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Molecular cloning and analysis of a novel teratocyte-specific carboxylesterase from the parasitic wasp, *Dinocampus coccinellae*

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Abstract

Teratocytes derived from the embryonic membrane (serosa) of parasitoids are released into the host hemocoel when the parasitoid eggs hatch, where they perform several functions during the post-embryonic stage. A full-length cDNA encoding a putative carboxylesterase was isolated from the teratocytes of *Dinocampus coccinellae* and was designated as teratocyte-specific carboxylesterase (TSC). It contained an open reading frame of 2571 bp coding for a protein of 857 amino acids with a calculated molecular mass of 89 kDa. The deduced amino acid sequence had many structural features that are highly conserved among serine hydrolases including Ser, Glu and His as a catalytic triad, carboxylesterase type-B (FGGNPNSVTLLGYSAG)/ lipase-serine (VTLLGYSAGA) active sites, and six *N*-glycosylation sites. Interestingly, the mRNA encoding the TSC gene was expressed exclusively in teratocytes but not in the parasitoid larva or in the non-parasitized host. Most notably, the TSC protein was distinguished by an insertion of 294 amino acids towards the N-terminal region and was flanked by carboxylesterase domains. Furthermore, sequence alignment and homology search revealed these additional amino acids to be unique to TSC and the insertion contributed significantly to its molecular mass resulting in a larger protein than other esterases. In addition to sequence analysis, the possible role of TSC in relation to the host (*Coccinella septempunctata*) and parasitoid (*D. coccinellae*) system is discussed. © 2005 Elsevier Ltd. All rights reserved.

Keywords: Dinocampus coccinellae; Coccinella septempunctata; Host-parasitoid relationship; Teratocytes; Carboxylesterase

1. Introduction

Parasitoids have evolved a high degree of nutritional, physiological, and behavioral interactions with their hosts. For survival in their hosts, parasitoid waspderived components such as venom, polydnavirus and teratocytes play important roles by manipulating the host's physiology (Beckage and Gelman, 2004). Teratocytes are unique cells originated from the serosal membrane of some endoparasitoid embryos which become dissociated after hatching (Dahlman, 1990; Lawrence, 1990; Buron and Beckage, 1997). They have nutritive and immunosuppressive roles in some species

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and can also regulate host growth and development in others (Kitano et al., 1990; Strand and Wong, 1991; Pennacchio et al., 1992; Dahlman and Vinson, 1993). Although several studies on teratocytes are available in literature, only one teratocyte-specific gene has been characterized at molecular level (Rana et al., 2002; Dahlman et al., 2003).

The braconid wasp, *Dinocampus coccinellae* parasitizes several aphidophagous lady beetle species. In a host beetle *Coccinella septempunctata*, teratocytes increase in size and decrease in number during parasitism indicating that *D. coccinellae* teratocytes primarily provide nutrition for developing parasitoid larvae in the host (Kadono-Okuda et al., 1995). The teratocytes synthesize a great amount of teratocyte-specific protein of 540 kDa, with a major subunit of 94 kDa, a hexamerin and is shown to be nutritive in function (Okuda and

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Kadono-Okuda, 1995). In addition it also exhibited a strong esterase activity (Kadono-Okuda et al., 1998). We now report the cloning and sequence analysis of the major teratocyte-specific gene that showed domain similarity to members of the carboxylesterase family. Although tetrocyte-specific carboxylesterase (TSC) exhibits homology to carboxylesterases, the presence of the 294 amino acids insertion flanked by carboxylesterase domains makes this protein structurally distinct from other esterases.

2. Materials and methods

2.1. Insects rearing and teratocytes sampling

Adults of *C. septempunctata*, which is the main host of D. coccinellae, were collected from the vicinity of Tsukuba and kept in the laboratory at 25 °C. After several days, parasitoid larvae came out from the hosts and made cocoons. Subsequently the emerged wasps were fed on 30% sucrose solution until used for parasitization. Unparasitized coccinellids were reared under short day (12L: 12D) at 25 °C, which are diapause-averting conditions (Okuda and Hodek, 1983). Coccinellids were parasitized 7 days after adult eclosion in a Petri dish under careful observation to avoid super-parasitism. Under such conditions, parasitoid larvae complete pre-pupal development within about 19 days without undergoing diapause. Parasitized hosts were submerged in ice-cold Ringer's solution (110 mM NaCl, 1.8 mM KCl, 1.1 mM CaCl₂, 2.4 mM NaHCO₃) and dissected. The teratocytes were distributed in the host body cavity and dislodged from host tissues by pipetting ice-cold Ringer's solution over the carcass several times. The teratocytes were then transferred to a watch dish and rinsed several times with the Ringer's solution to remove the host hemocytes. Following this they were homogenized in extraction buffer (0.1 M Tris-HCl (pH 8.3), 0.8 M NaCl, 2 mM PMSF, 0.2% DOC, and 0.2% Triton X), centrifuged at 13000 rpm in 1.5 ml tubes for 10 min at 4 °C. The supernatant was collected and used for SDS-PAGE and the demonstration of lipase activity.

2.2. Protein purification and amino acid sequencing

Teratocytes from day 12 after parasitization were used for protein sequencing. The 94 kDa band of the teratocyte protein was cut out from a 2.5–20% gradient SDS-PAGE gel (Fig. 1) and electroeluted. After acetone precipitation, the protein was purified by reverse phase HPLC (Shimadzu, Japan) with a C8 column (Tosoh, Japan) using 0.1% TFA and acetonitrile. Purified protein was dried up and dissolved in CNBr (in 70% formic acid) and digestion was carried out at room



Fig. 1. Profile of teratocyte protein analyzed by SDS-PAGE on a 2.5–20% gradient gel. M: Protein markers (Bio-Rad) TC: crude teratocyte extract of day 12 after parasitization. An arrow indicates a 94 kDa teratocyte-specific polypeptide.

temperature over night. Resultant fragments were isolated by reverse phase HPLC using the same conditions as above and amino acid residues were determined by a protein sequencer (Applied Biosystem 473 A).

2.2.1. cDNA cloning

Total RNA was extracted from the teratocytes using Isogen (Nippon Gene, Japan) and was used as template for first strand cDNA synthesis employing a 5'end adapter primer (SmartIIoligo, Clontech), an oligo dT primer (Amersham) and Superscript II reverse transcriptase (Invitrogen). The first strand cDNA was then used as template in PCR with degenerate primers designed from the amino acid sequence of the purified teratocyte protein. The primer set included the sense primer, GA(C/T)AA(C/T)CA(A/G)AA(C/T)CC(I)AA (C/T)CC(I)GT (DNQNNANPV) and the antisense primer. (A/G)TA(A/G)TG(A/G)TA(A/G)TT(A/G)TA(I)AC(I)GG(T/C)TG (QPVYNYHY). Three independent amplifications were carried out to avoid any nucleotide mutations. The resultant 2.1 kb PCR product was cloned into pGEMT-Easy vector (Promega) and nucleotide sequence was determined by an automated DNA sequencer (ABI 3100).

2.2.2. Rapid Amplification of cDNA Ends (RACE)

First strand cDNA was used as templates for 5' and 3' RACE PCR using adapter primers as above and the following gene specific primers: 5'RACE Primer: GTGCTTCAGCATAACGAATACCACGATACGCA RACE, and 3'RACE Primer: GCTATCACGGACTCT

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CTCAACTTATGCCGATGC. RACE products were processed and sequenced as above. Direct sequencing was also done for confirmation.

2.3. RT PCR

Total RNA was extracted from teratocytes (day 12), parasitoid larvae (day 12) and fat body of nonparasitized host beetles. The first strand cDNA was provided as template for RT-PCR using the following primers from both ends of the open reading frame of the cloned and sequenced cDNA: sense primer- ATGGA-GATGAAGATTTTGTTGGGATTGTTG; antisense primer- TTAGTAGTGATGCATATTGTTAATCGT-CATTTGC.

2.4. Protein expression

The TSC cDNA without its signal sequence was cloned into pIVEX2.3d plasmid (Roche) between Nco1 and Sac1 sites and in frame with the C-terminal His₆tag. After sequence verification, the constructed plasmid was used for expression in a cell free protein expression system (RTS100/500 Escheriachia coli HY kit from Roche). Chaperones were added to enhance the folding of the protein according to the Roche's application manual. A negative control reaction was performed using plasmid without the insert. Purification and detection of the recombinant TSC were done using His-Trap HP kit (Amersham), and AntiHis₆ antibody (Roche) followed by chemiluminescence detection system (WesternBreeze, Invitrogen), respectively. TNT System (Promega) was also used for TSC expression according to the manufacture's protocol. SDS PAGE and Western blot were done according to standard protocols.

2.5. Lipase activity of teratocytes

Hemolymph of non-parasitized coccinellids was collected by reflex bleeding, hemocytes removed and the lipophorin was isolated by potassium bromide (KBr) density gradient ultracentrifugation (Shapiro et al., 1984). High-density lipophorin (HDLp, density 1.078 g/ml), formed as a clear yellow band was collected, desalted and used immediately. Teratocyte extract of day 12 (protein concentration 5 mg/ml) was used for lipase activity as described by Kawooya et al. 1991. Three groups were used: (A) 1 ml of lipophorin plus 500 µl of extraction buffer, (2) 1 ml of lipophorin plus 500 µl teratocyte extract, (C) 1 ml of lipophorin plus 500 µl of extraction buffer containing 2-10 U of Pseudomonas Sp lipase (Toyobo, Japan). The reactions were incubated at 37 °C for 3h, terminated by keeping on ice and then subjected to KBr density gradient ultracentrifugation.

2.6. Other methods

Unless otherwise indicated, all molecular biology techniques were performed essentially as described in Sambrook et al. (1989). Protein concentration was measured using Bio-Rad protein assay kit. Triacylglyceride (TAG) was estimated using Iatroscan TLC Analyzer (Iatroscan, Japan). Signal sequence was predicted by the SignalP program (http://www.cbs. dtu.dk/services/SignalP/). NCBI (www.ncbi.nlm.nih. gov), PSORT II (http://psort.nibb.ac.jp/), ExPASy (http://au.expasy.org/prosite/) and PredictProtein (http://www.embl-heidelberg.de/predictprotein/predictprotein.html) programs were used for protein and nucleotide analyses. Phylogenetic analysis was done by ClustalX (Thompson et al., 1997).

3. Results

Using degenerative primers designed from the amino acid fragments of the purified teratocyte protein and subsequent PCR amplifications including RACE, we have obtained a full-length cDNA from the teratocytes of D. coccinellae (Fig. 2). The 2894 bp cDNA sequence revealed an open reading frame (ORF) of 2571 bp encoding a protein of 857 amino acid residues. There were two start codons at the 5' end positions at 122-124and 128-130. Based on the Kozak sequence, the first ATG was considered to be the translation initiation codon (Kozak, 1984). The stop codon was succeeded by a polyadenylation signal (AATAAA) 138 bp downstream and a Poly $(A)^+$ tail further downstream. The first 18 amino acids comprised a putative signal peptide and the next 14 amino acid residues exactly matched the N-terminal sequence determined by Edman degradation of the purified teratocyte protein. All other partial sequences obtained were also almost identical and were present in the ORF. The ORF predicted a protein with a molecular mass of 89 kDa and was consistent with the estimated molecular mass of 94 kDa for the fully processed native protein (Okuda and Kadono-Okuda, 1995). The predicted isoelectric point of the protein was 6.41. Further it was characterized by the presence of six *N*-glycosylation sites, a carboxylesterase type-B signature 2 motif (EDCLRLNVYT) at positions 101-110, another carboxylesterase type-B serine motif (FGGN PNSVTLLGYSAG) at positions 479-494, as well as a lipase serine active site (VTLLGYSAGA) at positions 486-495, and notably the catalytic triad, Ser492, Glu629, His752 which may form the charge-relay system. The sequence Gly Xaa-Ser-Xaa-Gly (Gly490-Tyr491-Ser492-Ala493-Gly494) is conserved in all esterases/lipases (Fig. 3). These data clearly showed that the inferred amino acid sequence of the cloned gene had all the features characteristic of an active

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CG TG CG TG CG CG TT CT AC		GGT GGC GGC GGC GGC GGC GGC N AAT N AAT CGT CC CC L AAT N AAT CTC L AAT	CAAG CAAG Q CAAG Q CAAG Q CAAG Q CAAG Q CAAG K CCAA K CCAA K CCAA P GTA	S ICT S CCT P P ICT S GCC A S GCT A ICC S GCT B CCT C P CCCC P P CCCC P P CCCC P P CCCC C P CCCC C P CCCC C P CCCC C P CCCC C P CCCC C P CCCC C P CCCC C CCCC C P CCCC C P CCCC C CCCC C P CCCC C CCCC C P CCCC C CCCCC C CCCCC C CCCCC C CCCCC C CCCCC C CCCCCC	GGT(GGT) GGT) GGT) GGT(GGT) GGT(GGT) GGT(GGA(GGA(GGA(GGA(GGA(GGA(GGA(S GGT(G AGC(S S GGT(G G G G G G G G G C C G G T T C C G G C C G G T T C C G G C C G G G T T C C G C G	P CAAN Q CAAT Q CAAT H CAAN Q CAAN Q CAAN Q CAAN G G CAAN G G CAAN C Q CAAN T AGC/	ACA(T T CCC(S CCT(P TTC(F TTC(F N TTC' F N TTC' F SCT(A A CCC(L A GAGA(S G G G G G G G G G G G G G	H GGCC G AATCO N AGCCO S GGCCO G CATCO S CATCO S CATCO S CATCO S CATCO CATC	P CAAC Q C CAAC Q C CAAC Q C CAAC Q C CAAC Q C CAC Q C CAAC Q C CAAC Q C CAC Q C CAC Q C CAC Q C CAC Q C CAC Q C CAC Q C CAC Q C CAC Q C CAC Q C CCC Q C C CCC Q C C CCC Q C C CCC Q C C CCC Q C C CCC Q C C CCC Q C C CCC Q C C CCC Q C C CCC Q C C CCC Q C C CCC Q C C CCC Q C C CCC Q C C CCC Q C C CCC Q C C CCC Q C	Y CCTC P GCTC A ICTC S CCTC P ICTC S G G G G G G G G G G G G G G G G G CGCC G R CGCC G G G CGCC G C G	W GGT(G G G G G G G G G G G G G G G G G	Y GGT G G AAT N AGC S G G AGT G AGT C A CAAA Q C C C C C C C C C C C C C	H CAAA Q CAAA Q CAAA Q CAAT H CAAA Q CAAA Q CAAA Q CAAA Q CAAA Q CAAA Q CAAA Q CAAA Q CAAA Q CAAA Q CAAA Q CAAA Q CAAA Q Q CAAA X A Q CAAA X A A Q Q CAAA X A Q Q CAAA X A A X A A A A A A A A A A A A A	S CCCC P TTC F GCT A TCT S CCT P TCC S TCC S CCT P TCC S CCT P TCC S CCT P TCC S CCT P TCC S CCT P TCC S CCC C C C C C C C C C C C C C C C	D GGA G G G G G G G G G G G G G G G G G	S GGCC G GGCC G GGCC G G AGCC S AGC S AGT S AAT T S G AAT T S C A C C T S G C C S S AGT S S AGT S S AGT S S S S S S S S S S S S S S S S S S S	Q CAA Q C CAA Q CCAA Q CCAA Q CCAA Q CCAA Q CCAA Q CCAA Q CCAA Q CCAA Q CCAA Q CCAA Q CCAA Q CCAA Q CCAA Q C CAA Q C CAA Q C CAA Q C CAA Q C CAA Q C CAA Q C CAA Q C CAA Q C CAA Q C CAA Q C CAA Q C CAA Q C CAA Q C C CAA Q C C CAA C C C C	S C P AC T IT F IC S C P IT F AC T AN S A S G IT L S C	280 1020 300 1080 320 1140 340 1200 360 1260 380 1320 400 1320 400 1320 400 1320 1440 420 1560 480 1620 500 1680
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Fig. 2. Nucleotide and inferred amino acid sequences for teratocyte-specific carboxylesterase of *D. coccinellae*. The putative signal peptide is shown in broken lines and the stop codon is indicated by an asterisk. A potential polyadenylation signal is in bold and the polyA tail in italics. The predicted *N*-glycosylation sites are shown in grey, carboxylesterase and lipase active sites are indicated in bold in the amino acid sequence. The amino acids determined by Edman degradation are underlined, whereas, unmatched residues are indicated below at their respective positions. The amino acids that are unique to TSC are boxed.

AGCO	CGAF	GAT	TAT	GCC	TAT	TCC	CTI	CCC		TTC	GCI	'GAA	TTC	CAC	GGI	'GAT	CCC	GTC	CT	1860
A	Ε	D	Y	A	Y	S	L	Ρ	K	F	A	Ε	F	Η	G	D	Ρ	V	L	580
CATO	TGG	ACC	CCA	GTC	GTI	GAA	CAG	AAT	'CTA	TCA	TCA	.GGC	AGT	AGC	'AAC	AAC	'AAT	GCA	GA	1920
I	W	Т	Ρ	V	V	Е	Q	N	L	S	S	G	S	S	Ν	Ν	Ν	A	Е	600
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A	F	I	S	A	Q	P	V	D	I	I	R	S	K	Q	A	N	F	V	P	620
TTTZ	ATC	ACC	GGC	'GTC	'AAC	AAA	GAT	GAA	CTC	GGT	'GGA	GTT	GTC	'ATA	GTC	GCT	GAG	GAA	CA	2040
L	I	Т	G	V	Ν	K	D	Е	L	G	G	V	V	I	V	A	Е	Е	Q	640
AGCI	CAA	AGT	GGA	AAC	'AGT	TCA	ATC	TAT	'GA'I	'GAA	TTC	AAC	AGC	'AAA	TGG	GAA	CAG	GTT	GC	2100
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L	Y	Α	D	Α	L	I	I	F	Q	G	Η	R	F	Е	R	L	М	А	N	720
CTAI	TCA	TCT	CAA	CCA	GTT	TAC	AAC	TAT	'CA'I	TAT	GTC	TAT	CCC	GCI	TGI	GAA	AGT	TTC	GC	2340
Y	S	S	Q	Ρ	V	Y	Ν	Y	Η	Y	V	Y	Ρ	A	C	Е	S	F	A	740
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K	W	S	N	G	S	н	F.	G	V	V	Н	Н	D	E	Ц	Ц	Ц	Ц	F.	/60
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CTAC	CTCC	AAC	GTT		TGG	ЮAA	.CCG	TCA	ACC	CAA	TAA	'CAT	'CCA	CAA	CAT	'TTG	GAA	ATT	GG	2580
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TTCC	TTT	TCT	TCC	TCT	TTT	TTC	TCT	TTT	TTT	TTT	TCT	CTT	CTT	TTAT	GGC	TAA	TTT	ТАА	AT	2820
TGTA	ATTI	ACA	TGA	ATA	AAT	'ATT	TTG	TTG	ATT	'GAA	TTA	AAA	AAA	ACC	CGA	AAA	AAA	ААА	AA	2880
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Fig. 2. (Continued)

carboxylesterase and hence it was termed TSC. However, TSC was distinguished by the insertion of 294 contiguous amino acids from position 111–404 towards its N-terminal and thus flanked by carboxylesterase domains. Alignment of the complete amino acid sequence of TSC with other insect carboxylesterases resulted in a long gap due to this insertion and a further homology search showed no similarity of this part to any known esterases, indicating that these inserted amino acids are unique to TSC.

RT-PCR analysis showed that the carboxylesterase gene was exclusively expressed in teratocytes (Fig. 4). No transcript was detected in parasitoid larva or in the non-parasitized host. Thus, the parasitoid larva that is basically from the same source as teratocytes did not seem to transcribe the TSC gene. The TSC cDNA without its signal sequence was cloned in an expression vector in frame with the C-terminal His₆-tag and expressed in a cell-free system. As shown in Fig. 5, the expressed protein was of expected size (90.1 kDa) in which the C-terminal His₆-tag and other linker amino acids contributed 1.1 kDa. This was in good agreement with the molecular mass of the native protein after glycosylation and other post-translational modifications as mentioned above. Although our data clearly indicate that the target gene was cloned from the teratocytes, the expressed protein from both cell free systems did not yield a functional enzyme (data not shown) indicating that it requires post-translational processing; especially glycosylation, and this could not be induced by the

Dinocampus ----MEMKILLGLLLCCAIAIATDNONNANPVVOTSAGTIOGSLWKTRLGKTIYAYRG 54 -----MKNTCGILLNLFLFIGCFLTCSASNTPKVQVHSGEIAGGFEYTYNGRKIYSFLG 54 Myzus Drosophila ----MNYVGLGLIIVLSCLWLGSNASDTDDPLLVQLPQGKLRGRDNGSYYSYES 50 ---MAVKWEMVGLAWAALLAFASFSAADNSVPVVHDTASGDLSGKFLTLTPNRTIEAYLG 57 Nilaparvata Lucilia MNFNVSLMEKLKWKIKCIENKFLNYRLTTNETVVAETEYGKVKGVKRLTVYDDSYYSFEG 60 Anisopteromalus ----MERPEVKTLSGOVRGLKOISVEGIGFYAFKG 31 Dinocampus IRYAEAPTGQNRFKQAIPV-KPHSGVYDATQDGPLCPQPVS----NNRIISEDCLRLNV 108 IPYASPPVQNNRFKEPQPV-QPWLGVWNATVPGSACLGIEF--GSGSKIIGQEDCLFLNV 111 Myzus IPYAEPPTGDLRFEAPEPYKQKWSDIFDATKTPVACLQWDQFTPGANKLVGEEDCLTVSV 110 IPYAQPPIGSRRFKDPEPF-GKWLGTFNGTKEPTKCLQVNGF-LPGKPVEGSEDCLYLNV 115 Drosophila Nilaparvata IPYAQPPVGELRFKAPQRP-TPWDGVRDCCNHKDKSVQVD---FITGKVCGSEDCLYLSV 11 IPYAKPPVGELRFKDPVPI-EPWQEVREATEFGPMAAQFD---VISKFSGGSDDCLYINV 87 ucilia 116 Anisopteromalus .:*** * **..* . **: . ----VVVFLHPGAFYSYSGTSNDFGPE-NLLDRDIVLVTVNYRLGSLG 153 Dinocampus YTPKLPQENSAGDLMNVIVHIHGGGYYFGEG--ILYQPHYLLDNNDFVYVSINYRLGVLG 169 YKPKNSKR----NSFPVVAHIHGGAFMFGAA--WQNGHENVMREGKFILVKISYRLGPLG 164 Myzus Drosophila Nilaparvata YTPSRNG-----VGYPVMVFIHGGGFVDGDGTSGFYGPDKLLLTKDIILVTIHYRLGFLG 170 YTNNLNPE----TKRPVLVYIHGGGFIIGENHRDMYGPD-YFIKKDVVLINIQYRLGALG 171 YTKKINSN----VKQPVMFYIHGGGFIFGSGNDFFYGPD-FLMRKDIVLVTFNYRLGVFG 142 Lucilia Anisopteromalus *: .:* *.: * . : ..: ÷... Dinocampus Myzus FASTGDG--VLPGNNGLKDOVAALKWIOONIVAFGGDPNSVTITGMSAGASSVHNHLISP 227 Drosophila FVSTGDRD--LPGNYGLKDQRLALKWIKQNIASFGGEPQNVLLVGHSAGGASVHLQMLRE 222 Nilaparvata FASLDDG--DFAGNYGLKDOSLALKWVKENIAKFGGDGDKVTVVGESAGAASAHFHILSP 228 FLSLNSEDLNVPGNAGLKDQVMALRWIKNNCANFGGNPDNITVFGESAGAASTHYMMLTE 231 Lucilia Anisopteromalus FLNLEHE--VAPGNQGLKDQVMALKWVRDNIANFGGDSENVTIFGESAGGASVHYLTVSP 200 **:*:::* ***** ***: :.: : * *** Dinocampus MSRGLFHRAIAMSGAVTNQNRLPSDQA-DLAKKQAQILGCPIDTYDNMFNCLYSKSAEDY 270 MSKGLFNRAIIQSGSAFCHWSTAENNA-QKTKYIANLLGCPTNNSVEIVECLRSRPAKAI 286 DFGQLARAAFSFSGNALDPWVIQKGARGRAFELGRNVGCESAEDSTSLKKCLKSKPASEL 282 Myzus Drosophila Nilaparvata QSQGLFQRAILLSGTADCPWAVSTAHQ-NGNLTAKMASLVNCSADTSATELLECLRKVEG 287 QTRGLFHRGILMSGNAICPWANTQCQH-RAFTLAKLAGYKGEDNDKDVLEFLMKAKPQDL 290 Lucilia Anisopteromalus LAKGLFHKAISOSGVFMNPWASVSGEP-RKKAYELCELLG--KKTTDPVEIVKFLRTVDT 257 Dinocampus AYSLPKFAEFHGDPVLIWTPVVEQNLSSGSSNNNAEAFISAQPVDIIRSKQANFVPLITG 330 AKSYLNFMPWRNFPFTPFGPTVEVAG-----YEKFLPDIPEKLVP----HDIPVLIS 334 VTAVRKFLIFSYVPFAPFSPVLEPSD-----APDAIITQDPRDVIKSGKFGQVPWAVS 335 Myzus Drosophila Nilaparvata SEFLIHN--EKFOTVWKGYSVPIVIFRPTIESHSGNAFITOESYKSO----SKKPMMIG 340 IKLEEKVLTLEERTNKVMFPFGPTVEPYQTADCVLPKHPREMVKTAWG----NSIPTMMG Lucilia Anisopteromalus MKLIEHQGELQIQELQKKCLSAFVPGVDDKSPNPFMPFSREVAVEQA----AHVPYLIG 312 Dinocampus VNKDĚLGGVVIVAEEQAQSGNSSIYDEFNSKWEQVAPISFSYERDTPRSSSISRDLKSFY 390 Myzus Drosophila IAODEGLIFSTFLGLENGFNELNNNWNEHLPHILDYNYTISNE---NLRFKTAODIKEFY 391 YVTEDGGYNAALLLKERKSGIVIDDLNERWLELAPYLLFYRDTKTKKDMDDYSRKIKQEY 395 ATSDEGALVLAILKRDKTRSLESALSEFDKRFTEIMPVEGDFL-DDPDHKERAEKIKTEY 399 NTSYEGLFFTSILKQMPMLVKELETCVNFVPSELADAER-----TAPETLEMGAKIK 398 Nilaparvata Lucilia Anisopteromalus YNDREGTLLYKIFENDDFESKNLRFEEFIHPNFAETLKR-----KKISLEDLKRMY 363 Dinocampus LRDQPVKQGSYHGLSQLYADALIIFQGHRFERLMANYSS-QPVYNYHYVYPACESFAKWS 449 FGDKPISKETKSNLSKMISDRSFGYGTSKAAQHIAAKNT-APVYFYEFGYSGNYSYVAFF 450 Myzus Drosophila IGNQRFDIESYSELQRLFTDILFKNSTQESLDLHRKYGK-SPAYAYVYDNPAEKGIAQVL 454 FGNSTISNETLPQLTKLYSDTYFLNGIKSTLSRHEGEKY-VYKFGYEGSYSISQLLSGDP 458 Nilaparvata Lucilia KAHVTGETPTADNFMDLCSHIYFWFPMHRLLQLRFNHTSGTPVYLYRFDFDSEDLINPYR 458 Anisopteromalus FKNKKISKETTGKFIDLFSDMYFIQGIHQVARVQAERNS-APTYMYQFTYDQGPNFSKGM 422 Dinocampus NGSHFG----VVHDELLLLFKMNKYPNVCNRDVKTLER-----LTGIIANFAKTGEP 498 DPKSYSRGSSPTHGDETNYVLKVDGFTVYDNEEDRKMIK-----TMVNIWATFIKSGVP 504 Mvzus Drosophila ANRTDYD-FGTVHGDDYFLIFENFVRDVEMRPDEQIISR-----NFINMLADFASSDNG 507 TYRN----GVCHADDLFYLFPMKPFLGLRVGSETEKD--KEISAKFVDLITNFVIEGNP Nilaparvata 511 Lucilia IMRSGRGVKGVSHADELTYFFWNQLAKRMPKESREYKT----IERMTGIWIQFATTGNP Anisopteromalus FSIDE---PGSTHMDELIYLFSMKFQETLNMEPIDKKSPHFRVMEQMVELWTNFAKYGRP 479 IPQNDAVNYSNVKWQPSTQNHPQHLEIGEELSIVNGPVYENRMNEWEKLFPLSTMQMTIN 558 Dinocampus Myzus DTENSEIWLP---VSKNPADLFRFTKITOOOTFEAREOSTMAIMNFGVAYHYONILNLMC 561 Drosophila SLKYGECDFKDSVGSEKFQLLAIYIDAARIGSMWNFRKLHE--548 Nilaparvata N---SKSEP--SIWTPSSK-DVDFLSISTEGNFEMKKNFPGA------547 YSNEIEGMEN-VSWDPIKKSDEVYKCLNISDELKMIDVPEMDKIKQWESMFEKHRDLF--Lucilia Anisopteromalus IPAPTELLP--VHWLPMND-GTVLRYLNIGEELRMEKVLNIEERYDYKLICHREKV----532 Dinocampus NMHH 562 Myzus OMT- 564

Fig. 3. An alignment using ClustalX program of the inferred amino acid sequence of TSC of *D. coccinellae* (AY498693) with five insect carboxylesterases: *M. persicae* (P35502); *D. melanogaster* (A28022); *N. lugens* (AAG40239); *L. cuprina* (AAB67728); and *A. calandrae* (AF064523). GenBank accession numbers are given in parentheses. Functionally important residues (catalytic triad) are indicated by arrows. Identical amino acid residues are shown by asterisks. The ":" and "·" indicate strong and weak group of amino acids that are conserved respectively. Amino acids from position 111 to 404 were removed from TSC sequence to avoid a long gap in the alignment (see text for details).

expression systems we used. Since the cloned TSC gene had both esterase and lipase motifs, and the esterase activity of this protein was already shown (KadonoOkuda et al., 1998), we were interested to know whether it also possesses lipase activity. To this point, we have used crude teratocyte extracts, as in the case of the



Fig. 4. RT-PCR analysis demonstrating TSC gene expression. RNA was isolated from teratocytes (TC), parasitoid larva (L) and fat bodies of non-parasitized host (NPH) reverse transcribed, amplified by PCR with TSC-specific primers and the amplification products were analyzed on a 0.8% agarose gel. Molecular size markers (M) (Invitrogen) are indicated at left.

esterase assay, together with the lipophorin (Lp) of the host beetle.

As in many insects the lipophorin (HDLp) of the host coccenellids is yellow in color due to carotenoids and its density was 1.078 g/ml. This HDLp formed a clear yellow band upon KBr density gradient centrifugation. The density of the Lp increased to 1.14 g/ml when it was incubated with teratocytes and analyzed after density gradient centrifugation. This indicates the conversion of HDLp to very high-density lipophorin (VHDLp) resulting in the band shift (Fig. 6). Increased density of Lp may be caused by the hydrolysis of lipids by the teratocytes. Lp incubated with bacterial lipase also showed a similar result and the disappearance of the yellow band was noticed when increased quantities of lipase or teratocyte extracts were used (data not shown).

We have observed a marked decrease in triacylglyceride (TAG) in the fat body of parasitized host compared to the TAG in the fat body of non-parasitized host (Fig. 7B). Further, TAG in the teratocytes showed a gradual increase from day 7 and reached a maximum on day 12 after parasitization. After this, the TAG content either reached a plateau or slightly decreased (Fig. 7A), with decrease in number of the teratocytes (Kadono-Okuda et al., 1995).

4. Discussion

Carboxylesterases are a group of serine esterases that catalyze the hydrolysis of a wide variety of ester



Fig. 5. Western blot analysis of TSC expressed in a cell- free protein expression system on a 7.5% SDS-PAGE gel. The TSC cDNA was cloned into pIVEX2.3d plasmid and expressed using RTS100 *E. coli* kit (Roche). TSC, TSC protein of molecular mass 90.1 kDa containing His₆-tag at C-terminal. C, negative control (plasmid without insert). Five microlitre of each reaction product was loaded. Protein markers (Bio-Rad) on the left.

and amide containing endogenous and xenobiotic compounds (Heyman, 1980). Some of them hydrolyze palmitoyl CoA, acyl-carnitine, and mono-and diacylglycerols and are thought to be involved in lipid metabolism (Satoh, 1987) while others are able to hydrolyze a broad range of substrates (Oakeshott et al., 1999). Furthermore, carboxylesterases are glycoproteins characterized by high mannose and N-glycosylation may play an important role in their catalytic activity (Kroetz et al., 1993). Previous work shows that teratocytes of D. coccinellae synthesize a teratocyte-specific polypeptide (Okuda and Kadono-Okuda, 1995), a high mannose containing glycoprotein, produced as a hexamer, and the molecular mass of its major subunit is 94 kDa (Kadono-Okuda et al., 1998). This is the most prominent protein in teratocytes observed when analyzed on a SDS-PAGE with Coomassie blue staining indicating its abundance (Okuda and Kadono-Okuda, 1995). Moreover, it exhibits a strong esterase activity but is devoid of juvenile hormone esterase activity (Kadono-Okuda et al.,



Fig. 6. Hydrolysis of lipids by teratocyte extract. HDLp incubated in the absence (Control) and in the presence of teratocyte extract (TC). Subsequent density gradient ultracentrifugation showing Lp band shift in the right tube (TC) due to the formation of VHDLp from HDLp.

1998). The deduced amino acid sequence of the teratocyte cDNA has the most characteristics of an active carboxylesterase enzyme. These include a catalytic triad, a conserved esterase/lipase motif, two carboxylesterase type-B (involved in lipid metabolism) motifs, a lipase-serine active site, and six N-glycosylation sites. Several lines of evidence indicate that we have cloned the major gene from the teratocytes of *D. coccinellae*. First, the cDNA sequence obtained would encode peptides similar to that of fragments obtained from Edman degradation of the purified teratocyte protein. Second, regions of the inferred amino acid sequence of TSC have esterase motifs and third, the predicted molecular mass of the protein (89 kDa) is in good agreement with that of its native protein (94 kDa). This difference in molecular mass is mainly due to glycosylation of the native protein and it is further supported by the presence of six *N*-glycosylation sites in the TSC sequence. The presence of a signal peptide and the absence of a consensus C-terminal endoplasmic reticulum retention signal, HXEL may indicate that TSC protein is secretory. Interestingly, TSC has a clear HDEL tetrapeptide (753–756), albeit 101 amino acids away from the C-terminal. Additionally, a secretory signal TEHT (Medda and Proia, 1992) found at the C-terminal of some carboxylesterases is absent in TSC. The PSORT II program predicts that TSC can be both cytoplasmic and extracellular in equal probability. Retention of rat liver carboxylesterase in the endoplasmic reticulum is still



Fig. 7. Triacylglyceride content (in mg per animal) in teratocytes and parasitoid larvae (A), fat bodies of parasitized and non-parasitized host (B) after different days of parasitization. (A) Open circle: total teratocytes in a host beetle, closed circle: a parasitoid larva (B) Open circle: fat bodies from a non-parasitized host beetle, closed circle: fat bodies from a parasitized host beetle, Values are mean \pm SE of four animals.

possible with a signal peptide and the absence of a retention sequence (Takagi et al., 1988). Taken together these data may thus agree with our previous report (Kadono-Okuda et al., 1998) that teratocyte protein largely accumulates in teratocyte cells with a little secretion.

While most insect carboxylesterases are implicated in insecticide resistance (Field et al., 1988; Karunaratne et al., 1993; Newcomb et al., 1997), many vertebrate carboxylesterases are reported to be involved in the hydrolysis of several lipids (Satoh, 1987). Our data do not seem to support a detoxification or xenobiotic degradation by TSC. The host beetles have a defensive alkaloid Coccinellin which is bitter in taste and indeed toxic (Tursch et al., 1971). However, the alkaloid does not contain ester or amide bonds to be digested by carboxylesterase. As mentioned above, TSC is marked by the presence of a lipase serine active site and two carboxylesterase type-B motifs that are all thought to be involved in lipid metabolism. TSC may be involved in the break down of host lipids following lipid accumulation in teratocytes (Fig. 7) as part of the utilization of fat as an energy source for the growing parasitoid larva. This is consistent with a nutritional role of *D. coccinellae* teratocytes (Okuda and Kadono-Okuda, 1995). Teratocytes serving as a nutrient source for developing parasitoid larvae are reported in several species such as *Aphidius ervi* (Falabella et al., 2000), *Microplitis mediator* (Quin et al., 2000), *Microctonus aethiopoides* (Barratt and Sutherland, 2001) and *Cotesia kariyai* (Nakamatsu et al., 2002) parasitoid systems.

Although the functions of teratocytes are not completely well understood, there is considerable published evidence to support their role in parasitoid development (reviewed by Beckage and Gelman, 2004). Microplitis croceipes teratocytes produce a 13.9 kDa protein (monomer, TSP14) that inhibits host protein synthesis that is linked to larval growth and development (Zhang and Dahlman, 1989; Dahlman, 1990). TSP14 appears to be the only teratocyte-specific gene cloned and characterized. It encodes a protein of 129 amino acids (including a signal sequence of 22 amino acids) and carries a cysteine-rich motif similar to that described from Campoletis sonorensis polydnavirus (Dahlman et al., 2003). Like native protein, the recombinant TSP14 inhibited host protein synthesis selectively in a dose dependent manner (Rana et al., 2002). However, there is no significant sequence similarity between TSP14 and TSC of the present study suggesting the existence of host-specific differences in the primary structure of major teratocyte-specific genes. Likewise, the biological role of teratocytes in hostparasitoid relationships may differ from one system to another (Dahlman, 1990).

It is interesting to note the distinct sequence difference of TSC and other carboxylesterases. Although TSC shares homologies with the members of the carboxylesterases and exhibits domain structures that are typical for this group, it significantly differs from them by a unique insertion of 294 amino acids. A NCBI conserved domain search (rpsblast) reveals a clear demarcation in the TSC protein domain structure. While the amino acid positions 27-110 and positions 405-829 display domain similarity (with E value ranging from 0.008 to 4e-77) to the members of the carboxylesterase family, the intervening amino acids (111-404) do not show any putative domain. In addition, an alignment of the complete amino acids of TSC with other insect carboxylesterases shows that the inserted 294 amino acids leaves a long gap in the alignment, indicating that this non-aligned sequence is unique to TSC and that they are flanked by carboxylesterase domains. The physiological significance of this insertion is uncertain. Interestingly this region is enriched with glutamine and glycine. BLAST search (protein-protein) of these unique amino acids alone shows no known protein domains but reveals similarity to immunoglobulin binding proteins. Since insects have no antigenic immunity, this non-aligned part of the sequence may function in host immune suppression through other means or be involved in other function(s) which warrants further investigation. While the size range of most esterases is 60–70 kDa (Cygler et al., 1993), TSC is 94 kDa and this relatively large molecular size invites speculation that in addition to a possible ester bond hydrolysis, TSC may also perform other function(s). Although the precise physiological functions of TSC remain unclear, our data on the cloning and sequence analysis clearly indicate the presence of a unique carboxylesterase in the teratocytes of a parasitic wasp.

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References

- Barratt, B.I.P., Sutherland, M., 2001. Development of teratocytes associated with *Microctonus aethiopoides* Loan (Hymenoptera: Braconidae) in natural and novel host species. J. Insect Physiol. 47, 257–262.
- Beckage, N.E., Gelman, D.B., 2004. Wasp parasitoid disruption of host development: implications for new biologically based strategies for insect control. Ann. Rev. Entomol. 49, 299–330.
- Buron, I.de., Beckage, N.E., 1997. Developmental changes in teratocytes of the braconid wasp *Cotesia congregata* in larvae of the tobacco hornworm, *Manduca sexta*. J. Insect Physiol. 43, 915–930.
- Cygler, M., Schrag, J.D., Sussman, J.L., Harel, M., Silman, I., Gentry, M.K., Doctor, B.P., 1993. Relationship between sequence conservation and three-dimensional structure in a large family of esterases, lipases, and related proteins. Protein Sci 2, 366–382.
- Dahlman, D.L., 1990. Evaluation of teratocyte functions: an overview. Arch. Insect. Biochem. Physiol. 13, 159–166.
- Dahlman, D.L., Vinson, S.B., 1993. Teratocytes: developmental and biochemical characteristics. In: Beckage, N.E., Thompson, S.N., Federici, B.A. (Eds.), Parasites and Pathogens of Insects, vol. 1. Academic Press, New York, pp. 145–165.
- Dahlman, D.L., Rana, R.L., Schepers, E.J., Schepers, T., Diluna, F.A., Webb, B.A., 2003. A teratocyte gene from a parasitic wasp that is associated with inhibition of insect growth and development inhibits host protein synthesis. Insect Biochem. Mol. Biol. 12, 527–534.

- Falabella, P., Tremblay, E., Pennacchio, F., 2000. Host regulation by the aphid parasitoid *Aphidius ervi*: the role of teratocytes. Entomol. Exp. Appl. 97, 1–9.
- Field, L.M., Devonshire, A.L., Forde, B.G., 1988. Molecular evidence that insecticide resistance in peach-potato aphid (*Myzus persicae*) results from amplification of an esterase gene. Biochem. J. 251, 309–312.
- Heyman, E., 1980. In: Jakoby, W.B. (Ed.), Enzymatic Basis of Detoxification, Vol. II. Academic Press, New York, pp. 291–323.
- Kadono-Okuda, K., Sakurai, H., Takeda, S., Okuda, T., 1995. Synchronous growth of a parasitoid, *Perilitus coccinellae* and the teratocyte with the development of the host, *Coccinella septempunctata*. Entomol. Exp. Appl. 75, 145–149.
- Kadono-Okuda, K., Weyda, F., Okuda, T., 1998. *Dinocampus* (= Perilitus) *coccinellae* teratocyte polypeptide: accumulative property, localization and characterization. J. Insect Physiol. 44, 1073–1080.
- Karunaratne, S.H.P.P., Jayawardena, K.G.I., Hemingway, J., Ketterman, A.J., 1993. Characterization of a B-type esterase involved in insecticide resistance from the mosquito *Culex quinquefasciatus*. Biochem. J. 294, 575–579.
- Kawooya, J.K., van der Host, D.J., van Heusden, M.C., Brigot, B.L.J., van Antwerpen, R., Law, J.H., 1991. Lipophorin structure analyzed by invitro treatment with lipases. J. Lipid. Res. 32, 1781–1788.
- Kitano, H., Wago, H., Arakawa, T., 1990. Possible role of teratocytes of the gregarious parasitoid, *Cotesia (= Apanteles) glomerata* in the suppression of phenoloxidase activity in the larval host, *Pieris rapae crucivora*. Arch. Insect Biochem. Physiol. 13, 177–185.
- Kozak, M., 1984. Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNAs. Nucleic Acids Res 12, 857–872.
- Kroetz, D.L., McBridge, O.W., Gonzalez, F.J., 1993. Glycosylationdependent activity of baculovirus-expressed human liver carboxylesterase: cDNA cloning and characterization of two highly similar enzyme forms. Biochemistry 32, 11606–11617.
- Lawrence, P.O., 1990. Serosal cells of *Biosteres longicaudatus* (Hymenoptera: Braconidae): ultrastructure and release of polypeptides. Arch. Insect Biochem. Physiol. 13, 199–216.
- Medda, S., Proia, R.L., 1992. The carboxylesterase family exhibits Cterminal sequence diversity reflecting the presence or absence of endoplasmic-reticulum-retention sequences. Eur. J. Biochem. 206, 801–806.
- Nakamatsu, Y., Fujii, S., Tanaka, T., 2002. Larva of an endoparasitoid, *Cotesia kariyai* (Hymenoptera: Braconidae), feed on the host fat body directly in the second stadium with the help of teratocytes. J. Insect Physiol. 48, 1041–1052.
- Newcomb, R.D., Campbell, P.M., Ollis, D.L., Cheah, E., Russel, R.J., Oakeshot, J.G., 1997. A single amino acid substitution converts a

carboxylesterase to an organophosphorous hydrolase and confers insecticide resistance on a blowfly. Proc. Natl. Acad. Sci. USA 94, 7464–7468.

- Oakeshott, J.G., Claudianos, C., Russel, R.J., Robin, G.C., 1999. Carboxyl/cholinesterases: A case study of the evolution of a successful multigene family. Bioessays 21, 1031–1042.
- Okuda, T., Hodek, I., 1983. Response to constant photoperiods in *Coccinella septempunctata bruckii* population from central Japan (Coleoptera: Coccinellidae). Acta Ent. Bohemoslov. 80, 74–75.
- Okuda, T., Kadono-Okuda, K., 1995. *Perilitus coccinellae* teratocyte polypeptide: Evidence for production of a teratocyte-specific 540 kDa protein. J. Insect Physiol. 41, 819–825.
- Pennacchio, F., Vinson, S.B., Tremblay, E., 1992. Host regulation effects of *Heliothis virescens* (F) larvae induced by teratocytes of *Cardiochiles nigriceps Viereck* (Lepidoptera: Noctuida-Hymenoptera: Braconidae). Arch. Insect Biochem. Physiol. 19, 177–192.
- Quin, Q., Gong, H., Ding, T., 2000. Two collagenases are secreted by teratocytes from *Microplitis mediator* (Hymenoptera: Braconidae) cultured in vitro. J. Invert. Pathol. 76, 79–80.
- Rana, R.L., Dahlman, D.L., Webb, B.A., 2002. Expression and characterization of a novel teratocyte protein of the braconid, *Microplitis croceipes* (Cresson). Insect Biochem. Mol. Biol. 32, 1507–1516.
- Sambrook, J., Fritisch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual, second ed. Cold Spring Harbor Lab. Press, New York.
- Satoh, T., 1987. Reviews in Biochemical Toxicology. In: Hodgson, E., Bend, J.R., Philpot, R.M. (Eds.), Reviews in Biochemical Toxicology, Vol. 8. Elsevier, New York, pp. 155–181.
- Shapiro, J.P., Keim, P.S., Law, J.H., 1984. Structural studies on lipophorin, an insect lipophorin. J. Biol. Chem. 259, 3680–3685.
- Strand, M.R., Wong, E.A., 1991. The growth and role of *Microplitis demolitor* teratocytes in parasitism of *Pseudoplusia includens*. J. Insect Physiol. 37, 503–515.
- Takagi, Y., Morohashi, K., Kawabata, S., Go, M., Omura, T., 1988. Molecular cloning and nucleotide sequence of cDNA of microsomal carboxylesterase E1 of rat liver. J. Biochem. 104, 801–806.
- Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 24, 4876–4882.
- Tursch, B., Doloze, D., Dupont, M., Hootele, C., Kaisin, M., Pasteels, J.M., Zimmermann, D., 1971. Coccinellin, the defensive alkaloid of the beetle *Coccinella septempunctata*. Chimia 25, 307.
- Zhang, D., Dahlman, D.L., 1989. *Microplitis croceipes* teratocytes cause developmental arrest of *Heliothis virescens* larvae. Arch. Insect. Biochem. Physiol. 12, 51–61.