Cloning of putative odorant-degrading enzyme and integumental esterase cDNAs from the wild silkmoth, *Antheraea polyphemus*

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Received 4 April 2002; received in revised form 7 July 2002; accepted 15 July 2002

Abstract

Odorant-degrading enzymes have been postulated to participate in the fast deactivation of insect pheromones. These proteins are expressed specifically in the sensillar lymph of insect antennae in such low amounts that, hitherto, isolation and protein-based cDNA cloning has not been possible. Using degenerate primers based on conserved amino acid sequences of insect carboxylesterases and juvenile hormone esterases, we were able to amplify partial cDNA fragments, which were then used for the design of gene-specific primers for RACE. This bioinformatics approach led us to the cloning of cDNAs, encoding a putative odorant-degrading enzyme (Apol-ODE) and a putative integumental esterase (Apol-IE) from the wild silkmoth, *Antheraea polyphemus*. Apol-ODE had a predicted molecular mass of 59,994 Da, pI of 6.63, three potential N-glycosylation sites, and a putative catalytic site Ser characterized by the sequence Gly195-Glu-Ser-Ala-Gly-Ala. Apol-IE gave calculated molecular mass of 61,694 Da, pI of 7.49, two potential N-glycosylation sites, and a putative active site with the sequence Gly214-Tyr-Ser-Ala-Gly. The transcript of Apol-ODE was detected by RT-PCR in male antennae and branches (sensillar tissues), but not in female antennae and other control tissues. Apol-IE was detected in male and female antennae as well as legs.

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Keywords: Bioinformatics; Sensillar esterase; Active site serine; RACE; RT-PCR

1. Introduction

While navigating en route to a pheromone source, a moth encounters intermittent chemical signals with short bursts of flux, separated by periods during which the flux is zero. The average duration of spikes within bursts is in the millisecond scale and it decreases as the animal comes closer to the pheromone source (Murlis et al., 2000). There is growing evidence in the literature that this remarkable temporal precision of the insect olfactory system is determined by early events (peripheral interactions) rather than by intracellular signaling processes (signal transduction) (Kaiselsing, 2001), but the literature is also dichotomous with respect to deactivation of chemical signals. One school favors the hypothesis that rapid inactivation is an enzymatic process regulated by odorant-degrading enzymes (ODEs) (Vogt et al., 1985), whereas the other school favors the hypothesis that chemical signals are first inactivated by odorant-binding proteins (OBPs) (Kaiselsing, 2001), with odorant-degrading enzymes participating afterwards in the catabolism (slow process) of pheromone degradation.

While recent structural studies unveiled some features of OBPs, in particular details of the protection (Sandler et al., 2000), binding, and release of pheromones (Horst et al., 2001), the molecular basis of pheromone inactivation is still terra incognita. Insect antenna-specific esterases from the wild silkmoth, *Antheraea polyphemus*, have been known for more than two decades (Klein, 1987; Vogt and Riddiford, 1981) and their ability to degrade pheromone has been demonstrated (Vogt et al., 1985). While sensillar esterase(s) is (are) specific to male antennae (Fig. 1), integumental esterase(s) is(are) present in male and female antennae and legs. Interestingly, each male antenna of the wild silkmoth has ca. 60,000 pheromone-sensitive sensilla trichodea and 10,000 sensilla basiconica (Keil, 1984; Meng et al.,...
2. Materials and methods

2.1. Tissue collection

Cocoon of the wild silkmoth, A. polyphemus, were purchased from various breeders. They were kept at 4 °C for two months, then transferred to 26 °C, 75% relative humidity, and 16L:8D photoregime and used three days after eclosion. For protein extraction, adults were anesthetized on ice and their antennae and legs were collected. Homogenization was performed in an ice-cold glass Dounce tissue grinder in 10 mM Tris-HCl, pH 8. Homogenized samples were centrifuged twice at 12,000 r.p.m (4 °C, 5 min). The supernatants were concentrated by centrifugation under vacuum and analyzed by native polyacrylamide gel electrophoresis (10% PAGE), with subsequent esterase visualization.

For cDNA cloning and RT-PCR, samples were collected at low temperature, with a Petri dish placed on a bed of ice or dry ice and set under a stereo microscope. Antennae and legs were cut with scissors. Branches of male antenna were separated from the flagellum with clean forceps. Fat body, hindgut, and male reproductive system were isolated. Integument was collected from the abdomen by removing fat body and the tracheal system. Muscles were collected from the thorax. Brain, subesophageal ganglion, and thoracic ganglion were collected and referred to as nervous system.

2.2. cDNA cloning and sequencing

Tissues were homogenized with TRizol (Invitrogen) in ice-cold Dounce tissue grinder (Wheaton) and total RNA was extracted according to the instruction manual. First strand cDNAs for 5’ and 3’-RACE were synthesized each from 1 µg of total RNA prepared from male antennae, female antennae and legs by using the SMART RACE cDNA Amplification Kit (Clontech) and SuperScript II (Invitrogen).

The following six degenerate primers (three forward and three reverse primers) were designed on the basis of three regions of conserved amino acid sequences in various insect esterases (Table 1) and used for obtaining partial sequences. F-1, 5’-CCIGTITA(C/T)TGTTT(A/T) (C/T)AT(A/C/T)CA(C/T)GGIGGIGGITT-3’; F-2, 5’-GGIAA(C/T)GGCGG(C/T)TGAAA(G/T)CA(C/T)CA-3’; F-3, 5’-GTCAATATT(T/C)GGCAA(C/T)GGG-3’; R-1, 5’-AAICCICCCIC(A/G)TG(A/G/T)AT(A/G) (A/T)AIACIA(A/G/T)1ACCGG-3’; R-2, TG(A/G)TC (C/T)TTIA(A/G)ICCIGC(A/G)TTCC; R-3, 5’-CCIGC (A/G)CTTTGICC(A/G)AATIGTIAAC-3’; ‘I’ indicates inosine. Taq DNA Polymerase (Roche) was used for PCR. Thirty-five cycles of amplification were carried out by combination of all degenerate primers and UPM (Clontech) by using various stepwise programs (94 °C
Table 1
Conserved regions of insect esterases. DmEST6, Drosophila melanogaster esterase-6 (Oakeshott et al., 1987); Estz21, Culex quinquefasciatus esterase α2-1 (Vaughan and Hemingway, 1995); LcE7, esterase from Lucilia cuprina (Newcomb et al., 1997); MpE4, Mycias persicae E4 esterase (Field et al., 1993); N1-EST1, Nilaparvata lugens carboxylesterase (Small and Hemingway, 2000b); CnumJHE, Choristoneura funebrana juvenile hormone esterase (JHE) (Feng et al., 1999); DmelJHE, D. melanogaster JHE (Campbell et al., 2001); HvirJHE, Heliothis virescens JHE (Hanzlik et al., 1989); MsexJHE, Manduca sexta JHE (Hinton and Hammock, 2001); TmolJHE, Tenebrio molitor JHE (Thomas et al., 2000)

<table>
<thead>
<tr>
<th>ESTERASE</th>
<th>Region-1</th>
<th>Region-2</th>
<th>Region-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>DmEST6</td>
<td>PVVAH1HGGA</td>
<td>GNYGLKDQQ</td>
<td>VLLVGHSAAG</td>
</tr>
<tr>
<td>Estz21</td>
<td>PVMLY1HGGA</td>
<td>GNAGLKDQQ</td>
<td>ITLFGESAG</td>
</tr>
<tr>
<td>LcE7</td>
<td>PVLVY1HGGA</td>
<td>GNAGLKDQQ</td>
<td>ITVFGESAG</td>
</tr>
<tr>
<td>MpE4</td>
<td>NVIVH1HGGA</td>
<td>GNGLKDQQ</td>
<td>VTITGMSAG</td>
</tr>
<tr>
<td>N1-EST1</td>
<td>PVMVF1HGGA</td>
<td>GNYGLKDQQ</td>
<td>VTVGESAG</td>
</tr>
<tr>
<td>CnumJHE</td>
<td>PVLVY1HGGA</td>
<td>GNGLRDIN</td>
<td>VTLAGQSAG</td>
</tr>
<tr>
<td>DmelJHE</td>
<td>PVMY1HGGA</td>
<td>GNGLRDIN</td>
<td>VTIFQGAS</td>
</tr>
<tr>
<td>HvirJHE</td>
<td>PIVLF1HGGA</td>
<td>GNGLRDIN</td>
<td>VTIQGQAS</td>
</tr>
<tr>
<td>MsexJHE</td>
<td>PVMVF1HGGA</td>
<td>GNGLRDIN</td>
<td>VTRMGSA</td>
</tr>
<tr>
<td>TmolJHE</td>
<td>PVMVF1HCCEF</td>
<td>GNGLKDQQ</td>
<td>VTIFQGAS</td>
</tr>
</tbody>
</table>
| Consensus      | PVLVY1HGGA    | GNAGLKDQQ     | VTIFQGAS      | --M--F--

for 1 min; 35–55 °C for 2 min; 72 °C for 3 min). PCR products were gel-purified by QIAquick Gel Extraction Kit (Qiagen) and ligated into pBluescript SK(+) (Stratagene) that had been digested with Eco RV (New England Biolabs). After purification of plasmid DNA by QIAprep miniprep (Qiagen), inserts were sequenced at an automated sequencing facility (Davis Sequencing). DNA sequences were analyzed by EditView 1.01 (Applied Biosystems) and MacVector (Accelrys). These PCR products were used to design gene-specific primers.

Both ends of cDNAs were amplified by RACE with gene-specific primers obtained from the partial cDNA sequences. PCR was conducted with Advantage-GC2 Kit (Clontech) to overcome the problem incurred by GC-rich regions. First, the TaqStart antibody attached to Taq polymerase was denatured at 94 °C for 3 min, then touchdown PCR was performed as follows: 5 cycles of 94 °C for 5 sec, 72 °C for 3 min; 5 cycles of 94 °C for 5 sec, 70 °C for 10 sec, 72 °C for 3 min; 30 cycles of 94 °C for 5 sec, 55 °C for 10 sec, 72 °C for 3 min; 72 °C for 3 min. Other procedures were carried out as mentioned above. Homology analyses were carried out using FASTA (http://www.ddbj.nig.ac.jp/). Amino acid sequences were compared by the ClustalW algorithm in MacVector. Signal sequence was predicted by SignalP V1.1 of World Wide Web Prediction Server in Center for Sequence Biological Analysis (http://www.cbs.dtu.dk/services/SignalP/) and N-glycosylation sites were predicted by NetNGlyc1.0 Prediction Server (http://www.cbs.dtu.dk/services/NetNGlyc/).

2.3. RT-PCR

cDNA templates for RT-PCR were synthesized as described above. The following primers Apol-ODE-F (5’-GTATAAGTTAAGCTCGCATT-3’), Apol-ODE-R (5’-CCCGGATCATTCCACTTTCC-3’), Apol-IE-F (5’-AGAGCTGCAGCGCCATATAAAGTAAGGGCCCTTT-3’) and Apol-IE-R (5’-CTGTAAGGGCTGCCTACCCATTT-3’) were designed for detection of the antennal and antenna-leg esterase transcripts. Touchdown PCR was carried out as described above. The quality of each template was checked by PCR with the following primers designed on the basis of conserved regions of insect actins: Actin-1, 5’-GCTATGATGAGAGGATGTCGCTG-3’ and Actin-2, 5’-CCGCGATCATTCCCAATAAAACTGAAGGAGGTA-3’ and Actin-1, 5’-AA(C/T)TGGA(C/T)GA(C/T)ATGGA(A/G)AA-3’ and Actin-2, 5’-CTGTAAGGGCTGCCTACCCATTT-3’. Photographs of agarose gels were taken by Gel Print 2000i (BioPhotonics) and cropped by Photoshop 6.0 (Adobe).

3. Results and discussion

Our bioinformatics approach was based on sequences of carboxylesterases and juvenile hormone esterases previously identified from insects. Although these esterases showed only moderate identity (28–40%), three short conserved regions have been identified (Table 1). PCR screening using templates from antennae or legs and with combinations of the six degenerate primers and universal primer mix (UPM) led to cloning and sequencing of a dozen partial cDNA sequences. The partial sequences were used for the design of gene-specific primers for RACE.

With gene-specific primers and UPM, we amplified 1 Kb of the 5’-region of the cDNA encoding an antennal esterase by 5’-RACE. Initial attempts to perform 3’-RACE by using gene-specific primers derived from the 1 Kb sequence were unrewarding. This was possibly due to high GC contents of this region. Utilization of Advantage GC-2 PCR (Clontech) overcame the problem and
led to amplification of a 1.4 Kb-long 3’-RACE product. The putative integumental esterase cDNA was cloned in the same manner. The cDNA of the putative antenna1 esterase from A. polyphemus was composed of 1,822 bp including GC-rich regions. SignalP V.1.1 predicted a signal sequence cleavage site between Arg-23 and Ile-24. The molecular mass of 59,994 Da (mature protein), calculated by direct translation of cDNA, was not in agreement with the molecular mass (55 KDa) of the pheromone-degrading sensillar esterase (Fig. 1) estimated by microgradient gels (Klein, 1987), particularly when considering possible post-translational modifications. The protein encoded by the antenna1 cDNA showed three potential N-glycosylation sites predicted by NetNGlyc1.0 Prediction Server (Fig. 2). The calculated pl (6.63) differed from the pl (3) of a sensillar esterase determined by ultrathin-layer isoelectric focusing gels (Klein, 1987). Although post-translational modifications may account for the different pls of native and deglycosylated proteins (Hanzlik and Hammock, 1987; Small and Hemingway, 2000a), it is not possible to conclude whether the cloned antennal cDNA encodes the previously characterized sensillar esterase (Fig. 1). The following findings, however, strongly suggest that the cloned cDNA encodes an odorant-degrading enzyme specific to male antennae. In the esterases of the α/β hydrolase fold family the active-site Ser is part of a conserved sequence: Gly-Glu(His)-Ser-Ala-Gly-Ala/Gly. This sequence, and in particular Gly-Xaa-Ser-Xaa-Gly, has been found in many other enzymes containing a catalytic triad (Cygler et al., 1993). Juvenile hormone esterases are no exception to this rule (Thomas et al., 2000). According to these criteria, the antennal esterase from A. polyphemus has a putative active site (Ser-197; starting from the signal peptide), characterized by the sequence Gly189-Glu-Ser-

Fig. 2. Alignment of putative esterases from the wild silkmoth, A. polyphemus: odorant-degrading enzyme (Apol-ODE; AY091503) and integumental esterase (Apol-IE; AY091504). The putative signal peptides are highlighted in italics and the potential glycosylation sites (NXT) are in italics and underlined. The sequences in the vicinity of the predicted Ser catalytic site are underlined. Asterisks and dots indicate identical and similar amino acid residues, respectively.
ods (GSPs) and templates from male antennae (MA), female antennae (FA), male legs (ML), integument (I), nervous systems (NS), muscle (M), fat body (FB), hindgut (HG), male reproductive system (RS), male antennae (MA), female antennae (FA).

320 bp-

Actin

Fig. 3. cDNA fragments amplified by RT-PCR with gene-specific primers (GSPs) and templates from male antennae (MA), female antennae (FA), male legs (ML), and branches (sensilla) from male antennae (MS). With GSPs from the sensillar esterase (ODE), the cDNA fragment (320 bp) of Apol-ODE was amplified specifically with templates from male antennae or branches. cDNA fragments (530 bp) from the integumental esterase (IE) were amplified with templates from male and female antennae and legs. The quality of the synthetic cDNAs was evaluated not only by the amplification of Apol-IE, but also by the amplification of an actin fragment (450 bp) with degenerate primers.

Meng et al., 1989). Detailed analysis of the expression of the antennal esterase in various tissues showed that the transcript was detected specifically in male antennae (Fig. 4). The antennal esterase cDNA was not detected in integument, nervous system, muscle, fat body, hindgut, and male reproductive system (Fig. 4). Therefore, we named this putative odorant-degrading enzyme Apol-ODE. Further characterization of the encoded enzyme, including its ability to degrade pheromone, must await functional expression of the protein. Also, it was not yet possible to determine accurately if the cloned cDNA encodes the same sensillar esterase (Fig. 1) that has been previously characterized (Klein, 1987; Vogt et al., 1985).

The cDNA of the antenna-leg esterase (putative integumental esterase) was composed of 1,891 bp, with an ORF of 1,683 bp. This cDNA encodes a protein with 544 amino acid residues, and a predicted signal peptide of 16 residues (Fig. 2). A molecular mass of 61,694 Da and pI of 7.49 were calculated from the predicted mature protein. Klein (1987) suggested that the antennae of the wild silkmoth possess two integumental esterases, with molecular masses of 65 and 90 KDa and pIs of 5.85–6 and 5, respectively. Considering the possible glycosylation of two potential N-glycosylation sites (Fig. 2), this antenna-leg cDNA may encode one of the previously characterized 65KDa integumental esterases (Klein, 1987). The potential active site Gly214-Tyr-Ser-Ala-Gly (Ser-216 starting from signal peptide) suggests that the antenna-leg cDNA encodes an esterase. Moreover, RT-PCR using cDNAs synthesized from the antennal-esterase gene in integumental esterase Apol-IE (Fig. 2).

This research is part of an on-going project aimed at getting a better understanding of the molecular basis of signal inactivation in insects. With a bioinformatics approach we were able to clone the cDNAs of two esterases from the wild silkmoth, one specific to the male antennae (putative odorant-degrading enzyme, Apol-ODE) and the other transcripted in antennae of both sexes and legs (putative integumental esterase, Apol-IE). We are now concentrating our efforts to compare Apol-ODE and Apol-IE to the sensillar and integumental esterases detected by the α- and β-naphthyl acetate assays (Fig. 1).

Acknowledgements

This research was supported in part by direct financial support from the department, college, and Chancellor’s office at UCD and by USDA grant No. 01-8500-0506-GR. We thank George Kamita and Andrew C. Hinton for their critique of an earlier version of the manuscript.

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