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

Dan-Dan Feng¹, Wei Yang¹, Wen-Juan Yang², Quan-You Yu¹* and Ze Zhang¹,²

¹The Institute of Agricultural and Life Sciences, Chongqing University, Chongqing, 400044, China.
²The Key Sericultural Laboratory of the Agricultural Ministry of China, Southwest University, Chongqing, 400716, China.

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,

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 chemoreceptor 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 degradation 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 SICXE10 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 assembly was completed (The International Silkworm Genome Consortium, 2008). Based on this new assembly, 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 converted 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 22±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, malphigian 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 cm², 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, haemolymph from 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}/OD_{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'-ATGTCAGCTTGACGCGCGGGG-3'; reverse (R): 5'-TACGACCATTTAGATTCCGTTAT-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 converted motifs or sites, amino acid sequence of
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 (pl) of Bmae33 were predicted by the computation tool of pl/Mw (http://web.expasy.org/compute_pl/). 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 were 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 (2x), 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’-GATTTCAAGGGACACTGCC-3’; R: 5’-ACGACTAAAGAAACAACCTC-3’. Duncan’s multiple range test in SPSS software was employed to test difference significances among different concentrations of the phoxin 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’-CCGGAATTCCAGGTGACGTAGGGGGCGAGGA-3’; R: 5’-ATAAGATGCGGCCGCTTTAAATCGCTTTTACTCTGGAC-3’). The amplified DNA did not include signal peptide coding sequence, and the underlines indicated EcoR I and Not I restriction enzyme sites, respectively. The purified PCR product and expression vector pET-28(a) were digested with EcoR 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 Ni²⁺ 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 Healthcare).

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 pl of Bmae33 were 62.15kDa and 5.87 kDa, respectively. Furthermore,
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. Multi-sequence alignment of Bmae33 and several other COEs showed that Bmae33 contained the catalytic triad (Ser196-His114-Glu327) and a conserved pentapeptide 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
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.
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 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.

REFERENCES


