.

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Conflict of Interest

The authors declare no conflict of interest.

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