African Journal of Biotechnology Vol. 11(42), pp. 9915-9923, 24 May, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.3814
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Molecular cloning, heterogenous expression and the induction profiles after organophosphate phoxim exposure of the carboxylesterase *Bmae33* in the silkworm, *Bombyx mori*

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Accepted 10 May, 2012

Carboxylesterases (COEs) are a multifunctional supergene family and some of them play important roles in hydrolyzing a wide variety of carboxylic acid esters. In insects, COEs are related to xenobiotic detoxification, pheromone degradation and developmental regulation. In the present study, one silkworm COE *Bmae33* gene, an ortholog to other Lepidopteran odorant-degrading esterases, was cloned and exogenously expressed in *Escherichia coli*. The results indicate that the *Bmae33* gene contained a 1, 656 bp open reading frame, encoding a protein of 551 amino acids. The molecular weight of predicted protein was 62.15 kDa, and the isoelectric point was 5.87. RT-PCR analysis showed that *Bmae33* was highly expressed in the head, fat body and integument of the silkworm larvae. Recombinant protein of Bmae33 was purified by His-tag affinity column, and its antibody was prepared. Western blotting analysis showed that the recombinant protein was purified successfully. In addition, the larvae and adults of the silkworm were exposed by volatile organophosphorus (OP) insecticide phoxim, respectively. After exposure, the expression of *Bmae33* gene could be up-regulated by phoxim in the silkworm larval head and adult antennae. The results presented in this study provided useful information for further understanding of the odorant detoxification roles.

Key words: *Bombyx mori*, carboxylesterase, recombinant expression, phoxim, induction.

INTRODUCTION

Carboxylesterases (COEs, EC 3.1.1.1) constitute a multifunctional gene family and are almost ubiquitous in animals, plants, and microbes (Bornscheuer, 2002; Marshall et al., 2003). COEs belong to the α/β-hydrolase's fold super family, which catalyses the hydrolysis of ester bonds of various substrates. The COEs with hydrolysis activities usually contain a catalytic triad (Ser-Glu-His, Asp instead of Glu sometimes), and a consensus sequence (Gly-x-Ser-x-Gly) around the active site Ser (Bornscheuer, 2002). According to sequence identity and substrate specificity, COE genes can be subdivided into eight subfamilies: α-esterase, β-esterase, juvenile hormone esterase, gliotactin, acetylcholinesterase, neurotactins, neuroligin and glutactin (Ranson et al., 2002; Yu et al., 2009).

In insects, the functions of COEs are highly diversified. It was proved that COEs play important roles in xenobiotic detoxification, sex pheromone or odorant degradation, neurogenesis and developmental regulation. Especially, insect COEs were highly related to detoxification of insecticides, such as organophosphorus,

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Abbreviations: COE, Carboxylesterase; **IPTG,** isopropyl-beta-D-thiogalactopyranoside; **PVDF,** polyvinylidene fluoride; **TBST**, Tris-buffered saline Tween-20; **OP,** organophosphorus.

pyrethroid and carbamate, etc. (Gao et al., 1998; Li et al., 2007). In addition, insect COEs also play important roles in detoxification of secondary metabolites of plant. It was shown that *Sitobion avenae* COEs could be induced by indole alkaloid gramine, and the activity of COEs was proportional to the consistency of gramine (Cai et al., 2009). Furthermore, COEs of *Helicoverpa armigera* and *Bemisia tabaci* could also be induced by quercetin, rutin and 2-tridaconone (Gao et al., 1998).

In insects, olfactory system is a highly specific and sensitive chemoceptor that plays important roles in the location of food sources and mates, avoiding damages from adverse environments and searching for oviposition sites, etc. (Bohbot and Vogt, 2005; Takken and Knols, 1999). However, the sex pheromones, plant secondary volatiles or other harmful odorants entered into olfactory system must be degraded in time; otherwise, they have harmful effects on insects. Presently, for the olfactory COEs, the degradations of sex pheromones were widely studied (Durand et al., 2009; Ishida and Leal, 2008; Kasang et al., 1989; Kaissling and Kasang, 1978). In addition, only one gene SlCXE10 expressed in the adult and larvae antennae of *Spodoptera littoralis* has also been proved to efficiently hydrolyze a kind of green leaf volatiles (Durand et al., 2010). Thus, previous studies of olfactory COEs mainly focused on the adults of Lepidopteran insects. Recently, it was found that multiple COE genes were expressed in the larval olfactory system (Yu et al., 2009). Whether the COEs in the larval olfactory system have the similar functions to those in the adults, such as degradation of plant secondary volatiles or other harmful odorants, need to be validated.

Bombyx mori (Bm) is an economically important insect and a Lepidopteran model. Recently, the new silkworm genome sequence assemble was completed (The International Silkworm Genome Consortium, 2008). Based on this new assemble, 76 putative COEs have been identified in the silkworm (Yu et al., 2009; Tsubota and Shiotsuki, 2010). Bm COEs were classified based on tissue specific expression, such as, midgut-, head and integument-, and silk gland-specific expression, furthermore, most of the COEs from head and integument-class were homologous to odorant-degrading enzyme (ODE) and antennal esterase and were also expressed in the larval antennae and maxillae (Yu et al., 2009). In previous studies, the research of olfactory system detoxification enzymes was mainly focused on the enzymes in adult olfactory system but seldom focused in larvae at present. To understand the functions of COEs in larval olfactory tissues, we studied the Bmae33, which was highly expressed in larval maxillae and might be orthologous to *Apol*-ODE and *Slit*-EST (Yu et al., 2009). We cloned the full-length coding sequence of *Bmae33*, expressed the recombinant protein in *Escherichia coli*, analyzed the conversed motifs, and furthermore, detected the induced expression pattern of Bmae33 by volatile organophosphorus (OP) insecticide.

MATERIALS AND METHODS

Insects and tissues

Larvae of silkworm strain *Dazao* were reared on fresh mulberry leaves under photoperiod of 12 h: 12 h (L:D) at 23±1°C and 72±5% RH. At the 3rd day of 5th instar, haemolymph from male and female larvae was mixed; testis, ovaries, integuments, heads, malpighian tubules, silk glands, fat bodies, midguts, tracheas from male and female larvae were dissected, respectively, immediately frozen with liquid nitrogen and stored at -80°C until use.

Experimental exposure to phoxim

Phoxim was used to induce the expression of *Bmae33* in the silkworm. For exposure experiment, 600, 300 and 100 µg phoxim (acetone as solvent and the whole volume was 60 µL) were loaded onto pieces of filter paper, approximately 2.0 × 2.0 cm^2, respectively. Then, the pieces of filter paper were separately put into the hermetical vessels, containing 3-day-old 5th instar larvae (six individuals) and fresh mulberry leaves, for 24 h. The pieces of filter paper with 60 µL acetone and without acetone were used as controls, respectively. The experiment was repeated three times. The exposure treatments of adults were similar as the larvae, while 1000, 600 and 300 µg phoxim were used and the total volume was 100 µL. In addition, 12 individuals were used in each treatment, and no mulberry leaves were put into the hermetical vessels. The heads of silkworm larvae and antennae of moths were dissociated after exposure to phoxim for 24 h, washed by ice-cold 0.75% NaCl, and immediately frozen with liquid nitrogen, and then stored at -80°C until use.

Total RNA extraction and first-strand cDNA synthesis

Trizol-reagent (Invitrogen) was used to prepare the total RNA according to the manufacturer's protocol. For RT-PCR analysis, each of the RNA samples was treated with DNase I (RNase free) (TaKaRa) to remove the genomic DNA contamination. The purity and quantity of extracted RNA were quantified by the ratio of $OD_{260/280}$ with an ultraviolet spectrometer and stored at -80°C. Then, the cDNA synthesis was performed using the reverse transcriptase M-MLV Kit (Promega) according to the manufacturer's instruction and 3.0 µg total RNA was used as template.

Cloning and sequencing of cDNA encoding *Bmae33*

The cDNA of the silkworm larvae heads was used to amplify the DNA fragment by PCR with the primers as follows: forward (F): 5'-ATGTCAGCTTGACCACGACGCGGGG-3'; reverse (R): 5'-TACGAACATTTATTAGATTCGTTAT-3'. PCR amplification was carried out in 50 µL of a total reaction volume, containing 2 µL of cDNA sample, 1 µL of each primer, 25 µL *Gotaq* Green Master Mix DNA polymerase (Promega), and 22 µL ddH2O. The conditions of PCR were: 94°C for 4 min, 30 cycles at 94°C for 30 s, 55°C for 45 s, 72°C for 90 s and final extension at 72°C for 10 min. The size of the expected PCR product was 1, 656 bp, and the PCR product was analyzed using agarose gel electrophoresis and purified using a Quick Gel Extraction Kit (TransGene). The purified DNA was ligated into pMD19-T simple vector (Takara) and several positive single-colonies were subjected to sequencing from both directions (Invitrogen).

Sequence analysis

To analyze the conversed motifs or sites, amino acid sequence of

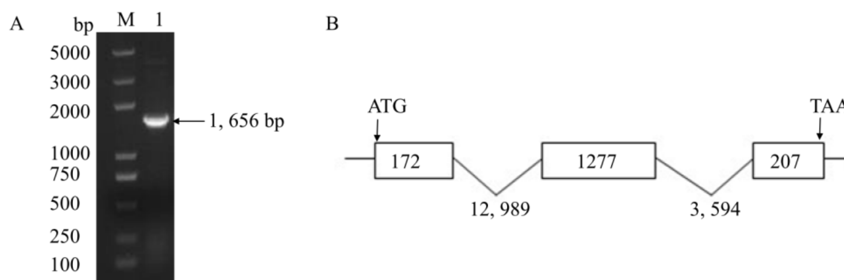

Figure 1. PCR amplification and gene structure of *Bmae33* in the silkworm. A) The agarose gel electrophoresis of *Bmae33* amplicon. M, DNA marker (DL5000); lane 1, PCR amplification product; Arrowhead, *Bmae33*. B) Structure of *Bmae33*. The boxes showed the exons, and the introns lie between these boxes. The numbers indicate the length of individual exon or intron.

Bmae33 and several COEs from other insects were aligned using Clustal X (Chenna et al., 2003). Phylogenetic tree was reconstructed using the neighbor-joining method in which distance was estimated by Poisson-corrected distance implemented in MEGA 4.0 program (Sudhir et al., 2008). The accuracy of the tree topology was assessed by bootstrap analysis with 1, 000 re-sampling replicates. N-terminal signal peptide sequence of *Bmae33* protein was predicted using the SignalP 3.0 program (http://www.cbs.dtu.dk/services/SignalP/), molecular weight and isoelectric point (pI) of *Bmae33* were predicted by the computation tool of pI/Mw (http://web.expasy.org/compute_pi/). N-glycosylation sites were predicted using NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/). In addition, the 3-D structure of mature *Bmae33* protein was modeled by Raswin (http://rasmol.org/OpenRasMol.html).

RT-PCR and quantitative RT-PCR

To determine the expression patterns of *Bmae33* gene, the total RNAs of tissues of the 3-day-old 5th instar larvae were used for RT-PCR analysis. The amplification reaction and programs were similar to the method of the cDNA cloning part. The total RNAs of the larvae heads and adult antennae were used to determine the expression level of *Bmae33* after exposure experiment through qRT-PCR. The products of reverse-transcription was diluted then used as template. The qRT-PCR was performed using Bio-Rad CFX Manager according to the manufacturer's instructions, and the reaction volume was 10 μL containing 5 μL SYBR premix *Ex Taq* II (2×), 0.3 μL of each primer, 0.2 μL ROXII, 1 μL template and 3.2 μL nuclease-free water. The housekeeping gene *Actin3* (*A3*) was used as an internal control; the amount of transcripts of *Bmae33* gene was normalized with that of *Bmactin3* gene, and the PCR condition for the *A3* gene was the same as *Bmae33*. The qRT-PCR protocol had an initial denaturation of 95°C for 3 min, followed by 39 cycles of melting: 95°C for 10 s, 55°C, for 30 s, and 95°C for 10 s extension. The primers used for qRT-PCR were as follows: F: 5'-GATTTCAGGCACCACTGCCC-3'; R: 5'-ACCACTAAAGAAACAACCTC-3'. Duncan's multiple range test in SPSS software was employed to test difference significances among different concentrations of the phoxim treatments and the controls.

Expression and purification of recombinant protein in *E. coli*

The ORF of *Bmae33* gene was amplified by PCR using a primer-pair (F: 5'-CCGGAATTCACCAGTGGACCAGTGAGGGGCAGGA-3'; R: 5'-ATAAGAATGCGGCCGCTTTAAATTCGGGTTTTACTTCGGAC-3'). The amplified DNA did not include signal peptide coding sequence, and the underlines indicated *Eco*R I and *Not* I restriction enzyme sites, respectively. The purified PCR product and expression vector pET-28(a) were digested with *Eco*R I and *Not* I, and the aimed products were ligated. Recombinant expression vector was transformed into *E. coli* BL21 strain, and then induced by IPTG with a final concentration of 0.5 mM at 16°C for 12 h. The recombinant protein was purified using His-tag Ni^{2+} affinity column, tested by SDS-PAGE gel and stained with Coomassie Brilliant Blue R-250.

Western blotting analysis

Polyclonal antiserum recognizing *Bmae33* was raised against the recombinant protein. The purified recombinant *Bmae33* expressed in *E. coli* was 1:1 mixed with Freund's complete adjuvant and then the mice were hypodermically injected at multiple sites. Antiserum was collected after three-boost immune injections and identified by western blotting. The purified recombinant protein used for western blotting was loaded on SDS-PAGE gel, after SDS-PAGE, the proteins were transferred to polyvinylidene difluoride (PVDF) membrane, blocked overnight by 5% non-fat milk at 4°C, and then the PVDF membrane was incubated with *Bmae33* antiserum for 1 h at 37°C. After washing in TBST, the membrane was incubated in secondary antibody [HRP-labeled Goat Anti-Mouse IgG (H+L)] (Beyotime) and then washed thoroughly in TBST. The binding was detected using ECL Plus Western Blotting Detection System (GE Headthcare).

RESULTS

Cloning and sequencing of *Bmae33*

The cDNA of heads of the 5th instar 3-day-old larvae was used as template to amplify the coding sequence of *Bmae33* by PCR, and a single band with the expected size was produced (Figure 1a). After cloning and sequencing, the full-length CDS of *Bmae33* was obtained, which consisted of 1, 656 bp and encoding 551 amino acids. The molecular weight and pI of *Bmae33* were 62.15kDa and5.87 kDa, respectively. Furthermore,

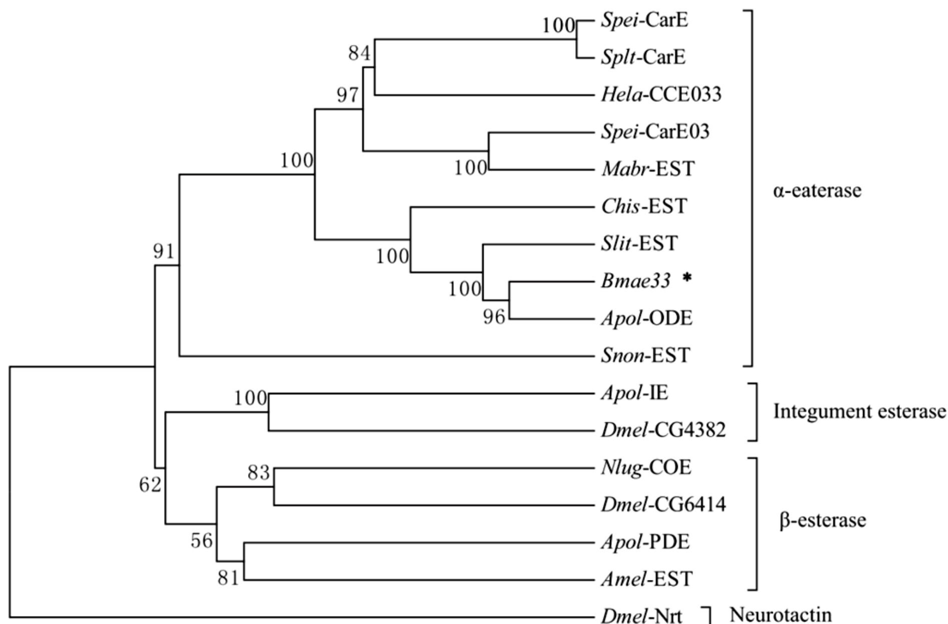

Figure 2. Neighbor-joining tree of *Bmae33* and other insect COEs. COEs and their GenBank accession numbers are as follows: *Spei*-CarE, *Spodoptera exigua* carboxylesterase, ADR64700; *Splt*-CarE: *Spodoptera litura* carboxylesterase, ADR64699; *Hela*-CCE033a: *Helicoverpa armigera* carboxyl/choline esterase CCE033a, ADF43492; *Spei*-CarE03: *Spodoptera exigua* carboxylesterase, ADR64703; *Mabr*-EST: *Mamestra brassicae* antennal esterase, AAR26516; *Chis*-EST: *Chilo suppressalis* esterase, ABD62774; *Apol*-ODE: *Antheraea polyphemus* odorant-degrading enzyme, AAM14415; *Snon*-EST: *Sesamia nonagrioides* antennal esterase, DQ680829; *Apol*-IE: *A. polyphemus* integument esterase, AAM14416; *Nlug*-COE: *Nilaparvata lugens* COE, AF302777; *Apol*-PDE: *Antheraea polyphemus* pheromone-degrading enzyme, AY866480; *Amel*-EST: *A. mellifera* antennal esterase, AB083009; *Dmel*-Nrt: *D. melanogaster* neurotactin, CG9704; *Slit*-EST: *Spodoptera littoralis* antennal esterase CXE11, ACV60238.

the cDNA was aligned with the silkworm genome. The results showed that *Bmae33* gene was located on scaf 2770 and the CDS of *Bmae33* gene contained two introns and three exons (Figure 1b).

Sequence analysis of *Bmae33*

A total of 17 representative esterase protein sequences from different organisms were used to reconstruct the phylogenetic tree (Figure 2). In previous studies, odorant-degrading esterases belonged to α-esterase, β-esterase and integument esterase, most of which were members of α-esterase (Yu et al., 2009; Vogt, 2005; Coisne et al., 2004). In the phylogenetic tree, *Bmae33* was grouped with α-esterase and phylogenetically related to *Apol*-ODE and *Slit*-EST, which shared 73.1.0 and 64.6% of amino acid identities with *Bmae33*, respectively.

A 24-amino acids signal peptide sequence was predicted, suggesting that *Bmae33* might be a secretory protein. Seven potential N-glycosylation sites in *Bmae33* were predicted using the NetNGlyc 1.0 server, including N189, N262, N313, N342, N365, N498 and N529, respectively. Multi-sequence alignment of *Bmae33* and several other COEs showed that *Bmae33* contained the catalytic triad (Ser196-His114-Glu327) and a conserved pentapepite characteristic of Gly-X-Ser-X-Gly at the serine active site (Oakeshott et al., 1999) (Figure 3a). The conserved domain of the *Bmae33* was also determined using http//www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi/ (Figure 3b). In addition, the Raswin software was used to model the three-dimensional structure of *Bmae33* protein. It indicated that 23 helices, 25 strands and 57 turns were found. Ser196, Glu327 and His114 were aggregated together and formed the catalytic triad (Figure 4). All of these analyses suggested that *Bmae33* protein could be an active hydrolase.

Tissue expression pattern of *Bmae33*

To detect and quantify the expression level of *Bmae33* gene, semi-quantitative RT-PCR was performed. *Bmae33* was highly expressed in head, fat body and integument, and there was no significant difference between male and female (Figure 5). The fat body and integument are

A

B

Figure 3. Conservative analysis of *Bmae33* protein sequence. A) Multiple sequence alignment of Bmae33 and carboxylesterases from other insects. The catalytic residues were marked with asterisk (*); the N-glycosylation sites are marked with " ", and the conserved pentapepite characteristic of Gly-X-Ser-X-Gly is marked with a box; B) conserved domains of *Bmae33*.

important detoxification tissues. *Bmae33* was highly expressed in these tissues, suggesting that it might play important roles in xenobiotic detoxification. Insect head contains various olfactory organs. The previous study indicate that *Bmae33* was also highly expressed in the larval maxillae (Yu et al., 2009). Thus, *Bmae33* might participate in odorant degradation.

Expression and purification of recombinant Bmae33, and preparation of antibody

The recombinant plasmid pET28 (a)-*Bmae33* was constructed and transformed into the *BL21* strain of *E. coli*. The recombinant protein expression was induced by IPTG and identified by SDS-PAGE. Compared with control, the induced products showed a 60.41 kDa band (Figure 6a). Ni^{2+} affinity column was used to purify the recombinant protein, and a single band with expected size was obtained (Figure 6a). The purified recombinant protein was injected into mice to prepare anti-*Bmae33* antibody. Afterwards, the antibody was also used to identify the purified protein through western blotting. Only one band was shown in the PVDF membrane (Figure 6b). This suggested that recombinant protein was purified, and the antibody was prepared successfully.

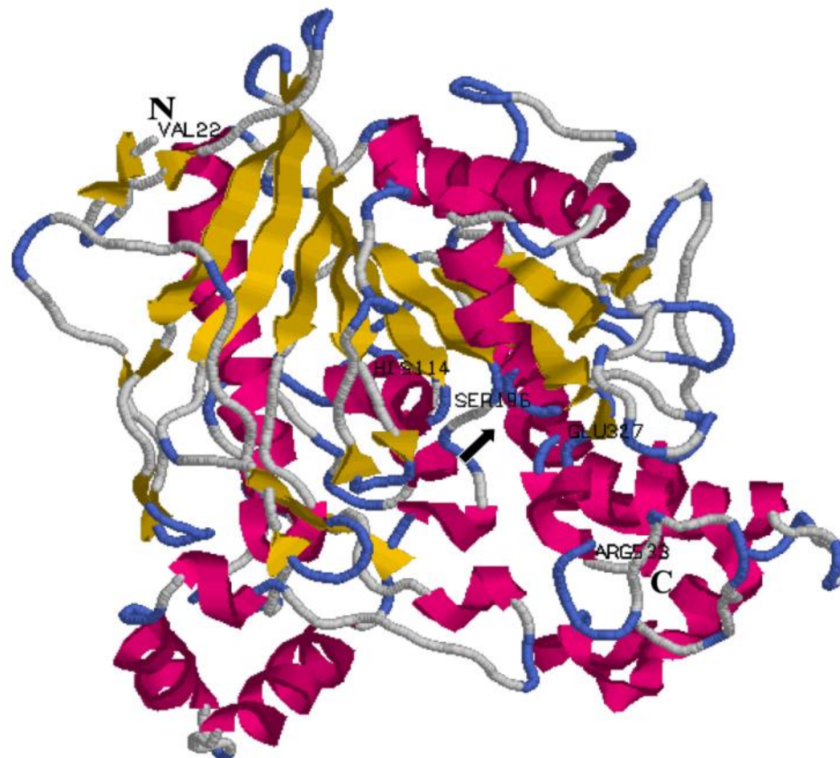

Figure 4. 3-D structure prediction of *Bmae33*. The arrows show the three amino acids (His, Glu and Ser), which composed the catalytic triad. The yellow arrows are shown as β-sheet, purple helices shown as α-helices, and the other parts of structure shown anomalous curl of protein.

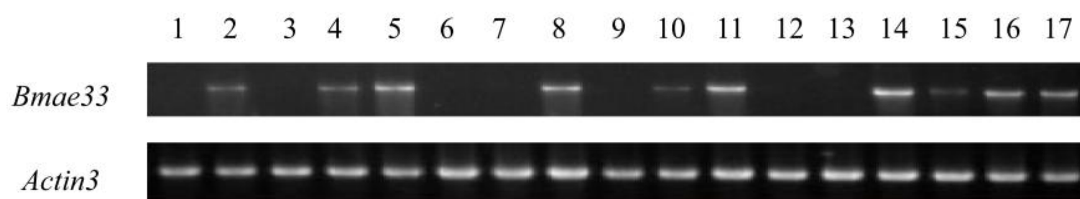

Figure 5. Analysis of expression of *Bmae33* gene in the tissues of 3-day old 5th instar larvae by RT-PCR. *Actin3* gene was used as the control gene.1, haemolymph; 2, testis; 3, ovaries; 4, female epidermis; 5, female heads; 6, female malpighian tubules; 7, female silk glands; 8, female fat bodies; 9, female midguts; 10, female tracheas; 11, male epidermis; 12, male malpighian tubules; 13, male silk glands; 14, male fat bodies; 15, male midguts; 16, male tracheas; 17, male heads.

larvae and antennae of adults were dissected, respectively. qRT- PCR was used to quantify the induced expression of *Bmae33*. The results show that the expression of *Bmae33* could be up-regulated in the head of 3-day-old 5th larvae when the dosage of phoxim reached 600 µg (Figure 7a), and it was 2.31 times to the control. In addition, the expression of *Bmae33* could also be induced in the antennae of male adults by 1000 µg phoxim (Figure 7b), and the expression level reached to 1.96-fold relative to the control. However, acetone also up-regulated the expression level of *Bmae33* weakly, but not significantly. The reason for this phenomenon was still unknown.

DISCUSSION

The cDNA sequence of *Bmae33* was cloned; its full-length CDS is 1,656 bp. The *Bmae33* gene is comprised of two exons and three introns; exons/introns boundary is

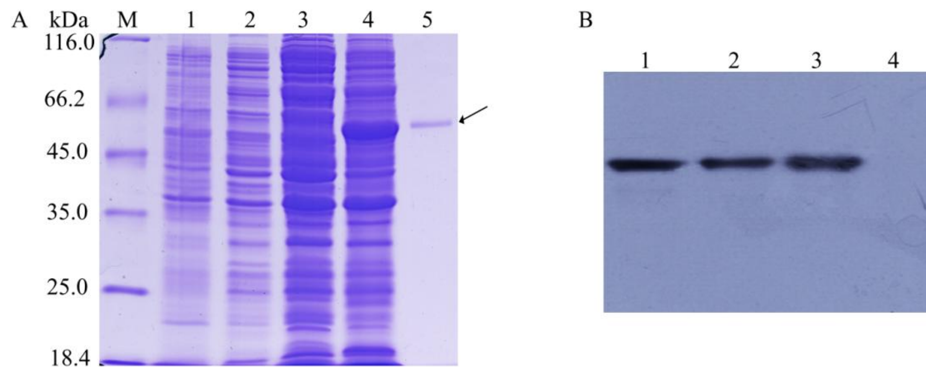

Figure 6. Heterogenous expression of Bmae33 and western blotting analysis. A) Expression of recombinant protein Bmae33 in *E.coli*. M, size marker proteins; arrowheads, *Bmae33*; 1, the product of vector pET28 (a) expressed by induction; 2, the recombinant vector *Bmae33*/pET28(a) expressed without induction; 3, the dissolved part of *Bmae33*/pET28(a) expressed by induction; 4, the undissolved part of *Bmae33*/pET28(a) expressed by induction; 5, the purified protein of *Bmae33*. B) Western blotting analysis of Bmae33 recombinant protein. 1, 250 mM imidazole elutropic products of the deposits from infected cell homogenate with pET28(a)-*Bmae33*; 2, 150 mM imidazole elutropic products; 3, 100 mM imidazole elutropic products; 4, the deposits of the infected cell homogenate with pET28(a) plasmid without insert.

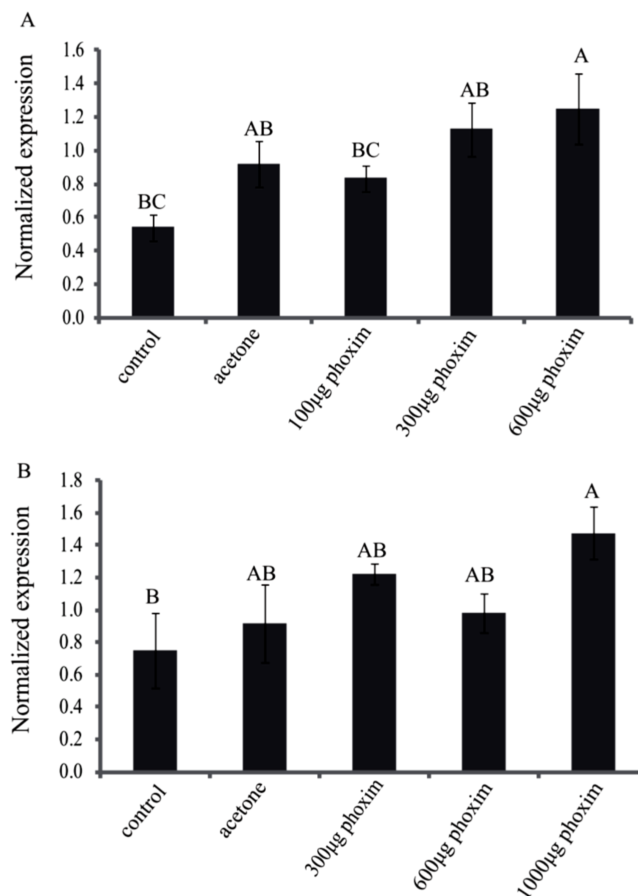

Figure 7. qRT-PCR expression analysis of *Bmae33* induced by phoxim. A) Expression changes in the head of larvae after exposure. B) Induction of *Bmae33* in the adult antennae after exposure.

Effect of phoxim exposure on *Bmae33* expression level

To understand the putative function of odorant degradation, the silkworm larvae and adults were exposed to volatile phoxim. After exposure, the head of in accord with GT-AG rule. The *Bmae33* gene encodes 551 amino acids, including a 24 amino acids signal peptide. Phylogenetic analysis showed that *Bmae33* belonged to α-esterase, and it might be the orthologous gene of *Apol*-ODE and *Slit*-EST, which shared 73.1 and 64.6% amino acid identities, respectively. Multiple sequence alignment indicated that Bmae33 protein contained the three amino acid residues Ser196, Glu327 and His114, which were required for hydrolytic activity (Figure 3). Furthermore, the 3-D model structure of *Bmae33* mature protein showed that the catalytic residues could aggregate together (Figure 4). Thus, these results suggest that *Bmae33* might be an active hydrolase.

COEs are widely distributed in the olfactory tissues, and function as odorant-degrading enzyme. Previous studies showed that *Apol*-ODE played important roles in the degradation of sex pheromone, and *Slit*-EST was involved in detoxification of insecticide/xenobiotics and digestion of dietary esters (Ishida, 2002; Durand et al., 2009; Campbell et al., 2003; Strode et al., 2008; Ishida and Leal, 2002). Bmae33 was found to be expressed in antennae and maxillae of the silkworm (Yu et al., 2009). However, sex pheromone of female silkworm moth is composed of bombykol and bombykal; and bombykal was degraded by antenna specific aldehyde oxidase (Kasang et al., 1989). Thus, *Bmae33* and its orthologous genes (*Apol*-ODE and *Slit*-EST) might have diversified. In fact, the host plant mulberry leaves contains analogous products of ester sex pheromone such as (Z)-3-hexenyl acetate and (E)-2-hexenyl acetate, and they could be potential odorants that attract insects to seek food (Ishida and Leal, 2005; Tanaka et al., 2009). Our study and previous observations showed that *Bmae33* gene was expressed in the head antennae and maxillae of 3-day-5th instar larvae (Figure 5) (Yu et al., 2009). Thus, we speculate that *Bmae33* may take part in degrading volatile ester odor molecules of mulberry leaves.

Previous studies indicate that COEs play important roles in detoxification of organophosphorous insecticides. In *Culex*, carboxylesterase *B1* exhibited a high degradation activity against organophosphates, such as malathion and chlorpyrifos (Zheng et al., 2007). In *N. lugens*, carboxylesterase *Nl-EST1* showed the activities of OP insecticides degradation (Small and Hemingway, 2000). In addition to the degradation activities of sex pheromone and volatile ester odor molecules of host plant, COEs in olfactory tissues might have other functions, such as detoxification of volatile xenobiotics (Poupardin et al., 2008 Li et al., 2007). To understand the putative detoxification function, the larvae and adults of the silkworm were exposed by volatile OP insecticide phoxim, respectively. After phoxim exposure, the target tissues must be dissected in time; however, the dissection of the larval antennae and maxillae was relatively difficult. Thus, the larval head was used to detect the induction expression of *Bmae33*. The results of qRT-PCR showed that the expression of *Bmae33* gene could be up-regulated by phoxim in the silkworm larval head and adult antennae. This suggests that *Bmae33* might play important roles in detoxification of volatile OP insecticides in the olfactory tissues of the silkworm.

CONCLUSION

In general, we cloned the *Bmae33* gene, analyzed the expression pattern, and conducted heterogenous expression and purification of recombinant protein. The induction profiles after organophosphate phoxim exposure were also investigated. The result suggests that *Bmae33* might contain the catalysis function for volatile insecticides, such as phoxim. Previous studies indicate that olfactory COEs could hydrolyze the volatiles of host plant (Durand et al., 2010), whether *Bmae33* also contain similar function or not. The present study has provided the foundation for functional study of the *Bmae33*. The localization of *Bmae33* protein in the tissues of silkworm and the detoxification mechanism of *Bmae33* will be further studied in future.

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