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Full Length Research Paper

Molecular characters and recombinant expression of the carboxylesterase gene of the meadow moth *Loxostege sticticalis* L. (Lepidoptera: Pyralidae)

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Insect carboxylesterases are enzymes that catalyze the hydrolysis of ester and amide moieties, which play important roles in insecticide resistance, specifically allelochemical tolerance and developmental regulation. We obtained the cDNA encoding carboxylesterase gene of *Loxostege sticticalis* (*LstiCarE*) by a cDNA library screen. The full cDNA of *LstiCarE* is 1,980 bp in length, containing an open reading frame (ORF) of 1,875 bp, which encodes a preprotein of 625 amino acid residues. The *LstiCarE* contains the catalytic triad (Ser-His-Glu), the pentapeptide GxSxG motif and GxxHxxD/E motif, which are typical characteristic of esterases. The GxSxG and GxxHxxD/E motifs of *LstiCarE* are modified as GCSAG and GxxHxxQ, respectively. The 3-D model structure of *LstiCarE* showed that Ser197, His440 and Glu321 are aggregated together, which form the catalytic triad. The recombinant *LstiCarE* were successfully expressed in BL21 cells using recombinant plasmid DNA, and showed high carboxylesterase activity. However, the biochemical and physiological functions of carboxylesterase gene in *L. sticticalis* requires further investigation.

Key words: Carboxylesterase gene, *Loxostege sticticalis,* recombinant expression.

INTRODUCTION

Carboxylesterases (CarEs, EC 3.1.1.1) belong to the α/β hydrolase's fold superfamily that catalyzes the hydrolysis of ester bonds of various substrates (Oakeshott et al., 1999). The key role of insect CarEs is hydrolyzing esters of carboxylic acids. Carboxylesterases also play important roles in insecticide resistance, allelochemical tolerance and developmental regulation (Oakeshott et al., 2005). Furthermore, carboxylesterases can participate in other functions, such as pheromone degradation in moths (Li et al., 2007) and hydrolysis of the neurotransmitter acetylcholine and juvenile hormone (JH) (Taylor and Radic, 1994; Riddiford et al., 2003).

Insect *CarE* genes can be subdivided into eight subfamilies based on sequence homogenous and substrate specificity: α-esterase (ae), β-esterase (be), juvenile hormone esterase (jhe), gliotactins (gli), acetylcholinesterases (ace, AChE), neurotactins (nrt), neuroligins (nlg) and glutactin (glt) class (Ranson et al., 2002). The subfamilies of α-esterases, β-esterases, acetylcholinesterases and juvenile hormone esterase account for the majority of the catalytically active COEs (Ranson et al., 2002). To date, the gene encoding carboxylesterase family has been well characterized. For example, the genes encoding JHEs or putative JHEs have been identified in 13 insects, including the species from Lepidopteran,

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Abbreviations: CarE, Carboxylesterases; **JH,** juvenile hormone; **ae,** α-esterase; **be,** β-esterase; **jhe,** juvenile hormone esterase; **gli,** gliotactins; **nrt,** neurotactins; **nlg,** neuroligins; **glt,** glutactin; **PM,** plasma membrane; **BLAST,** Basic Local Alignment Search Tool; **PCR,** polymerase chain reaction; **IPTG,** isopropyl-beta-D-thiogalactopyranoside; **PBS,** phosphate-buffered saline; **PVDF,** polyvinylidene fluoride; **DAB,** diaminbenzidine.

Coleopteran, Orthopteran, Dipteran, Hymenopteran and Hemipteran (Tsubota et al., 2010). In *Spodoptera littoralis*, 19 putative esterase genes were identified from the antennae (Durand et al., 2010). Based on the genome sequence, 76 putative *CarEs* were identified in *Bombyx mori*, and α-esterases were significantly expanded in the silkworm. Most of the *CarEs* are tissue specific and mainly expressed in the midgut, head, integument and silk gland (Yu et al., 2009).

The beet webworm, *Loxostege sticticalis* (Lepidoptera: Pyralidae) is a key pest in Asia, Europe, and North America, and is a voracious defoliator of crops and weeds. In China, the species cause serious economic damage almost every year and intermittent outbreaks over a wide region of the north China (Jiang et al., 2010a, b). The beet webworm in China showed some high resistance to organophosphate insecticides (Jiang et al., 2010a, b). Studies on insect carboxylesterases have been mainly focused on mediating insecticide resistance. In the present study, we identified a gene encoding carboxylesterase from *L. sticticalis* (*LstiCarE*) larvae by cDNA library screening. The molecular characters and conserved motifes of *LstiCarE* were analysed, and the recombinant *LstiCarE* was successfully expressed in *E. coli*.

MATERIALS AND METHODS

Insect larvae

A laboratory colony of *L. sticticalis* was reared at 22±1°C, 70–80% RH and L:D 16:8 photoperiods on LB Agar (Yin et al., 2010). The midgut for analysis was isolated from 5th instar larvae and was stored at −70°C until use.

Cloning and sequencing of *LstiCarE*

A cDNA expression library of *L. sticticalis* peritrophic midgut (Yin et al., 2010) was screened by subtractive immunoscreening with antibodies made against a collection of *Spodoptera exigua* midgut plasma membrane (PM) proteins (Zhang et al., 2008). The screening procedure was according to the descriptions by Wang et al. (2004) and Guo et al. (2005). The positive cDNA clones were subjected to sequencing using the dideoxynucleotide chain termination method (Takara Co., Dalian, China). The sequences from 282 clones were BLASTed in GenBank. The sequence of clone 92 was similar to the known carboxylesterase genes, and the full sequence of clone 92 was obtained after other 3 sequence reactions.

Molecular modeling of *LstiCarE* construction

For modeling the 3D structure of mature *LstiCarE*, we used the HMMSTR/I-sites /Rosetta Prediction Server (http://www.bioinfo.rpi.edu/~bystrc/hmmstr/server.php) (Bystroff and Shao, 2002; Ling et al., 2008).

Expression of recombinant *LstiCarE* in *E. coli*

To construct pET30-*LstiCarE*, the open reading frame (ORF) of *LstiCarE* was amplified by polymerase chain reaction (PCR) using primers CarE-MFP (5´- GGAATTCCAGGACACTGGCATAACT AACACGC -3´) and CarE-MRP (5´- CCGCTCGAGAAAGCTAAT AAAATGACAACAGAAT -3´). The PCR conditions were as follows: after 5 min at 94°C, 30 cycles of 30 sec at 94°C, 30 sec at 55°C, and 120 sec at 72°C, then 10 min at 72°C. The expected PCR products were excised from the agarose gel and purified using a DNA gel extraction kit (Takara, Japan). Then, the obtained PCR products were cloned into pET30 with *Eco*R I and *Xho* I sites, and transformed into *E. coli*, BL21 strain. After a 3 h preincubation, the recombinant *LstiCarE* was induced by adding isopropyl-beta-D-thiogalactopyranoside (IPTG) to give a final concentration of 0.5 mM for 4 h. The cells (1L) were harvested by centrifugation, and the pellets were homogenized in phosphate-buffered saline (PBS, 0.04 M, pH 7.0). After centrifugation at 12,000 g for 20 min at 4°C, the supernatants were dried and stored at −70 °C until use.

Measurement of carboxylesterase activity

The cells (1L) after IPTG induction were harvested by centrifugation, homogenized in 100 mL PBS, (0.04 M, pH 7.0), and centrifuged at 12,000 g for 20 min at 4°C. The supernatants were used to measure the carboxylesterase activity according to the method described by Moores et al. (1996). 200 µL of mixture solution [100 µM α-naphthyl acetate and 6 g/L Fast Blue RR salt (diazotized 4-(benzoylamino)-2, 5-dimethoxyaniline/ZnCl2] was added to 50 µL of the supernatant. After 30 min, the optical density (OD) value at 450 nm was measured with a microplate reader (Spectra Rainbow; TECAN, Austria). Based on the standard curve, the activity of the carboxylesterases was determined (Moores et al., 1996).

Western blot analysis

The western blot was performed following the description of Wei et al. (2005). The extracted proteins from the cells before or after induction with IPTG were mixed with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, boiled for 10 min, and immediately loaded on the 8% SDS-PAGE gel. After SDS-PAGE, the proteins were blotted on a polyvinylidene fluoride (PVDF) membrane (Hybond-P, Amersham), and the membrane was incubated with antibodies to 6×His for 2 h at 37°C. After washing in PBS-Tween, the membrane was incubated in secondary antibodies (HRP-conjugated goat anti-rabbit IgG, dilution 1/2000) for 2 h at 37°C, and then washed thoroughly in PBST. The binding was detected using a diaminbenzidine (DAB) stock stain kit (Sino-American Biotechnology Co., China).

RESULTS

Cloning the carboxylesterase cDNA of *L. sticticalis*

From two rounds of screening the PM cDNA expression library, 282 positive clones were obtained and sequenced. The sequences were BLASTed in GenBank for homologous sequences. The number 92 clone named Lsti92, was similar to the known carboxylesterase genes, making Lsti92 a candidate for encoding carboxylesterase The nucleotide sequence reported here is deposited in GenBank under the accession number EU339106.

The full-length *LstiCarE* cDNA consists of 1,980 nucleotides including the poly (A) tail. The open reading frame

(ORF) is 1,875 bp in length, and encodes a predictive precursor protein containing 625 amino acids (Figure 1). The ORF is terminated by a TAA stop codon followed by an 83 bp 3′ untranslated region exclusive of the poly (A) tail. The putative consensus polyadenylation sequence (AATATA) is found 55 bp from the stop codon (Figure 1).

Amino acid sequence analysis displayed the residues of Ser 197, His 440, and Glu 321 formed the catalytic triad (Ser-His-Glu) in *LstiCarE*, which is the conserved serine active site. The pentapeptide GxSxG motif is a typical characteristic of esterases. Interestingly, the GxSxG motif of *LstiCarE* is encoded as GCSAG (Figure 1). Sequence analyses also show that there are three potential N-glycosylation sites in *LstiCarE*, located at N279, N412 and N519, respectively (Figure 1 and 2). One potential O-glycosylation site was predicted using the NetOglyc 3.1 server, which is located at the C-terminal end of *LstiCarE* (Figure 3). Six cysteines are distributed at position 77, 94, 155, 196, 290 and 441 in *LstiCarE*, which is important for the stable structure of carboxylesterases (Figure 1).

Characterization and model building of the 3-D structure of *LstiCarE*

We used the HMMSTR Prediction Server (http://www.bioinfo.rpi.edu/~bystrc /hmmstr/server.php) to model the 3-D structure of *LstiCarE*. There are 22 helicas, 25 strands and 56 turns (Figure 4). The Ser 197, His 440 and Glu 321 are aggregated together, and form the catalytic triad (Figure 4).

To determine the retained important motifs, we compared the *LstiCarE* with other *CarEs*, and found that there are 11 *CarEs* that share some conserved structures, including GxSxG and GxxHxxD/E motifs (Figure 5). Among the 11 *CarEs*, the GxSxG motifs of seven *CarEs* appear as GESAG. The GxSxG motif of *BmanCarE*, *BmorCarE*, *CquiCarE* and *LstiCarE* were modified to GISAG, GQSAG, GNSAG and GCSAG, respectively. The 7th amino acid residue of GxxHxxD/E motif in *LstiCarE* is Q, which is different from the others (Figure 5).

Expression of recombinant *LstiCarE* in *E. coli*

Using primers CarE-MFP and CarE-MRP, the ORF of *LstiCarE* was amplified (Figure 6), and inserted into the vector pET30. The recombinant plasmid DNA was transformed into the BL21 strain of *E. coli*, and induced to express. The cells were frozen, thawed and disrupted by sonication. To confirm that the expressed product was the expected recombinant protein, western blot was performed using the anti-6×His antibody. The induced products that were transformed with the recombinant plasmid showed a 75 KD band, however, no band was present in the one that was transformed with pET30 not containing insert DNA (Figure 7). The cell extractions were also used to measure the carboxylesterases activity. The supernatants transformed with recombinant pET30-LstiCarE showed high carboxylesterases activity (16.68 pM/μL), which is significantly higher than that of the cell transformed with pET30 (0.31pM/μL) (Figure 8).

DISCUSSION

In the present study, we obtained a cDNA that encodes carboxylesterase in the midgut of *L. sticticalis*. The ORF of *LstiCarE* is 1,875 bp in length, and encodes a predictive precursor protein containing 625 amino acids, which is shorter than that of *BmorCarE*, but longer than that of other sequenced insect *CarEs*, including *ArosCarE* (*Athalia rosae*, BAD91555), *BmorCarE* (*Bombyx mori,* BAI66483), *BmanCarE* (*Bombyx mandarina,* ABY57297), *CsupCarE* (*Chilo suppressalis*, ABD62772), *CquiCarE* (*Culex quinquefasciatus,* XP001868403), *SexiCarE* (*Spodoptera exigua*, ABQ59309), *SlitCarE* (*Spodoptera litura,* ABE01157), *HarmCarE* (*Helicoverpa armigera*, ABQ42338), *AgosCarE* (*Aphis gossypii,* AAS15643) and *AaegCarE* (*Aedes aegypti,* XP001656534). The *LstiCarE* contains the catalytic triad (Ser-His-Glu), GxSxG motif and six cysteines, which are the typical molecular characters of insect carboxylesterase. By comparison, the amino acid sequences of *LstiCarE* with the other known 10 insect *CarEs*, the GxSxG and GxxHxxD/E motifs are conserved, which confirmed that the *LstiCarE* gene we obtained is one member of the carboxylesterase family. It is interesting that the GxSxG and GxxHxxD/E motifs of *LstiCarE* are modified as GCSAG and GxxHxxQ, respectively. These changes may be attributed to the evolution of the carboxylesterase family genes. The amino acid residues in the motif of the carboxylesterase family of genes also show some variety in the same species. The GxSxG motifs of *CarEs* of *B. mori* include three types, GHSAQ, GESAG and GQSAG (Tsubota et al., 2010).

The 3-D model structure of *LstiCarE* showed that Ser197, His440 and Glu321 are aggregated together, which maybe the format of the catalytic triad. The recent crystal structures of mammalian *CarEs* have greatly contributed to our understanding of the enzyme mechanism of *CarE*. The general catalytic mechanism involves a catalytic triad, consisting of a Ser, His and either a Glu or Asp residue (Wheelock et al., 2005). However, site-directed mutagenesis analysis showed that there is a potential fourth catalytic serine residue in humans, *CarE* (Stok et al., 2004). The crystal structures of human carboxylesterase is comprised of three domains and two ligand binding sites, including the regulatory domain, the α/β domain, the catalytic domain, sucrose Z-site and soman active site (Fleming et al., 2007). The predicted 3-D model structure of *LstiCarE* is similar to the crystal structures of human carboxylesterase, but needs further

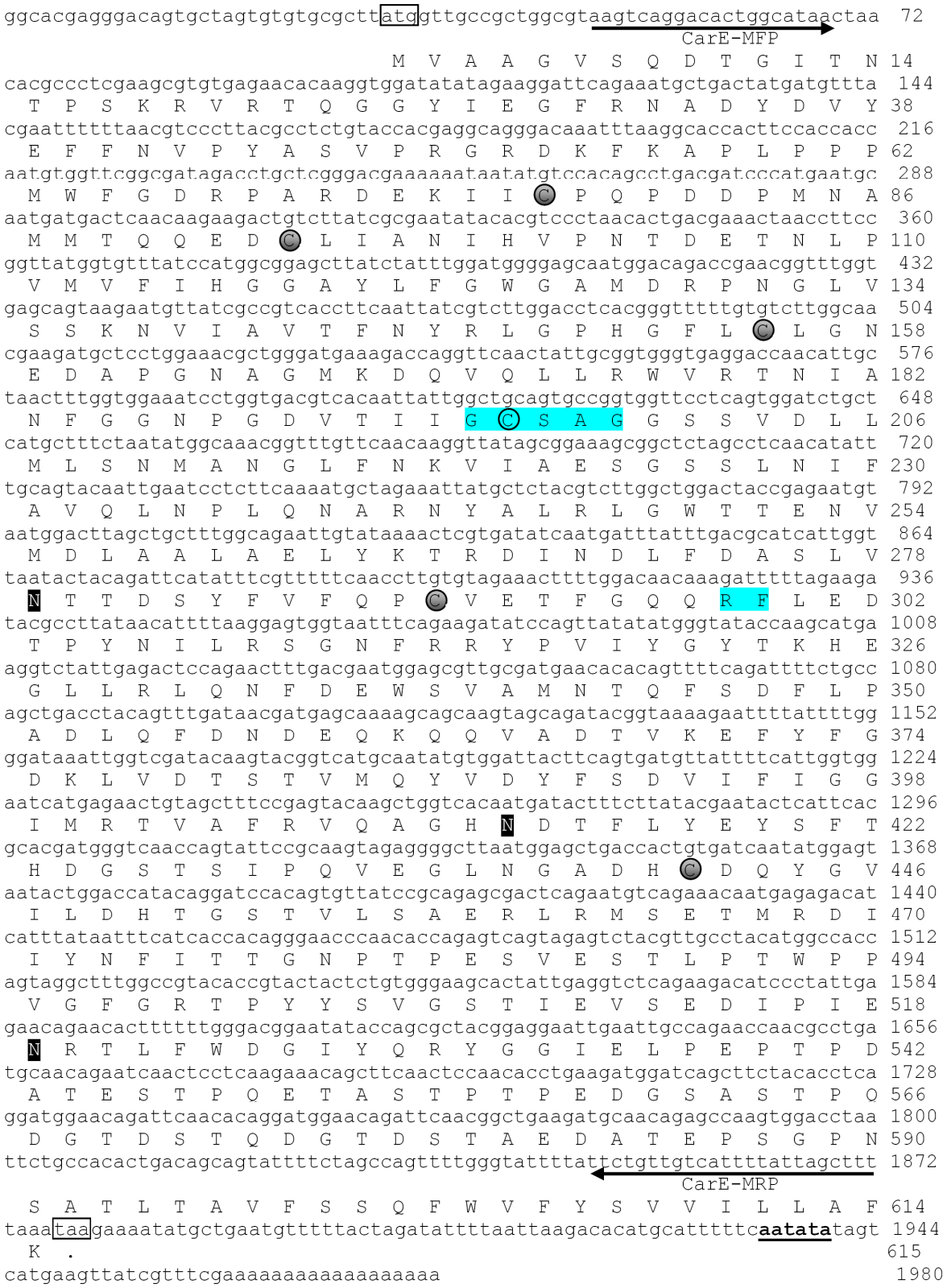

Figure 1. Nucleotide sequence and deduced amino acid sequence of carboxylesterase cDNA in *L. sticticalis*. The suggested start codon ATG and stop codon TAG are indicated in boxes. A putative polyadenylation signal is underlined. The predicted conserved motifes were shaded with turquoise. The N-glycosylation sites are shaded with black. The six cysteine residues are shown by a circle. Arrows under the nucleotide sequences represent the position of the different synthetic primers used in recombinant expression.

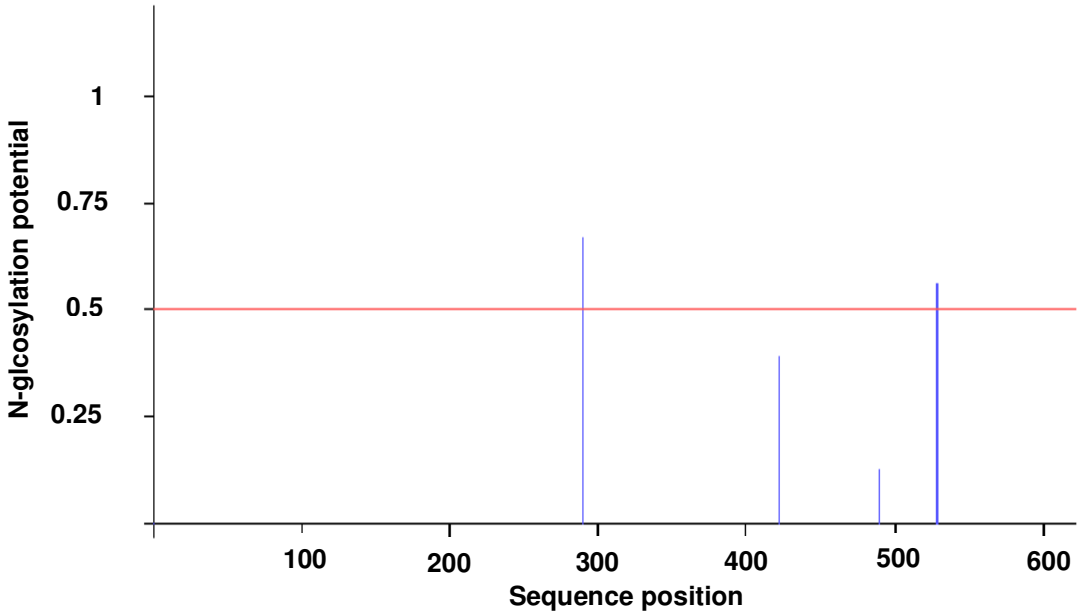

Figure 2. Prediction of N-glycosylation sites in *LstiCarE*.

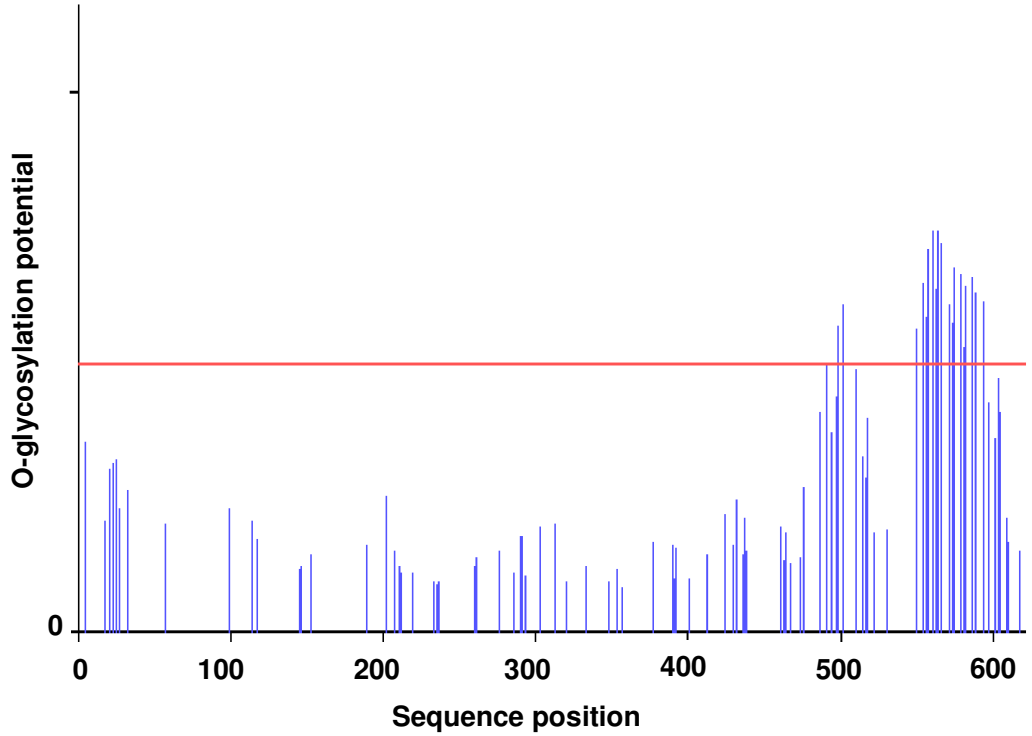

Figure 3. Prediction of O-glycosylation sites in *LstiCarE*.

studying to confirm.

Using *E. coli* expression system, we expressed the recombinant carboxylesterase in BL21 cells. The cell extracts from the recombinant *LstiCarE* showed high carboxylesterase activity, but the blank plasmid showed almost no activity. This confirmed that the *LstiCarE* we cloned was the member of carboxylesterase genes. The results of the electrophoresed SDS-PAGE and western

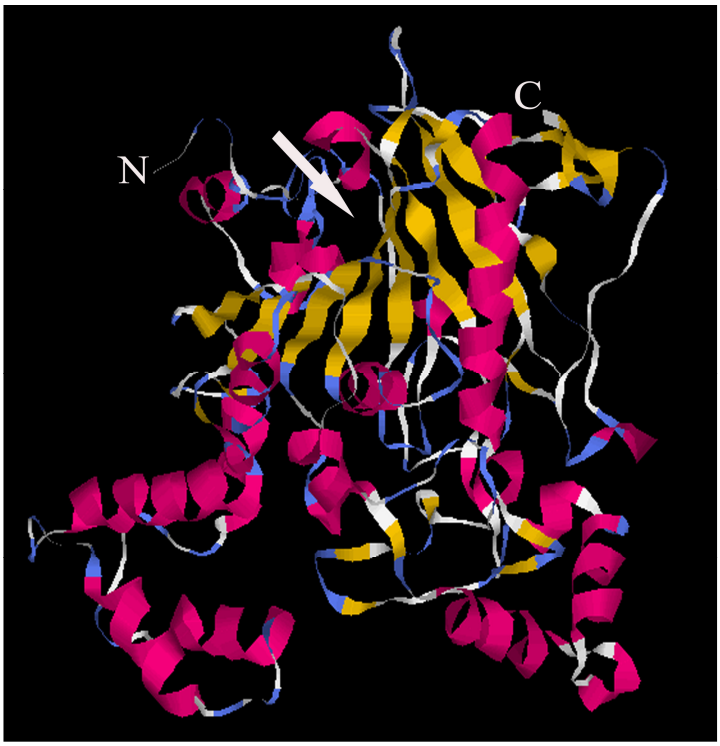

Figure 4. Predicted 3 D structure of *LstiCarE*. The arrow shows the catalytic triad.

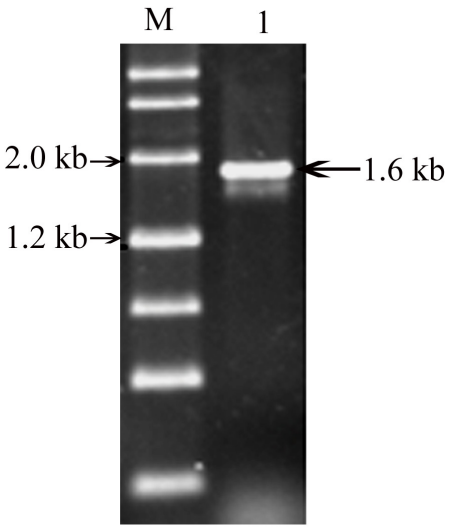

Figure 6. PCR product of amplification of the ORF of *LstiCarE*.

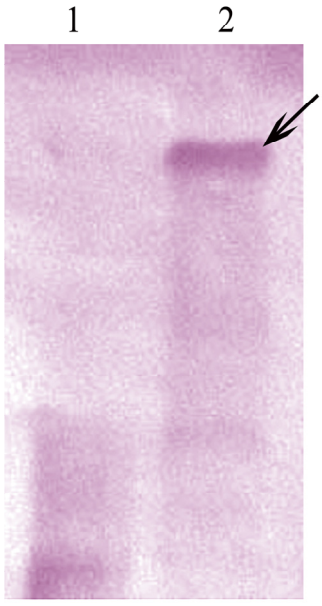

Figure 7. Western blotting analysis of expressed *LstiCarE* in BL21 cells. Lane 1, the result of western-blotting of the extracts from the infected cell homogenate with PET30 plasmid without insert; lane 2, the result of western-blotting of the infected cell homogenate with recombinant plasmid.

blotting demonstrated that we obtained the expected recombinant *LstiCarE*. The predicted molecular weight of *LsticarE* is 68.1 kDa. However, the recombinant *LstiCarE* is about 75 kDa, which can be attributed to the 6-7 kDa fused protein from the vector. Insect carboxylesterases play multiple functions in insecticide resistance, allelo-

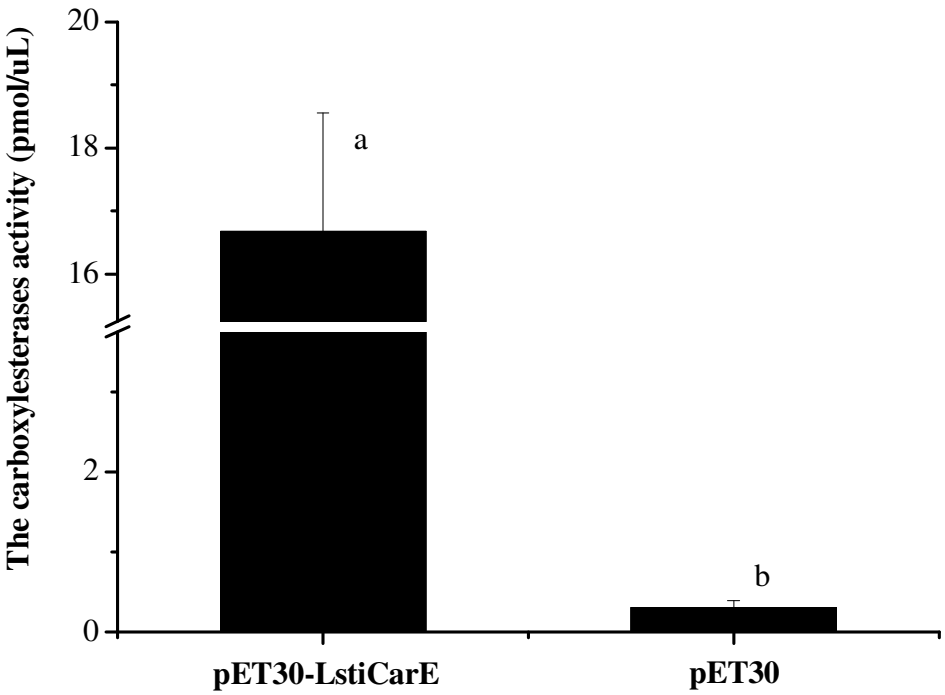

Figure 8. The carboxylesterases activity of recombinant *LstiCarE*. The cells were transformed with recombinant PET30-*LstiCarE* and PET30, respectively. The letter in the columns indicate significant differences level (different letter indicate that $P < 0.01$).

chemical tolerance and developmental regulation. Thus, we can use the recombinant *LstiCarE* to analyze the function of the carboxylesterases in *L. sticticalis*. In the present manuscript, we mainly concentrated on the molecular characters of the carboxylesterase gene of the meadow moth *L. sticticalis,* including sequence analysis, conserved motif analysis and recombinant expression of *LstiCarE*. However, the biochemical and physiological functions of *LstiCarE* need to be further elucidated.

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