Thesis for the Degree of Master of Science

Molecular cloning and characterization of two Juvenile hormone esterase-like carboxylesterases cDNAs from the Morotoge shrimp, *Pandalopsis japonica*

by

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The Graduate School

Pukyong National University

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(물렁 가시 붉은 새우로부터 두개의 Juvenile hormone esterase-like carboxylesterases cDNAs 클로닝 및 특징 연구)

Advisor: Prof. Hyun-Woo Kim

by

Sun Ok Lee

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(Chairman) Dr. Chul-Woong Oh

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February 25, 2011

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Molecular cloning and characterization of two Juvenile hormone esterase-like carboxylesterases cDNAs from the Morotoge shrimp, *Pandalopsis japonica*

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Abstract

Methyl farnesoate (MF), a crustacean juvenile hormone (JH) analog, plays important roles in the regulation of a number of physiological processes such as molting, metamorphosis, reproductive maturation. Understanding its metabolic pathways is a key for various potential applications in crustacean aquaculture including artificial seed production and facilitation of growth and molt. Synthetic pathway of MF has been relatively well established but little has been known about its degradation and recycling pathways in crustacean. In insect juvenile hormone esterase (JHE) appeared to be responsible for JH metabolism. In order to expand our knowledge about MF metabolism, we isolated two cDNAs encoding JHE-like carboxylesterases (CXEs) from the hepatopancreas and ovary of *Pandalopsis japonica* by combination of the *in-silico* data mining from the expressed sequence tag (EST) database and traditional PCR-based cloning strategy. Full length of Pj-CXE1 (2084bp) and Pj-CXE2 (1985bp) cDNAs encoded a protein composed of 584 and 581 amino acids residues respectively. Structural analysis with other known carboxylesterases from various species revealed that the active sites and domain organizations of Pj-CXEs were well conserved suggesting that they may act on the similar substrate as shown in other carboxylesterases. Monophylic relationship among Pj-CXEs and other JHEs and acetylcholine esterases (AchEs) was not constructed by phylogenetic analysis suggesting that evolutional relationship may be more complicated than our thoughts. End-point RT-PCR results showed that Pj-CXE1 was predominantly expressed in the gonad whereas Pj-

CXE2 was highly expressed in both the hepatopancreas and hindgut. Quantitative PCR results found that expression of Pj-CXE1 was upregulated in the gonads from the 7 days post eyestalk ablation (ESA) group. In contrast, no significant expressional change of Pj-CXE2 has been identified in neither hepatopancreas nor gonad. Our study provided two novel decapods JHE-like CXE cDNAs and expression of Pj-CXE1 is related with factors from X-organ/sinus gland complex. Further study will be needed to expand our knowledge in MF metabolism and its role in crustaceans molting and reproduction.

Introduction

Unlike other fish species, a year-round seed production is one of the most challenging tasks in crustacean aquaculture because of the limited knowledge of crustacean reproductive endocrinology. Eyestalk ablation (ESA) is only the experimentally-proven method to induce gonad development and spawning (Emmerson 1980; Choy 1987; Que-Tae Jo 1999). Although this technique turned out to be effective in facilitating gonadal development among decapod crustaceans, it sometimes causes several accompanying problems including high death rate after surgical operation and lower healthy seed ratio (Emmerson 1980; Choy 1987; Que-Tae Jo 1999). Facilitation of gonad maturation by ESA is based on the knowledge that 20-hydroxy-ecdysone (20E) is one of the major steroid hormone for molting and reproduction in decapods crustacean (Subramoniam 2000). Hemolymph 20E level increases by eliminating X-organ/sinus gland complex. This neuroendocrine organ is major source for variety of neuropeptides, which have inhibitory effects on ecdysterogenesis in Y-organ. Among those peptide hormones, molt-inhibiting hormone (MIH), appear to be responsible for decreasing 20E secretion from Y-organ. In fact, MIH is member of CHH subfamily II (or the MIH/GIH subfamily) and its structural characteristics and functions in ecdysteroid production are not clearly discernable from other members. In addition to MIH, another neuropeptide from X-organ/sinus gland complex, Mandibular organ-inhibiting hormone (MOIH) exerts inhibitory effects on mandibular organ suppressing the production of MF, which is thought as another

gonad-stimulating factor in crustacean (Wainwright et al. 1996; Borst et al. 2002). Recent accumulating evidences suggest that MF is endogenous hormone in decapod crustaceans (Homola et al. 1997; Nagaraju 2007).

MF is sesquiterpenoids, which is believed to be unique to the arthropods including insects and crustaceans (Tobe et al. 1999). However, insect appears to posses additional forms of sesquiterpenoid hormones, Juvenile hormone (JHs). JHs are expoxidated forms of MF and play important roles in the regulation of a number of physiological processes such as molting, metamorphosis, reproductive maturation and pheromone biosynthesis (Tsubota et al. 2010a). Similar to JHs in insects, MF appears to be involved in several important biological roles in decapod reproduction and molting. The Major site for MF production appears to be the mandibular organ (MO) which is analogous to the insect corpora allata and is located in the mandibulo-maxillary region (Borst et al. 1987; Nagaraju et al. 2004; Nagaraju 2007). MF treatment stimulated ovarian maturation in most decapods crustacean species including infraorder Cardiea, *Macrobrachium rosenbergii* (Wider et al. 1995), infraorder brachyuran, *Libinia emarginata*, (Jo et al. 1999), and infraorder astacidea, *Procambarus clarkii* (Laufer et al. 1998). Those induced ovarian maturation is believed to be from direct binding of MF to its specific receptor. Recent study showed that daphnia retinoid X receptor homolog binds MF and several related compounds but no direct evidence for its activation generated from the interaction (Wang et al. 2009). Growing evidences showed that those stimulatory effects were not limited to female

reproduction and MF appears to be involved in testicular development (Brody et al. 1989; Reddy et al. 2004). In addition to reproduction, MF is also involved in molting cycle. Administration of MF accelerated molting in the two crayfish, *C. quandricarinatus* and *P. clarkia* and one crab *Oziotelphusa senex senex* supporting its role in inducing molting (Abdu et al. 2002; Nagaraju et al. 2004; Laufer et al. 2005).

Overall synthetic and metabolic pathway for sesquiterpenoid hormones such as juvenile hormone III (JHIII) and MF, appears to be conserved both in insects and crustaceans. One major difference is that crustaceans appear to lack epoxiase and S-adenosyl-methionine (SAM) -dependent methyltransferase (JHAMT), which convert farnesoic acid (FA) to JH III (Hui et al. 2010). Therefore, crustacean lacks JH III and MF is the end product in sesquiterpenoid hormone system. Biosynthesis of MF appears to be composed of two steps. First, farnesyl pyrophosphate (FPP) is generated via mevalonate pathway, which is universal in both vertebrates and invertebrates. In arthropod-specific second step, Farnesoic acid (FA) is activated by P450 monooxygenase and farnesoic acid O-methlytransferase (FAMeT) producing MF (Goldstein et al. 1990; Holford et al. 2004). Crustacean FAMeT catalyzes methylation of FA to MF and cDNAs for this protein have been isolated from various crustacean species including lobster, shrimp, and crab and its expression was induced by the eyestalk ablation (Gunawardene et al. 2002; Holford et al. 2004; Kuballa et al. 2007).

As shown in its biosynthetic pathway, degradation pathway of sesquiterpenoid hormones appears to be similar each other between insects and crustacean. In insect

species, JHs are degraded by two groups of enzymes: juvenile hormone esterase (JHE) and juvenile hormone epoxide hydrolase (JHEH). JHE, secreted enzyme, converts JH to JH acid reversibly whereas JHEH, nonsecreted enzyme, convert JH to JH diol as irreversible product (Hammock 1985; Gilbert et al. 2000). Although precise mechanism is not clearly established in crustaceans, degradation of MF appears to occur through ester hydrolysis by specific carboxylesterases like insect species. However, crustaceans lack JH-III and substrate for the hydrolysis reaction must be MF producing farnesoic acid (FA). In fact, it is known that the major product of FA is the hepatopancreas suggesting the major site for MF esterase is hepatopancreas. In fact, MF esterase activity has been identified both in the hepatopancreas and gonads of *L.emarginata, H. americanus*(Chang 1997) and *P. clarkia*. (Homola et al. 1997; Tamone et al. 1997). Despite the evidence of MF esterase, any nucleotide or protein information, which is responsible for the activity, has not been identified in crustacean. In the present study, we isolated full size of cDNAs encoding two carboxylesterases (Pj-CXE1 and 2), which exhibited the highest amino acid similarity to insect JHE from decapods crustaceans, *Pandalopsis japonica*. Tissue distribution analysis and their expressional effects by ESA were also carried out to estimate their role in MF metabolism.

Materials and Methods

Experimental animals

Live shrimps were purchased from a local seafood market, and held in a tank with 5 L of circulating aerated seawater for 3−5 days 4 ℃ for acclimatization before experiments. Right after dissection, tissues were frozen in liquid nitrogen and stored at -70 ℃ until used for total RNA extraction. The gonad-somatic index (GSI = gonad weight/body weight) was determined for each individual. Eyestalk ablation (ESA) was carried out .

After 3 days acclimatization in a tank with circulating aerated autoclaved seawater, ablation was done by searing the base of eyestalk with a hot iron after cutting the eyestalks. 3 days and 7 days after the ablation, the animals were sacrificed and each tissue was stored in the deep freezer(-70 ℃) until used for RNA extraction and dead or unhealthy ones were excluded from the analysis.

Cloning of full length of Pj-CXEs cDNAs

Full length of two Pj-CXEs cDNAs was determined by combination of bioinformatic analysis and traditional PCR-based cloning strategy. Total three partial cDNA sequences from the cDNA database constructed from the neuronal tissues of

Pandalopsis japonica exhibited high similarity to JHEs from insect species. Since none of the sequences contained full length of open reading frame (ORF), the rest cDNA sequence for each fragmental sequence was determined by traditional PCR-based traditional cloning strategy. Since there was no information about the tissue where each sequence was expressed, PCR was carried out using the sequence-specific primers designed for each partial cDNA sequence.

In order to determine full size of each fragmental sequence, traditional RT-PCR was carried out. Since all three partial sequences were expressed in the hepatopancreas and gonad, total RNA was purified from those tissues using Trizol reagent according to the manufacturer's instructions (Invitrogen, USA). Purified total RNAs were subjected to be qualified by formamide gel electrophoresis, quantified by absorbance at 260 nm (Nanodrop Technologies, Inc., USA) and stored at -70 ℃. cDNA was synthesized in a reaction containing reverse transcriptase and oligo-dT primer. A 12 μl mixture containing 3 μg total RNA, 1 μl 20 μM oligo dT primer and 4 μl dNTPs was heated to 70 ˚C for 5 min and chilled on ice for 2 min. First-strand buffer (5 X, 4 μl), 2 μl 0.1 M DTT and 1 μl RNaseout were added to the mixture, which was incubated at 37 ˚C for 2 min. Finally, MMLV reverse transcriptase (1 μl) was added and the mixture was incubated at 37 ˚C for 50 min. Synthesized cDNA was quantified, aliquoted and stored in -20 ℃.

Rapid amplification of cDNA ends (RACE) technique was used to obtain full-length cDNA. All the primers used for each PCR were shown in table 1. 5' upstream

region was determined by DNA Walking SpeedUp™ Kit (Seegene, Korea) according to the manufacturer's instructions. Briefly, first, 50 ng cDNA, 2 μl of 5 μM DW2-ACP (one of DW2-ACP from 1 to 12), 5 μM specific primer 1, 10 μl 2X SeeAmp™ ACP™ master Mix II and 6 μl distilled water were mixed. The mixture was placed in a preheated (94℃) thermal cycler and the PCR was carried out for one cycle (94℃ for 5 min, 42℃ for 1 min and 72℃ for 2 min), for 30 cycles (94℃ for 30 sec, 60℃ for 30 sec and 72℃ for 100 sec) and for one cycle (72℃ for 7 min). The PCR products were purified using a PCR purification Kit (Geneall Inc., Korea) to remove the DW-ACP and specific primer1 present in the first PCR reaction. Second, 20 μl of a mixture containing 3 μl purified first PCR products, 2 μl 5 μM DW2-ACPN, 1 μl 5 μM specific primer2, 10 μl 2X SeeAmp™ ACP™ master Mix II and 4 μl distilled water was preheated (94℃) thermal cycler. The second PCR was carried out for one cycle (94℃ for 3 min), for 35 cycles (94℃ for 30 sec, 60℃ for 30 sec and 72℃ for 100 sec) and for one cycle (72℃ for 7 min). Third, a mixture containing 2 μl second PCR products, 1 μl 5 μM UniP2, 1 μl 5 μM specific primer, 10 μl 2X SeeAmp™ ACP™ master Mix II and 6 μl distilled water was placed in a preheated (94℃) thermal cycler. The third PCR was carried out for one cycle (94℃ for 3 min), for 30 cycles (94℃ for 30 sec, 65℃ for 30 sec and 72℃ for 100 sec) and for one cycle (72℃ for 7 min). PCR products were purified, cloned, and sequenced as described above.

3' end of the full size of mRNA was identified by 3' RACE. cDNA was

synthesized using a reverse primer (Table 1). The reaction (12μl) containing 3 μg total RNA, 1 μl 20 μM 3' RACE primer and 4 μl dNTPs (2.5 mM) was heated to 70 ˚C for 5 min and chilled on ice for 2 min. First-strand buffer (5x, 4 μl), 2 μl 0.1 M DTT and 1 μl RNase out were added to the reaction mixture, which was incubated at 42 ˚C for 2 min. MMLV reverse transcriptase (1 μl) was added and then the mixture was incubated at 42 ˚C for 50 min. PCR was carried out with two sequence-specific forward primers and linker primers. Reactions (30 μl) contained cDNA (100 ng), primers (1 μl 100 μM), Takara Ex Taq polymerase (0.2 μl), each dNTP (2.5 mM), and 3 μl 10x buffer (Takara Bio Inc., Japan). PCR conditions were 1 min at 94 ˚C; 40 cycles at 94 ˚C for 1 min, 50 ˚C for 30 sec, and 72 ˚C for 30 sec; and post-extension at 72 ˚C for 5 min.

PCR products were separated with 1.5% agarose gel electrophoresis and stained with ethidium bromide. The PCR products with expected size were purified using Gel Extraction Kit (Geneall Inc., Korea), ligated into TA plasmid vector with the pGEM-T Easy Cloning Kit (Promega, USA), and transformed into One Shot Top 10 *E. coli* strain (Invitrogen, USA). Nucleotide sequences of the cloned cDNAs were determined using the automated DNA sequencer (ABI Biosystem, USA). The nucleotide sequence similarities were examined by BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/).

Obtained fragmental 5' and 3' sequences from RACEs were assembled together with partial cDNA sequence corresponding to each fragmental sequence. Computer-aided assembled sequences were reconfirmed by RT-PCR using two

sequence-specific primers, which aimed at each end of the full sequence. Open reading frame (ORF) was identified and deduced amino acid sequence was determined by ORF finder (http://www.ncbi.nlm.nih.gov/projects/gorf/).

All primers used this experiment were designed using IDTSciTools program (http://www.idtdna.com/SciTools/SciTools.aspx) and were synthesized by Bioneer Company, Korea (Table 1).

Expression analysis of Pj-CXEs

Qualitative and Quantitative Expression analysis of Pj-CXE was performed by PCR-based strategies. Tissue-specific expression profile was examined using endpoint RT-PCR. Total RNAs from gill, gonad, hepatopancreas, abdominal extensor and flexor muscle, heart, abdominal ganglia, and epidermis were isolated and quantified as described above. Genomic DNA was removed by treatment with DNase I (Promega, USA). cDNA was synthesized as described above, except that random hexamers were used as primers for the reverse transcription. Reactions (20 µl) contained 1 µl cDNA (100 ng), 2 µl 4 µM sequence-specific primers (Table 1), 0.2 µl Ex Taq polymerase (Takara Bio Inc., Japan), 4 µl dNTP (2.5 mM each), and 4 µl buffer (5X). PCR conditions were 1 min at 94 °C, followed by 25 cycles at 94 °C for 30 s, 63 °C for 30 s, and 72 °C for 30 s. The sequence-specific primers for each target gene were designed using the PrimerQuest tool (http://www.idtdna.com) and 18S rRNA primers were used as a positive control (Table 1).

In order to know expressional change of each carboxylesterases by ESA, quantitative PCR was carried out using the DNA Engine Chromo4 Real-Time Detector (Bio-Rad, USA) and SYBR Green premix Ex TaqTM (Takara Bio Inc., Japan). All the procedures for cDNA synthesis and PCR were same as used for endpoint RT-PCR except that 30 cycles, rather than 25 cycles. Standard curves were constructed to confirm the efficiency of primers and to quantify copy numbers as described previously (Kim et al. 2008). Pj-CXEs copy numbers were normalized to the 18S rRNA copy number according to the equation: (actual copy numbers of Pj-CXE1 or Pj-CXE2 / actual copy number of 18S rRNA) x 10,000.

Data analysis and statistics

Multiple amino acid sequence analysis was performed using ClustalW software (http://www.ebi.ac.uk/Tools/clustalw2/index.html) and represented by GeneDoc software (http://www.nrbsc.org/gfx/genedoc/index.html).

The signal peptide sequence was predicted by SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/de). Molecular mass and PI of Pj-CXEs were estimated by the Compute PI/Mw tool (http://www.expasy.ch/tools/pi_tool.html). A phylogenetic diagram of carboxylesterases from the shrimp was generated by minimum-evolution method and the bootstrapping test was performed with 1,000 replications using Molecular Evolutionary Genetics Analysis (MEGA4) (Tamura et al. 2007). The level of significance of Pj-CXEs in each tissue were evaluated statistically

by comparing the means, using Sigma Plot. The results were considered significant at $P < 0.05$.

Table 1. Primer list of Pj-CXE1 and Pj-CXE2

Name	Sequence (5′-3′)	Description
3RT	CTGTGAATGCTGCGACTACGATTTTTTTTTTTTTTTT	Primer for RT reaction for 3′ RACE
RACE R1	ACGCTGTGAATGCTGCGACTAC	First primer for RACE
RACE R2	GCTGTGAATGCTGCGACTACGA	Second primer for RACE
CXE1 F1	GTCAGCCACGGAGATGAGCTC	FWD first primer for 3' RACE
CXE1 F2	ACTACTTATTCCGTGGTGGTCCA	FWD second primer for 3' RACE
CXE1 R1	TGACCTTGTTGTATCGTCCATCT	1 RVS 5'walking primer of Pj-CXE1
CXE1 R2	TGCATGAGTCTGATTGGATGGTCA	2 RVS 5'walking primer of Pj-CXE1
CXE1 R3	CATCTTTACTGAGCAGGACATGGGGTAG	3 RVS 5'walking primer of Pj-CXE1
CXE1 R4	CCTCCATGGATAAAGACCATCACTGGG	4 RVS 5'walking primer of Pj-CXE1
CXE1 RT F1	CCTCCAATGGGTGCAAAGGAACAT	FWD primer for CXE1 expression
CXE1 RT R1	TGATTGGATGGTCAGGCAGGAAGT	RVS primer for CXE1 expression
CXE2 F1	GTTCCTTACGGGGCAGCTGTTATG	FWD first primer for 3' RACE
CXE2 F2	CCGCCAAAGAATTACCAGGAAAAGAAG	FWD second primer for 3' RACE
CXE2 RT F1	CCAGTTGATTGTTTGTGCGGGTGA	FWD primer for CXE2 expression
CXE2 RT R1	ACCATCTCTCACACCCTTCCACTT	RVS primer for CXE2 expression
18SrRNA-F	ATGAGAGTGCTCAAAGCAGGCTACTC	FWD primer for 18SrDNA expression
18SrRNA-R	GGCGAATCGCTAGTCAGCATCGTT	RVS primer for 18SrDNA expression
M13F(-40)	CAGGAAACAGCTATGAC	Vector FWD primer for DNA sequencing
M13R(-20)	GTAAAACGACGGCCAG	Vector RVS primer for DNA sequencing

Results and discussion

Cloning of full size of two Pj-CXE cDNA

From the obtained partial sequences, 5' and 3' RACEs were carried out using sequence-specific forward and reverse primers. First, 2084bp of a full-size of carboxylesterases cDNA (Pj-CXE1, GenBank Accession number: HQ406776) was identified after two consecutive 5' and single 3' RACEs (Fig. 1). The other full length of Pj-CXE2 (GenBank Accession number: HQ406777) cDNA sequence (1985bp) was also identified by single 3' RACE (Fig. 2). Full length of each Pj-CXE was reconfirmed by RT-PCR using gene-specific primers, which was aimed for amplifying full ORF sequence. ORF analysis showed that cDNA of Pj-CXE1 encoded a protein, which is consisted of 584 amino acids and its estimated molecular weight was 64.28KDa. Full length of Pj-CXE2 cDNA encoded a protein of 581 amino acid residues and estimated molecular weight was 64.06 KDa, which was similar to Pj-CXE1. Polyadenylation sequence (AATAA) was found only in Pj-CXE1. PI analysis results revealed that both Pj-CXE1 and 2 were acidic (PI = 5.02 and 5.79 respectively), which is similar to those of JHs in insect species (Keith D. Wing 1984; Yehia A.I. Abdel-Aal 1988). Signal peptide prediction program identified a putative signal peptide region, which would be cleaved between 19 and 20 residues with more than 99% probability in both Pj-CXEs (Fig.3). This result suggests Pj-CXE1 and 2 are

secreting protein as other carboxylesterases in insect species. However, it is interesting to know that MF esterase activity is conspicuously absent from the hemolymph of the species studied to date, which is different from insect JHEs (L. E. King 1995; Chang 1997). Possible explanation is either inactivation of MF esterase activity during circulation or tissue-specific regional expression after secretion.

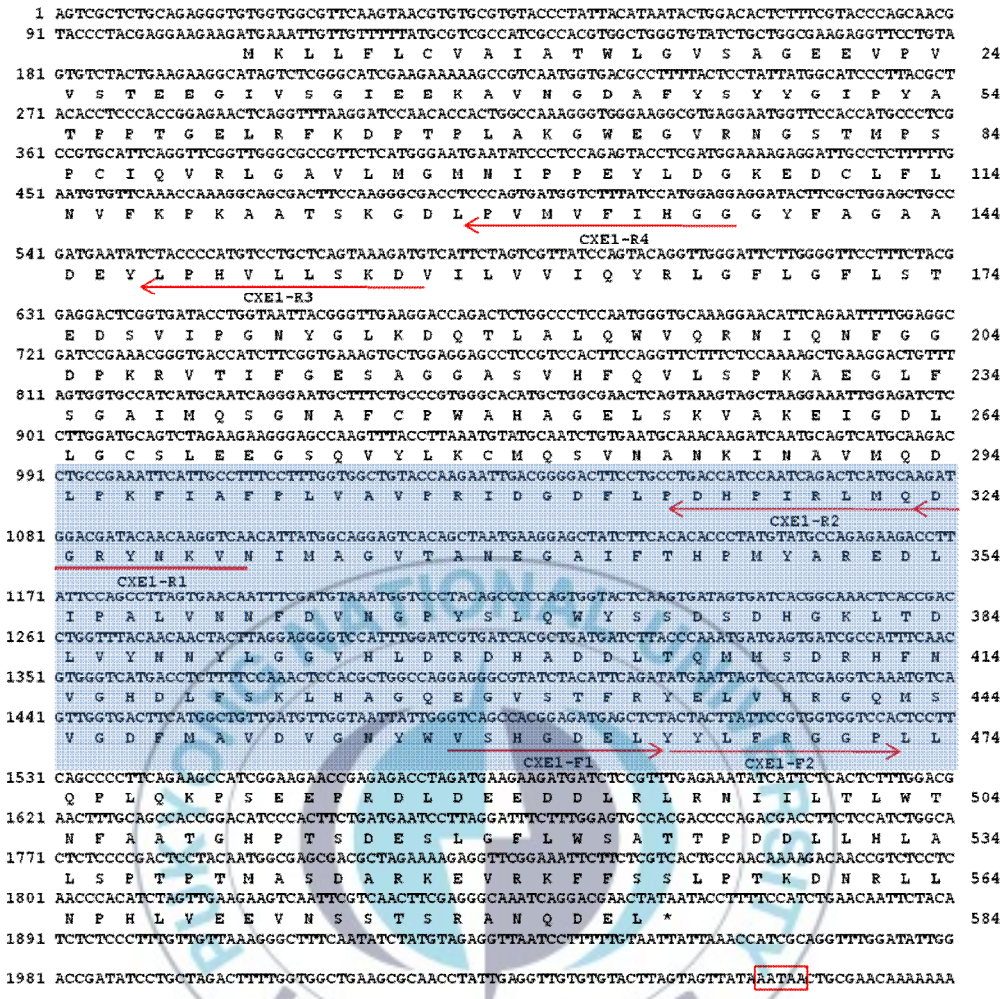

Fig. 1. Full-sized cDNA sequence and deduced amino acid sequence of Pj-CXE1 from *Pandalopsis japonica*. Nucleotide numbers and animo acid residues were shown left and right respectively. Arrows indicate sequence specific primers. Poly A (AATAA) signal was boxed.

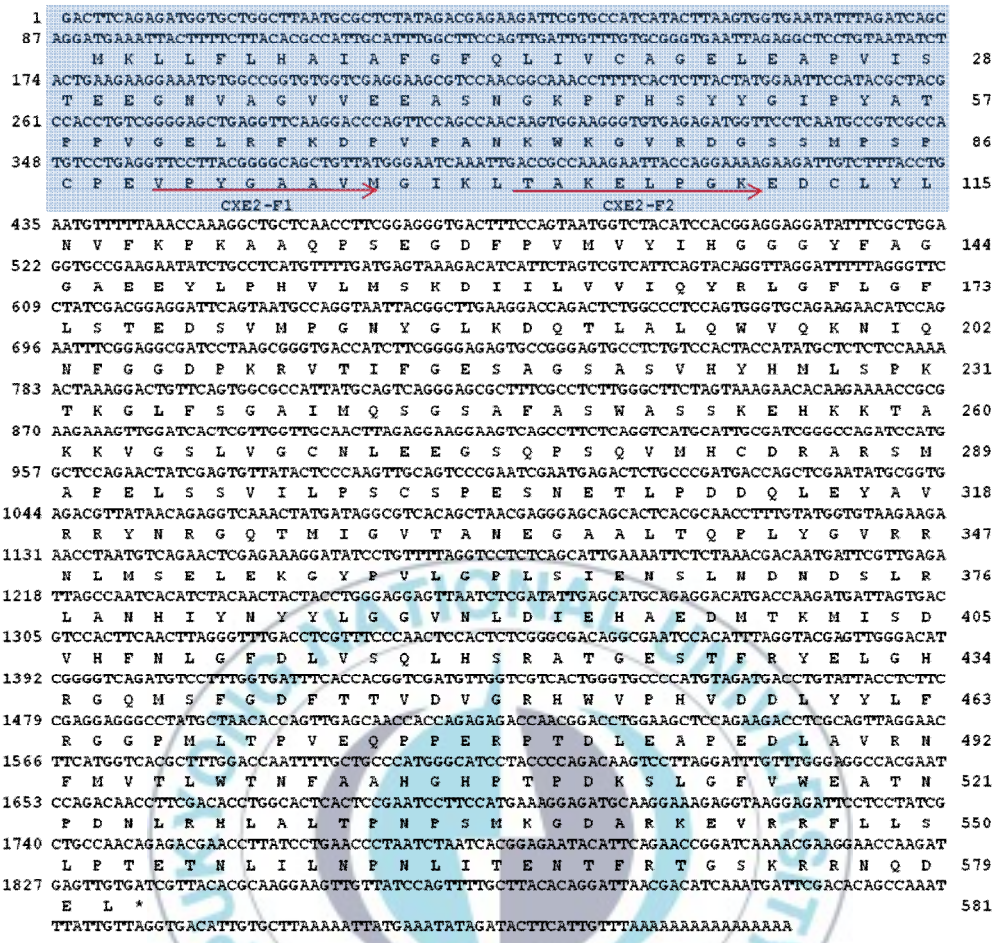

Fig. 2. Full-sized cDNA sequence and deduced amino acid sequence of Pj-CXE2 from *Pandalopsis japonica*. Nucleotide numbers and animo acid residues were shown left and right respectively. Arrows indicate sequence specific primers.

Structural analysis of Pj-CXEs

Amino acid sequence similarity analysis of two Pj-CXEs to database in NCBI showed that isolated Pj-CXEs exhibited the highest amino acid similarity with JHEs from various insect species including *Acyrthosiphon pisum* (GenBank accession number: XP_001950765), *Athalia rosae* (GenBank accession number: BAD91554), and *Apis mellifera* (GenBank accession number: BAC54130). No crustaceans homolog corresponding to any Pj-CXE was identified from the database suggesting both Pj-CXE1 and Pj-CXE 2 were firstly reported JHE-like carboxylesterases from crustacean species. Both Pj-CXEs exhibited similar amino acid similarity to JHEs from insect species (50%). It is interesting to know that *lepidopteran* JHE, *Manduca sexta*-JHE, exhibited only 30% identity to the JHEs of *T. molitor* (Hinton et al. 2003) and *D.melanogaste*r (Campbell et al. 2001). Two Pj-CXEs showed higher similarity to some insect JHEs than other JHEs from several insect species. Relationship between amino acid similarity and substrate specificity (especially, MF or JHE III) is not clear, however, and further study is needed. Amino acid identity between Pj-CXE1 and 2 was 60% and similarity was 75% each other.

In order to estimate their function, the deduced amino acid sequences of two Pj-CXEs were aligned with JHEs from several insect species including fruit fly, mosquito, and honey bee (Fig. 3). Multiple alignment of Pj-CXEs with insect JHEs revealed that two Pj-CXEs exhibit typical domain organization of carboxylesterases, catalytic N-terminal region and variable C-terminal region. In addition to the domain organization,

conserved characteristics or carboxylesterases were also well identified. First, three amino acid resides consisting the catalytic triad, Ser-Glu-His, were well conserved in both Pj-CXEs (Thomas et al. 1999). In addition, carboxylesterases-specific GxSxG motif was also conserved where Ser residue, one of catalytic triad, is included. However, typical GQSAG motif of insect JHEs was replaced by GESAG in both Pj-CXE1 and 2. It has been known that GQSAG motif is characteristic sequence of insect JHE. However, a few exceptional sequences within the motif have been reported. For instance, GQSAG motif was not found in any isolated carboxylesterases from *A. melifera* (Claudianos et al. 2006) . More recently, another JHE-like gene was identified, which has GLSAG rather than GQSAG motif and its suppression by gene-silencing technique resulted in increase of JH titer suggesting GQSAG may not be obligatory motif for its activity (Mackert et al. 2008). In addition, GQSAG motif turned out not to be a necessary and sufficient condition for JHE activity. In *A.aegypti*, only one of three putative JHE genes, which contain GQSAG motif, showed functional activity (Bai et al. 2007). In one of moth species, *B. mori*, two genes exhibited ability to degrade JHE out of five GQSAG-containing carboxylesterases (Tsubota et al. 2010c). Since there was no JH III in decapod crustacean species and given similar structure of MF and JHIII, functional study is needed to know if one of isolated crustaceans CXE exhibit esterase activity for MF. In addition to GQSAG motif, additional typical motifs of JHE were compared with Pj-CXEs (Fig. 3). Two motifs (RF, DQ, and E) were well conserved except for GxxHxxD/E motif, in which Gly was substituted by Trp in both Pj-CXEs.

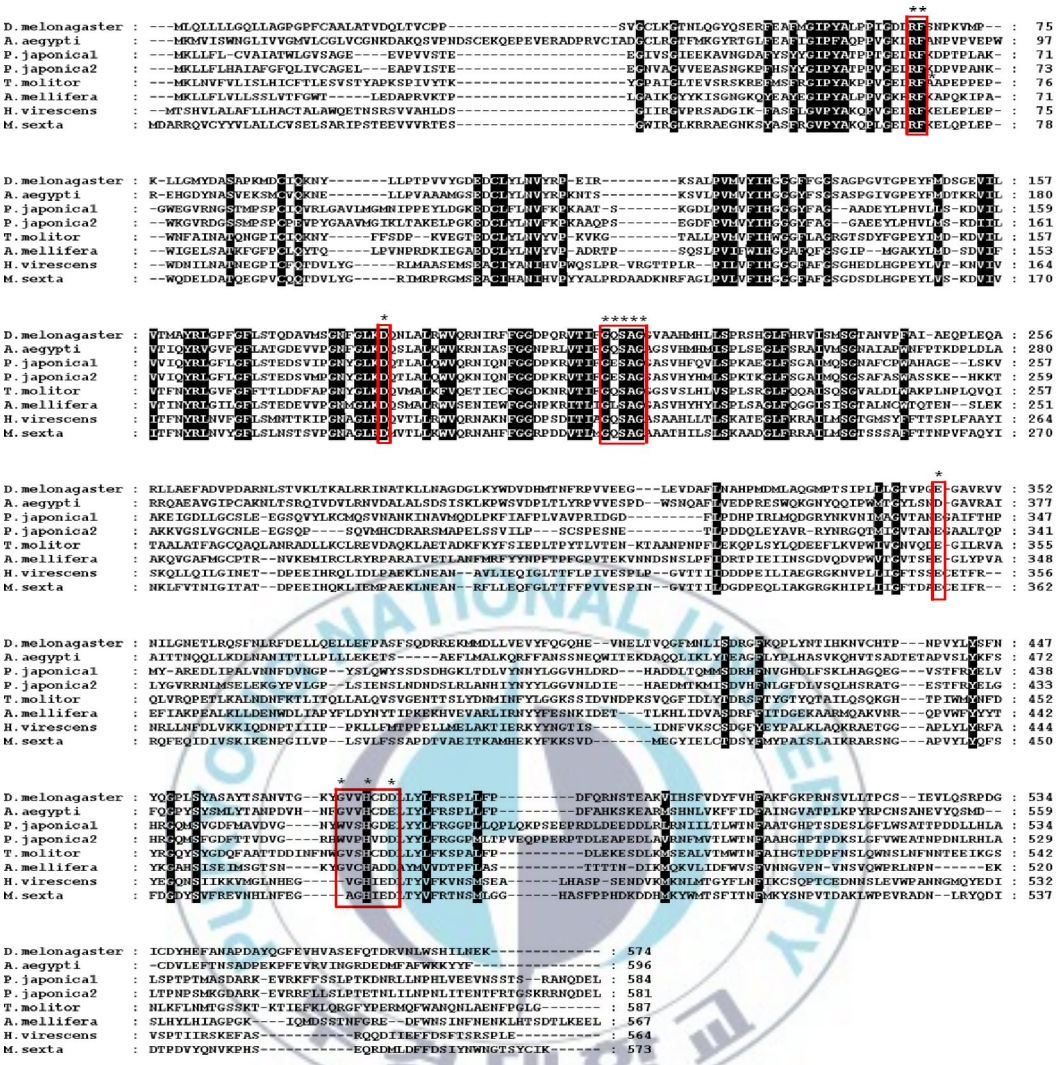

Fig. 3. Multiple alignment of amino acid sequence of Pj-CXE1 and Pj-CXE2 with JHEs from insect species. GenBank Accession numbers are following: *Drosophila melonagaster* (AAK07833.1) *Aedes aegypti* (EAT43357.1) *Heliothis virescens* (P12992.2) *Tenebrio molitor* (AAL41023.1) *Manduca sexta* (AAG42021.2) *Apis mellifera* (NP_001011563.1) *Pandalopsis japonica* 1,2 (HQ406776, HQ406777)

Phylogenetic analysis was also carried out to understand relationship of Pj-CXEs with other carboxylesterases from various insect species. Until now, JHE and AChE were only two carboxylesterases that their endogenous substrates have been clearly demonstrated and other carboxylesterases were named from the species and numbers. Since C-terminal region was variable in length among different carboxylesterases, conserved catalytic N-terminal region was used for the analysis.

Phylogenetic analysis result revealed that Both Pj-CXEs were clustered with other non-lepidopteran JHEs. Interestingly, Pj-CXEs were more closely related to JHEs from the non-lepidopteran species than those from the lepidopteran insects. Evolutional distance of JHEs between the non-lepidopteran and the lepidopteran insects agree with the previous study (Tsubota et al. 2010c). In contrast JHEs, AChEs from both non-lepidopteran and lepidopteran were clustered together. Pj-AChE, putative acetylcholine esterase from *P. japonica*, was also clustered together with other insect AChEs, which indicates that crustacean AChE is the most closely related with AChEs from insect species. Although Phylogenetic analysis result suggests that Pj-CXE1 and 2 are more closely related to JHE and AChEs than other carboxylesterases, its substrate specificity may not be as simple as those result. Recent X-ray crystal structure of JHE from *Manduca sexta* (MsJHE) revealed substrate-binding pocket (Wogulis et al. 2006; Kamita et al. 2010). In their finding, JHE has an unusual narrow deep binding pocket and surface solvent is not accessible. In contrast, AChE from the Pacific electric ray and rabbit liver carboxylesterase show substrate binding pockets that are accessible to

surface solvent, in spite of their similarity to JHE in primary structure and other charicteristics in active sites. From those reasons, it appears to be impossible to estimate substrate specificity just by sequence similarity and other Phylogenetic analysis and eventual functional study should be made, which would be third specific endogenous substrate for various carboxylesterases. At least, MF is structurally very identical to JH but the epoxide group, we can expect that substrate-binding pocket would be as deep as JHE for docking the hydrophobic substrate, MF.

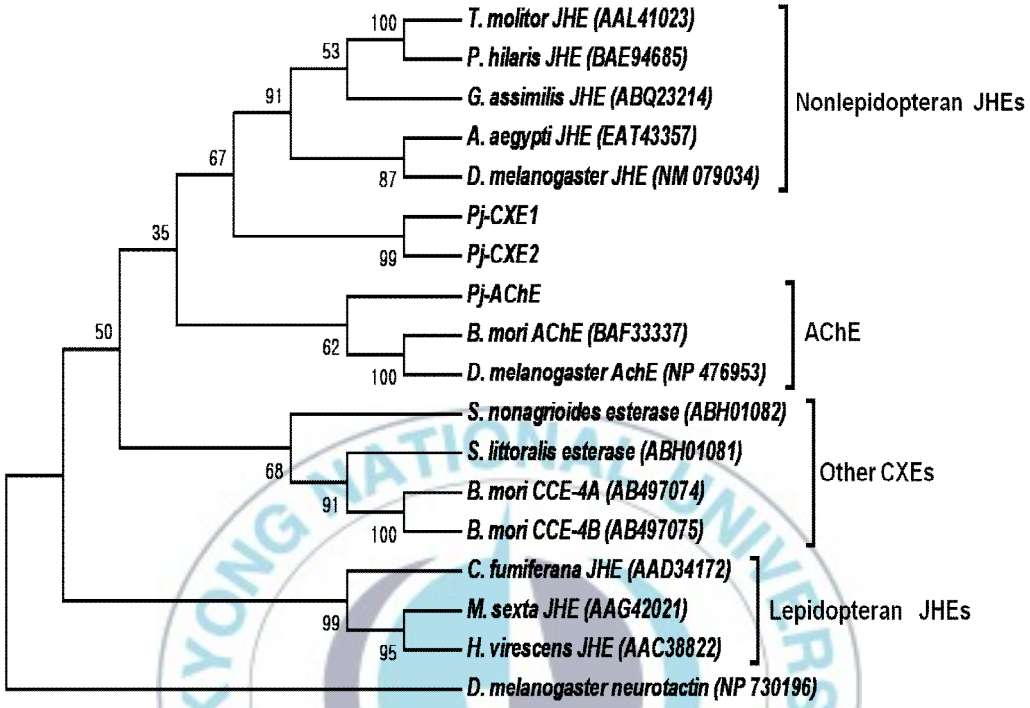

Fig. 4. Phylogenetic analysis of various carboxylesterases from arthropod species. Diagram was generated by the neighbor joining method with MEGA 4.0 program. Bootstrap replications were 1000. GenBank Accession Number was shown for each protein

Expression analysis of Pj-CXEs

End-point RT-PCRs were carried out to know tissue distribution of two Pj-CXEs (Fig. 5). 30 rounds of PCR reaction result produced a strong 18S rRNA amplicon in all examined tissues suggesting synthesized cDNAs were good. Pj-CXE1 was expressed predominantly in gonad and moderately in hepatopancreas and hindgut, whereas Pj-CXE2 was strongly expressed in hepatopancreas and hindgut and moderate expression was also detected in gonad and abdominal ganglia. No detectable expression was identified in muscular tissues including deep abdominal and heart. This result is corresponding to the Previous results that major MF esterase activity has been identified both in the hepatopancreas and gonads of *L.emarginata, H. americanus* (Chang 1997) and *P. clarkia*. (Homola et al. 1997; Tamone et al. 1997). However, difference in major production site of each Pj-CXE brings the possibility that MF esterase activity in hepatopancreas and ovary may come from different gene products. Although not all species are accepted, major production site for insect JHE transcript appear to be fatbody (Liu et al. 2008). Unlike JHE, in which its activity has been largely found in various tissues including hemolymph, no MF esterase activity found in hemolymph (L. E. King 1995; Chang 1997). From that reason, it may be possible that each gene product in hepatopancreas and gonad may be responsible for the activity respectively in crustacean and further study is needed.

In order to understand transcriptional effects by factors from sinus X-

organ/gland complex, quantitative RT-PCR was carried out after ESA (Fig. 6). Copy number of Pj-CXE1 in the gonad was about 14 folds higher than in the hepatopancreas of the control group, whereas copy number of Pj-CXE2 in the hepatopancreas was 4.7 folds higher than in the ovary. 7 days after ESA, Pj-CXE1 transcript numbers increased up to 3.4 folds than control group whereas there was no significant difference in its transcript 3 days after ESA. This result suggested that expression of Pj-CXE1 is transcriptionally under control of components from X-organ/sinus gland complex. On the contrary to the Pj-CXE1, similar Pj-CXE2 mRNA transcript numbers were found both in the hepatopancreas and gonad. In addition, ESA did not affect on Pj-CXE2 transcription until 7^{th} days.

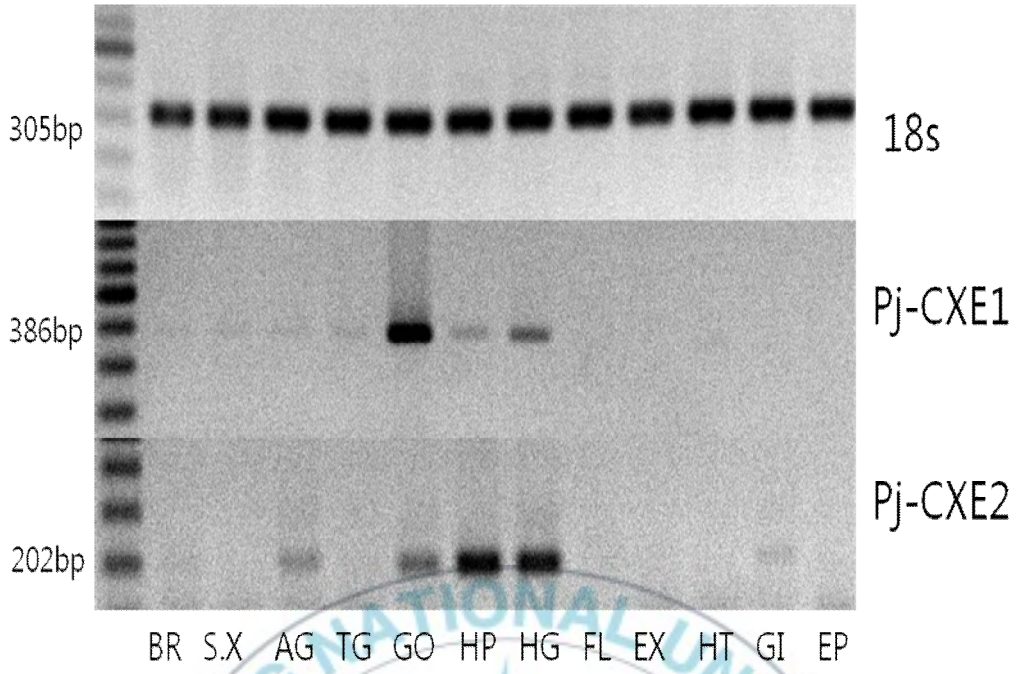

Fig. 5. Expression of Pj-CXE1 and Pj-CXE2 in various tissues. End-point RT-PCR was carried out for 30 cycles and products were separated on 1.5% agarose gel. The 18S rRNA was used as a positive control.

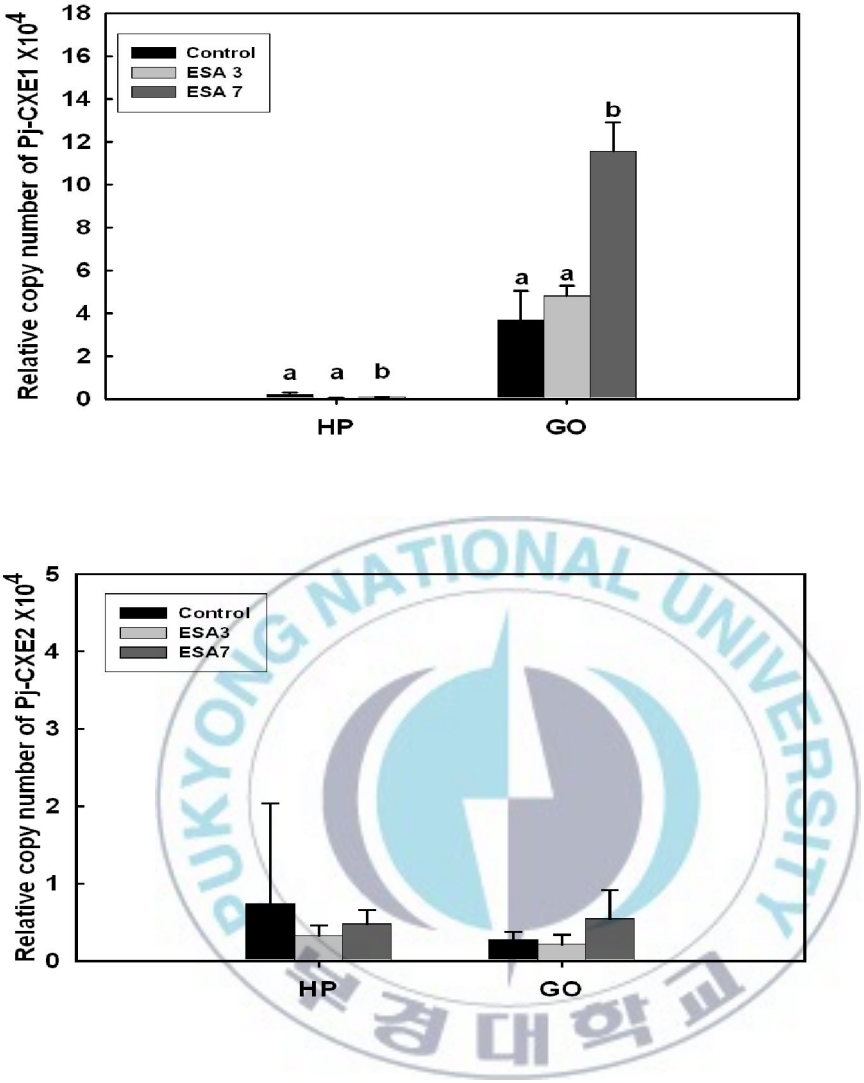

Fig. 6. Relative copy numbers of Pj-CXE1 and Pj-CXE2 between hepatopancreas and gonad from *Pandalopsis japonica.* Copy numbers were normalized by the number of 18S rRNA. Statistical significance was accepted only when P <0.05. a and b indicate the statistical significance of each other.

MF esterase in crustacean species

In present study, we isolated two JHE-like carboxylesterases cDNAs (Pj-CXE1 and 2) from the decapods crustaceans for the first time. In addition, we still do not exclude the existence of the additional JHE-like or MF-specific carboxylesterases genes. If we know how many carboxylesterases genes exist in each insect species, it would not be surprised to have additional carboxylesterases in crustacean. Several insect genome or EST projects have churned out numerous carboxylesterases nucleotide sequence information. Total twenty one carboxylesterases genes have been identified from the honey bee, *A. mellifera* (Mackert et al. 2008) and ten different carboxylesterases genes were also isolated from moss, *B. mori* (Tsubota et al. 2010b). Sixteen different carboxylesterases genes were identified from *D.melanogaster* (Erica J. Crone 2007). Interestingly, only one gene out of those numerous genes has exhibited JHE activity. As shown in insect JHEs and other carboxylesterases, no structural characteristics, which is JHE-specific or different from other carboxylesterases, has been identified. Our similarity and phylogenetic analysis failed to provide any structural or evolutional characteristics of two Pj-CXEs for suggesting potential MF activity. Until now, only functional-expression assay would be the answer for screen MF esterase gene given our nucleotide sequence information.

Tissue distribution profiles revealed that two Pj-CXEs exhibited different expression pattern, in which Pj-CXE1 is gonad-specific gene and Pj-CXE2 was hepatopancreatic gene. Our results suggested that MF esterase activities from the

hepatopancreas and from the gonad may have come from the different gene products but further study is needed. Present study also showed that only gonadal expression of Pj-CXE1 was induced by ESA and no other significant expressional change has been identified in neither hepatopancreatic nor in gonadal Pj-CXE2 transcripts. In insect species, expression profiles of JHE and JH-like carboxylesterases vary and change according to their developmental and molting stages (Durand et al. 2010; Tsubota et al. 2010c). However, MF esterase from decapods crustaceans appears to be different from insect JHEs. Previous experiment using juvenile *H. americanus*, showed that MF esterase activity in the hepatopancreas is constitutive and the specific activity did not significantly changed with respect to molt stage, eyestalk ablation and exogenous MF (Chang 1997). However, other crustaceans such as *L. emarginata* showed seasonal differences in MF esterase activity in both the hepatopancreas and gonads and ESA appeared to induce MF esterase activity (Takac et al. 1995). At least our finding is that one JHE-like carboxylesterases gene is transcriptionally regulated by factor(s) from X-organ/sinus gland complex and may be responsible for MF activity in gonad. Further isolation of JHE-like carboxylesterases genes and functional expression study would give us better knowledge about MF esterase and its role in molting and reproduction.

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