

# cDNAs Encoding Chitin Synthase from Shrimp (*Pandalopsis Japonica*): Molecular Characterization and Expression Analysis

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## Abstract

Crustacean growth occurs via molting, the periodic shedding of the exoskeleton. Understanding the genes involved in chitin metabolism associated with the periodic molt cycle is important for various applications to decapod crustacean aquaculture. Chitin synthase is an important enzyme in the chitin biosynthetic pathway that plays a major role in synthesis of new cuticle after molting. In this study, we isolated a full-length cDNA encoding chitin synthase (PajCHS) from *Pandalopsis japonica* through a combination of PCR (Polymerase chain reaction)-based cloning and bioinformatics analysis. The identified PajCHS encodes a transmembrane protein with 1525 amino acid residues (175 kDa). Comparison with other CHSs from insects revealed that PajCHS contains three domains: N-terminal domain A, catalytic domain B, and C-terminal domain C. Three conserved motifs (EDR, QRRRW, and SWGTR) were also well conserved within and near the catalytic domain B, suggesting that Paj-CHS is functionally active. Variation in the transmembrane helix within the N-terminal and C-terminal domains suggested that the orientation of each CHS may be different. Phylogenetic analysis suggested that PajCHS is an ortholog of CHS1 group members from insect species. However, tissue expression profiles indicated that epidermis, hepatopancreas, intestine, and gill were the major production sites for PajCHS transcript, which is considerably different from insect CHS1. qPCR results showed that eye stalk ablation and 20 hydroxyecdysone (20E) injection increased the expression level of PajCHS mRNA, suggesting that the expression of PajCHS1 may be controlled by endogenous 20E.

**Keywords:** Decapod; *Pandalopsis japonica*; Chitin; Molting; Epidermis

## Introduction

Chitin, a linear homopolymer of  $\beta$ -(1,4)-N-acetyl-D-glucosamine (GlcNAc), is a major component of the exoskeleton and peritrophic membrane (PM) in arthropods [1,2]. For decapods, the exoskeletons of which are mainly composed of chitin, its metabolism is one of the most crucial processes for growth and development during which the periodic shedding of the old exoskeleton and replacement with a new one is required [3]. Understanding chitin metabolism has various industrial applications, including growth enhancement and the production of soft-shell products.

Chitin formation is catalyzed by the membrane protein chitin synthase (CHS, UDP-N-acetyl-D-glucosamine: chitin 4- $\beta$ -N-acetylglucosaminyltransferase; EC 2.4.1.16), which is a glycosyltransferase family 2 protein [4,5]. Since the first cDNA encoding an insect CHS was identified in the sheep blowfly, *Lucia cuprina* [6], several CHS genes have been reported by various genome projects on insects, including *Anopheles gambiae*, *Aedes aegypti* [7], *Drosophila melanogaster* [8], *Manduca sexta* [9,10], and *Tribolium castaneum* [11]. Insect genomes possess two copies of CHS genes (CHS1 and CHS2) as a result of gene duplication from the ancestral CHS gene [12].

The expression and functions of CHS1 and CHS2 appear to be distinct from one another. CHS1 genes are exclusively expressed in the epidermis underlying the cuticular exoskeleton and are related to ectodermal cells, such as tracheal cells, whereas CHS2 genes are expressed during the synthesis of chitin in the peritrophic membrane (PM) of the gut [6,9,11,13,14]. CHS1 genes also contain exons that lead to the production of two alternatively spliced variants, CHS1A and CHS1B [9,11,14]. The distribution of these in tissue, and their expression, differ during development, suggesting that they may have different biological functions [9,11,14].

It is essential to understand the enzymes involved in chitin metabolism in crustaceans. At least four groups of chitinase genes have been identified and characterized in decapods [15], and the expression of some of these is influenced by the molting hormone ecdysone. However, there is little information on CHS genes and their functions in decapod crustaceans. In the present study, we identified and characterized the full-length cDNA (PajCHS) encoding a CHS from the decapod crustacean *Pandalopsis japonica*, which is an important fishery resource in East Asian countries including Korea and Japan and is a good model system in which to investigate the physiology of crustacean molting [16,17]. To determine the transcriptional effects of CHS gene expression during the molting cycle, both quantitative and qualitative expressional analyses were performed after eyestalk ablation (ESA) and 20-hydroxy ecdysone (20E) injection.

## Materials and Methods

### Experimental animals

Adult *P. japonica* were purchased from a local seafood market and acclimatized to culture tanks (50 L each). The physicochemical characteristics of the water, including salinity (33PPT), dissolved O<sub>2</sub> (~6 ppm), and water temperature (6°C), were maintained as described

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**Received** November 06, 2014; **Accepted** November 21, 2014; **Published** January 24, 2015

**Citation:** Uddowla H, Kim AR, Park WG, Kim HW (2015) cDNAs Encoding Chitin Synthase from Shrimp (*Pandalopsis Japonica*): Molecular Characterization and Expression Analysis. J Aquac Res Development 6: 298. doi:10.4172/2155-9546.1000298

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previously (NFRDI, 2009). Fifteen shrimp were kept in each tank and dead shrimp and molted exoskeletons were removed as quickly as possible to avoid water pollution. As a food source, peeled frozen shrimp (*L. vannamei*) and scallops were supplied once a day at a rate of 5% body weight. Every 2 days, waste and uneaten feed were removed by siphoning. Half of the water was exchanged by adding fresh seawater every 5 days (it was properly aerated and a similar temperature was maintained). Molting stages were identified based on setal development in the uropod [18] and intermolt shrimp were used for the experiments.

### ESA and 20E injection

The animals were divided into three groups: control, ESA group, and 20E-injected group. ESA was performed as described previously [15]. Untreated animals were used as controls. Tissues obtained from individuals in each group were frozen directly in liquid nitrogen and stored at  $-70^{\circ}\text{C}$  until used for RNA extraction. To determine the levels of CHS mRNA expression in different tissues, premolt [18]. *P. japonica* were injected with 20E (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Premolt animals were divided into two groups: one was administered 20E, and the other (control) received the same volume of PBS. A single injection of 20E at a concentration of 10 ng/g body weight was administered (hemolymph volume was assumed to be 30% of the wet body weight and ecdysteroid level was 30 pg/ $\mu\text{L}$  during the intermolt stage [19,20]. Injections (20 ng/ $\mu\text{L}$  stock solution of PBS containing 10% ethanol) were made into the sinus at the base of the fifth walking leg. After injection, the shrimp were kept in aerated plastic tanks and sacrificed at different time intervals: 12 h, 36 h, 72 h, day 5, and day 7. Tissues were collected and stored at  $-70^{\circ}\text{C}$  until further use.

### Cloning of the full-length PajCHS cDNA

A BLAST search of a shrimp EST database [21] identified a partial sequence (2237 base pairs [bp]) as being homologous to insect CHS. To

obtain the full-length cDNA, a conventional cloning strategy and rapid amplification of cDNA ends (RACE) were applied. All primers used in the experiment were designed using the IDT SciTools program (<http://www.idtdna.com/SciTools/SciTools.aspx>) and were synthesized by Bioneer Corp. (Daejeon, Korea) (Table 1).

Total RNA was purified from different tissues using Trizol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA), quantified by spectrophotometry (Nanodrop Technologies, Wilmington, DE), and stored at  $-70^{\circ}\text{C}$ . cDNA was synthesized in a reaction (10  $\mu\text{L}$ ) containing 2  $\mu\text{g}$  total RNA, 0.5  $\mu\text{L}$  DNaseI, and 1  $\mu\text{L}$  10 $\times$ DNase buffer. The total reaction was kept at  $37^{\circ}\text{C}$  for 30 min followed by  $70^{\circ}\text{C}$  for 10 min. After adding 1  $\mu\text{L}$  3Race dT (TTTTTTTTTTTTTTTT) (20  $\mu\text{M}$ ) and 4  $\mu\text{L}$  dNTPs (2.5 mM each), the reaction was terminated by heating at  $70^{\circ}\text{C}$  for 5 min and chilling on ice for 2 min. Then, 4  $\mu\text{L}$  first-strand buffer (5 $\times$ ), 2  $\mu\text{L}$  DTT (0.1 M), and 1  $\mu\text{L}$  RNaseOUT (Invitrogen) were added, and the reaction mixture was incubated at  $37^{\circ}\text{C}$  for 2 min. Finally, 1  $\mu\text{L}$  MMLV reverse transcriptase (Invitrogen) was added and the reaction mixture, which was incubated at  $37^{\circ}\text{C}$  for 50 min. The enzyme was inactivated at  $70^{\circ}\text{C}$  for 15 min. cDNA was quantified and stored at  $-20^{\circ}\text{C}$  until use.

5' RACE was performed to obtain the 5' upstream region using a DNA Walking SpeedUp™ kit (Seegene, Seoul, Korea) until the full ORF was identified. PCR conditions were as described previously [16]. To obtain the 3' UTR sequence including the polyadenylation signals, 3' RACE was performed according to a previously described procedure [15]. Amplified PCR products were separated by 1.5% agarose gel electrophoresis and stained with ethidium bromide. Amplicons of the expected size were cut out from the gel with a razor blade and purified using a gel extraction kit (GeneAll Biotechnology, Seoul, Korea), ligated into the vector using a pGEM-T Easy Cloning Kit (Promega, Madison, WI), and transformed into One Shot Top 10 *E. coli* (Invitrogen). Fragmented sequences were joined together using the Align Sequences

Primer name	Sequences (5'-3')	Description
PajCHS PCF1	AACAATCTTGAGCTGCTTGTGGAC	Forward primer for EST sequence confirmation
PajCHS PCF2	GGACTTATGGTATGGTATCAGATG	Forward primer for EST sequence confirmation
PajCHS PCR1	CCTAGACTGACGCTTTCTTGGAC	Reverse primer for EST sequence confirmation
PajCHS PCR2	CGTCTTCTTGGACCACAGTCTCG	Reverse primer for EST sequence confirmation
PajCHS5R R1	TGAACAGTCTCTGTAGTCTTGC	Specific reverse primers for 5' region
PajCHS5R R2	GTACATAATGACGAGCTTGGCTA	Specific reverse primers for 5' region
PajCHS5R R3	TAAGGAGTTGTACATTGGAGAGACAAA	Specific reverse primers for 5' region
PajCHS5R R4	TGCGATCCATGTTTGGGAAAGTAACCAAAA	Specific reverse primers for 5' region
PajCHS DEGF1	GARACNAARGGNTGG	Degenerate forward primers for 5' region
PajCHS DEGF1-1	GGNTGGGAYGTNTTY	Degenerate forward primers for 5' region
PajCHS DEGF2	TTYWSNTAYGCNTTYCCN	Degenerate forward primers for 5' region
PajCHS DEGF3	CARGGNTTYWSNTAYGCN	Degenerate forward primers for 5' region
PajCHS3R F2	CATTTATCCAGAGGAATGCTGTTG	Specific reverse primers for 3RACE
PajCHS3R F3	CAAGCAATCGGAACGTTGGACGTGGC	Specific reverse primers for 3RACE
PajCHS DW5R R5	CCCGAACTAGCACAAACCCGAAGGT	5'RACE reverse specific primer
PajCHS DW5R R6	CATCGAGGAACTTTTGGTTGCTCTC	5'RACE reverse specific primer
PajCHS DW5R R7	GTTGTCTCCGAGAGAGAACCCTGGAC	5'RACE reverse specific primer
PajCHS RTFD	TGTACTGTTGCTTCAGCGAGGT	PajCHS Forward primer for qPCR
PajCHS RTRD	AGCTACAAAGGCACCCACCAACAT	PajCHS Forward primer for qPCR
PajCHS full con F1	GAAGTTACTGAGGAATCTTAAAGGATC	Forward primer for ORF confirmation
PajCHS full con F2	AGGATCATTGTGCTCGGATGC	Forward primer for ORF confirmation
PajCHS full con R1	ATTGTACATATAAATAATGAAAAGCCTG	Reverse primer for ORF confirmation
PajCHS full con R2	ATAATGAAAAGCCTGTCTAGGAGAG	Reverse primer for ORF confirmation
M13F (-40)	CAGGAAACAGCTATGAC	Vector FWD primer for DNA sequencing
M13R (-20)	GTAAAACGACGGCCAG	Vector RVS primer for DNA sequencing

Table 1: List of primers used for the cloning of PajCHS and qPCR.

Nucleotide BLAST program (www.ncbi.nlm.nih.gov). A single PajCHS transcript was reconfirmed by RT-PCR using the primers for the 3' and 5' ends (Table 1), and nucleotide sequences were determined with an automated DNA sequencer (Applied Biosystems, Foster City, CA).

### Bioinformatics analysis of PajCHS

The theoretical isoelectric point (pI) and molecular weight of PajCHS were calculated using the web-based program Compute PI/Mw (http://au.expasy.org/tools/pi\_tool.html). Signal peptide and putative cleavage sites were predicted by SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/). Multiple amino acid sequence alignment was performed using ClustalW, which is offered by the European Bioinformatics Institute (http://www.ebi.ac.uk/clustalw/), and the results were presented using the GeneDoc program (http://www.nrbsc.org/gfx/genedoc/index.html). Phylogenetic tree was constructed using the minimum-evolution method of Molecular Evolutionary Genetics Analysis (MEGA4) with 1000 bootstrapping replicates [22]. Coiled-coil domains were identified using the program COILS (http://www.ch.embnet.org/software/COILS\_form.html). Protein sequences were analyzed for transmembrane helices (TMH) using the TMHMM v.2.0 software (available at http://www.cbs.dtu.dk/services/TMHMM/). NetNGlyc 1.0 Server was used to identify glycosylation sites of this enzyme (http://www.cbs.dtu.dk/services/NetNGlyc/).

### Expression analysis of PajCHS

Expression analysis of PajCHS was performed by qualitative and quantitative PCR strategies. The tissue distribution of PajCHS transcripts was analyzed by end-point RT-PCR. Total RNA was isolated from gill, epidermis, gonad, hepatopancreas, deep abdominal flexor and extensor muscles, heart, thoracic and abdominal ganglia, brain, X-organ/sinus gland complex (XO/SG), and intestine. cDNA was synthesized under conditions similar to those used for cloning PajCHS except with random hexamers instead of the oligo-dT primer. Synthesized cDNA was treated with DNaseI (Promega), quantified, and aliquoted until it was used in reactions. PCR mixtures (20 µL) contained 1 µL cDNA (100 ng), 1 µL 4 µM sequence-specific primers (Table 1), 0.2 µL Ex Taq polymerase (Takara Bio Inc., Kyoto, Japan), 2 µL dNTPs (2.5 mM each), and 2 µL buffer (10×). PCR conditions were 3 min at 94°C, followed by 30 cycles of 94°C for 30s, 60°C for 30s, and 72°C for 30s. 18S rRNA primers were used as a positive control.

Quantitative PCR (qPCR) was carried out using the DNA Engine Chromo 4 Real-Time Detector (Bio-Rad, Hercules, CA) to compare PajCHS transcript levels between control and ESA groups. Six individual samples were analyzed in each group. SYBR Green premix Ex Taq™ (Takara Bio Inc.) was used with 50 ng cDNA as a template. qPCR was carried out under the same conditions used for end-point RT-PCR as described above, except 40 cycles were performed. Standard curves

were constructed to quantify copy numbers as described previously [23] and to confirm the efficiency of each primer set. Calculated copy number was normalized relative to the copy number of 18S rRNA. The relative copy number was calculated as follows: actual copy number of PajCHS/actual copy number of 18S rRNA. Statistical analysis of PajCHS was performed by a Student's t test using SigmaPlot (Systat Software, Inc., Richmond, CA). The effects of 20E on PajCHS gene expression in different tissues, such as the epidermis and gill, were quantified by qPCR as described above.

## Results

### Cloning and characterization of PajCHS

Based on the results of bioinformatics analysis and the traditional cloning strategy, the full-length chitin synthase cDNA was identified (PajCHS). The obtained PajCHS cDNA (4578 bp) encoded a protein of 1525 residues and had 81 bp and 402 bp untranslated regions (UTRs) at the 5' and 3' ends, respectively. PajCHS exhibited the highest sequence similarity (76%) to *T. castaneum* (TcCHS, NM\_001039402) followed by 75% sequence similarity to *A. gambiae* (AgCHS, XP\_321336) and *L. migratoria* (LmCHS, ACY\_38589). BLAST analysis of PajCHS also showed 76% similarity to and 62% identity with an unannotated sequence from the cladoceran *Daphnia pulex* (EFX76951). To determine the evolutionary relationship between PajCHS and cladoceran CHS, we considered *Daphnia* CHS in our study.

PajCHS is a large protein with an estimated molecular mass of approximately 175 kDa and a slightly acidic pI of 6.12, similar to insect CHS (Table 2). To compare the structural characteristics between PajCHS and other known CHSs from insect species, multiple alignments were performed (Figure 1). Similar to other CHSs, PajCHS exhibited the conserved primary structure of chitin synthase composed of three domains, A, B, and C [6]. In domain A, a signal-anchoring sequence was identified between Gly14 and Ser15 residues, indicating that PajCHS is a membrane-bound protein. Insect signal-anchoring sequences range in length from 15 to 22 residues (data not shown). Secondary structure analysis predicted that PajCHS may contain 16 TMHs, each of which is composed of 19–23 amino acid residues. The number of TMHs in insect species varies from 14 and 17 (Table 2). Nine TMHs were predicted in domain A of PajCHS, whereas 7–9 TMHs were identified in the CHS from insect species (Figure 1). The B domain of PajCHS is similar in size to insect CHSs (~400 residues) and contains the catalytic center of the protein (Figure 1). Two unique conserved motifs (EDR and QRRRW) within the catalytic domain are present in all CHS and are therefore considered signature sequences. The catalytic activity of *D. melanogaster* CHS (DmCHS2) was confirmed by its expression in Schneider 2 cells with radiolabeled N-acetyl-D-glucosamine from uridine diphospho-N-acetyl-D-glucosamine. Two

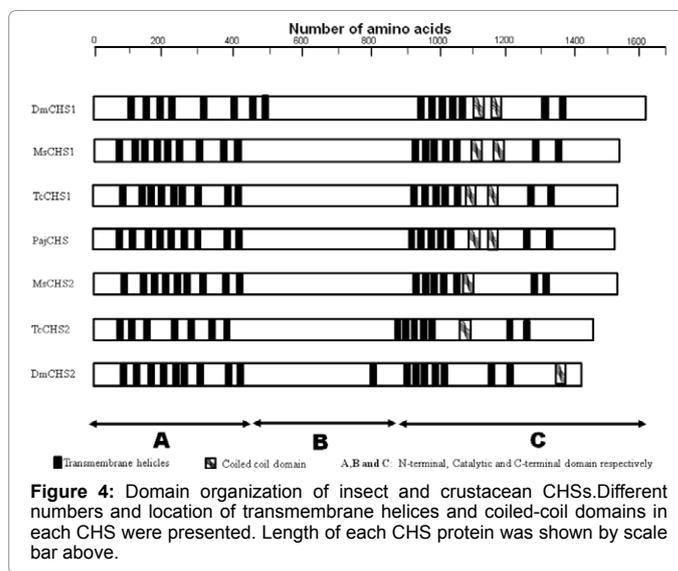
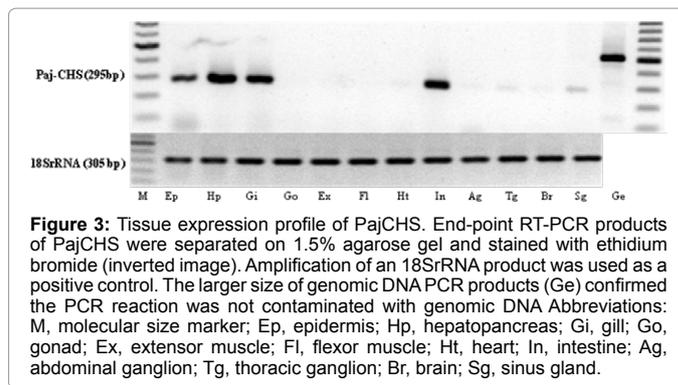
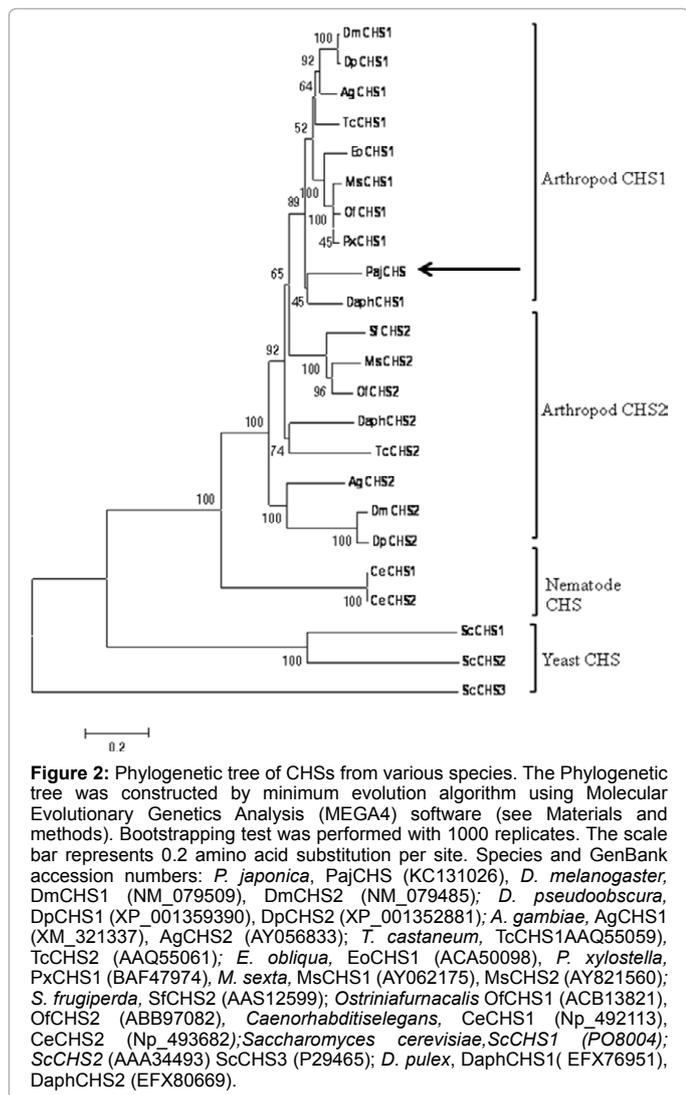
	PajCHS	TcCHS1	TcCHS2	MsCHS1	MsCHS2	DmCHS1	DmCHS2	Daph CHS1	Daph CHS2
<b>Organisms</b>	<i>P. japonica</i>	<i>T. castaneum</i>	<i>T. castaneum</i>	<i>M. sexta</i>	<i>M. sexta</i>	<i>D. melanogaster</i>	<i>D. melanogaster</i>	<i>D. pulex</i>	<i>D. pulex</i>
<b>GenBank Accession number</b>	KC131026	AAQ55059	AAQ55061	AY062175	AY821560	NM_079509	NM_079485	EFX76951	EFX80669
<b>Class</b>	1	1	2	1	2	1	2	1	2
<b>Molecular Mass (kDa)</b>	175	177.76	167.67	178.56	174.42	182.8	161.39	182.46	
<b>PI</b>	6.12	6.66	5.93	6.56	6.02	6.37	6.66	6.27	6.19
<b>TMH</b>	16	16	14	16	16	15	17	16	16
<b>Position of Coiled-coils</b>	1080-1116 1151-1181	1061-1106 1133-1164	1098-1133	1063-1106 1134-1164	1058-1092	1079-1122 1153-1181	1347-1375	1069-1112 1138-1174	-

**Table 2:** Characteristics of some properties of insects and crustacean CHS. PI, isoelectric point, TMH, Transmembrane helices; (see, materials and methods, Bioinformatics analysis).



motifs in the catalytic domain, EDR and QRRRW, were predicted to be oriented to the cytoplasmic side, and a third motif in the C-terminal domain, SWGTR, was predicted to face the extracellular side of the membrane (Figure 1).

Five TMHs were predicted to be located just after the central catalytic domain (Figure 1). This is similar to that of cellular synthase, where it constitutes a pore in the membrane through which newly synthesized carbohydrate polymers may be extruded [24]. Seven potential N-glycosylation sites were identified throughout the sequence (data not shown) using PROSCAN [25], suggesting that this protein can be glycosylated. In addition, two coiled-coil sites were predicted from amino acid positions 1080–1116 and 1151–1181 (Figure 1), which are potential sites for protein–protein interactions and/or signals for vesicular trafficking [26]. TMHs in the N-terminus showed different patterns among the insect species, while the C-terminal TMHs were remarkably conserved both with respect to their locations and spacing between adjacent TMHs (Figure 1). Among the seven C-terminal TMHs, five were found in clusters immediately following the catalytic domain and two more sequences were located closer to the C-terminus (Figure 1). A phylogenetic tree was generated to determine the evolutionary relationships among PajCHSs (Figure 2). Three yeast

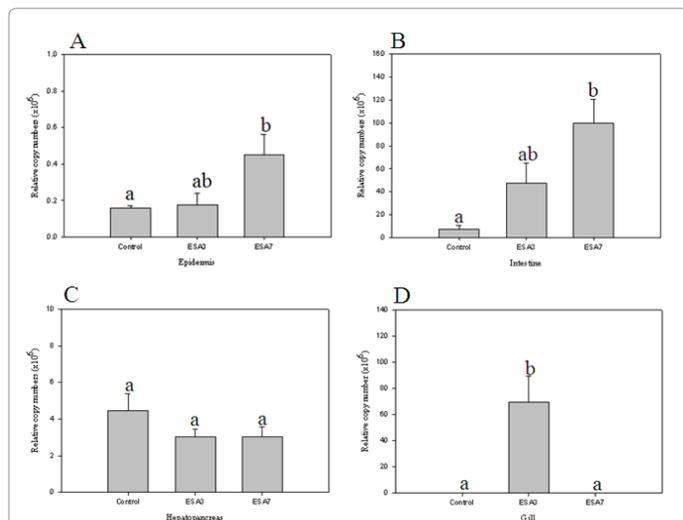


CHS clustered as outgroup members and nematode CHSs were also grouped together. PajCHS was clustered together with the insect CHS1 group, suggesting that an arthropod CHS gene duplication event may have occurred before the divergence of insects and crustaceans. Insect CHSs were further subdivided into CHS1A and CHS1B.

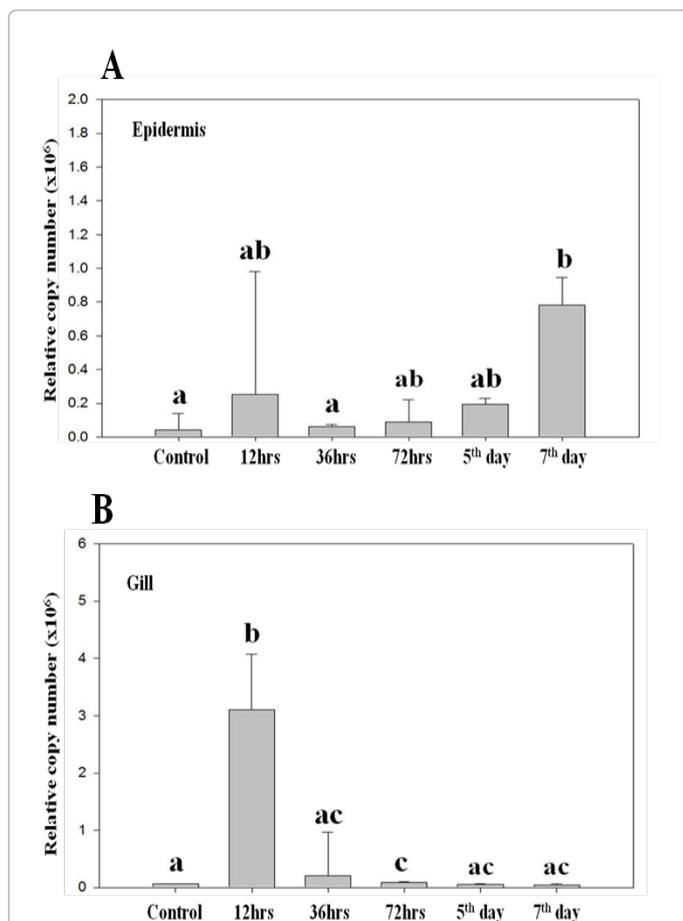
### Transcriptional analysis of PajCHS

The distribution of the PajCHS transcript in tissue was determined by end-point RT-PCR and subsequent agarose gel electrophoresis of PCR products (Figure 3). Homogeneous 18S rRNA PCR products indicated that cDNA synthesis was successful, and the synthesized cDNA was free from genomic contamination. The transcript was mainly detected in epidermis, hepatopancreas, intestine, and gill (Figure 4). PCR product was also identified in the sinus gland/X-organ complex (SG); however, PajCHS transcript was not detected in the gonad, heart, brain, thoracic and abdominal ganglia, or deep abdominal flexor and extensor muscles.

qPCR was used to determine the effects of molt induction by ESA on the expression of PajCHS (Figure 5). The levels of PajCHS mRNA expression were significantly increased on day 7 after ESA in the epidermis and intestine compared to the control group. In the epidermis, mRNA expression level increased up to 2.8-fold on day 7 but reached 13.7-fold in the intestine compared to the control group. In the gill, PajCHS transcript level increased significantly on day 3 (1047-fold) and decreased to the basal level on day 7. However, no significant



**Figure 5:** Effect of eyestalk ablation (ESA) on expression of mRNA levels of PajCHS. mRNA level was measured by qPCR from various tissues including (A) epidermis (B) intestine (C) hepatopancreas and (D) gill after ESA and data were normalized by the copy number of 18S rRNA. The data represents mean values  $\pm$  SE (n=6). Means that share the same letter are not significantly different (P>0.05)



**Figure 6:** Effects of 20E on the PajCHS gene expression in (A) epidermis and (B) Gill. mRNA levels of PajCHS were quantified in epidermis and gill by qPCR and data were normalized by the copy number of 18S rRNA. Means that share the same letter are not significantly different (P>0.05).

effects of ESA were observed in the hepatopancreas. Similarly, PajCHS expression was also increased in the epidermis and gill after injection with 20E (Figure 6). In the epidermis, the expression was increased by 8.7-fold compared to the control group on day 7 after 20E injection and that in the gill was significantly increased to 53-fold at 12 h (Figure 5).

## Discussion

Chitin degradation and synthesis are common phenomena in arthropods and are directly related to proper development and growth. Understanding genes involved in chitin metabolism is the first step for its applications in decapod aquaculture. Although CHS is a pivotal enzyme in the chitin biosynthesis pathway, no full-length CHS gene in a decapod crustacean has previously been reported, mainly due to their large size (~5 kb). To the best of our knowledge, PajCHS is the first full-length cDNA encoding CHS reported from a decapod crustacean. Although two partial sequences have been reported from *L. vannamei* [27] and *H. americanus* (NCBI database, GQ169704), they are too small to compare to PajCHS.

The primary structure and overall domain organization of PajCHS are similar to those from insect species (Figure 1). In addition, signature motifs (EDR, QRRRW, and SWGTR) are also well conserved. Site-directed mutagenesis of EDR and QRRRW motifs in fungal CHS results in loss of activity, suggesting that these two motifs are essential for CHS activity [28]. The (S/T)WGTKG motifs face the extracellular environment and play an important role in chitin translocation rather than synthesis [29]. These results indicate that PajCHS encodes the catalytically active chitin synthase. Despite the overall structural similarity, the number of TMHs varies among CHSs from different species (Figure 1); the N-terminal end contains 9–10 TMHs, whereas the C-terminal end contains 7–8 TMHs (Figure 1). Different numbers of TMHs have been proposed in some CHSs from insect species, including *D. melanogaster* and *T. castaneum*. Domains A and C of PajCHS were predicted to have 9 and 7 TMHs, respectively. The number of TMHs affects the orientation of CHSs, and further studies are required to determine whether differences in the number of TMHs may be responsible for the differences in protein orientation.

Due to the large sizes of CHSs (~160–180 kDa) and their membrane-bound functional forms, it has been difficult to study their structure and activity in both insect and crustacean species. Recent knockdown experiments using RNA-silencing techniques have revealed that each CHS gene plays an important role in growth and developmental stages [30,31]. In a previous study, injection of dsRNA corresponding to MsCHS1 cDNA into fifth-instar larvae (*M. sexta*) resulted in severe head deformities and death of pupae, whereas controls showed normal development [12]. In another study, the injection of dsRNA into *Locusta migratoria* corresponding to LmCHS1 or either of its two variants, LmCHS1A/LmCHS1B, showed a nymph mortality rate ranging from 50% to 95%; however, phenotypic deformities were similar to those observed when LmCHS1 or LmCHS1A were used, whereas the use of LmCHS1B led to a crippled-cuticle phenotype [31]. These results indicate that both of the variants perform essential functions for insect growth and development. Moreover, in vivo treatment of fourth-instar larvae of *Spodoptera exigua* with siRNA-silencing CHS1 causes abnormalities and disordered cuticle formation [30]. This indicates that CHS plays an important role in the proper growth and development of arthropods, and RNA-silencing techniques should be applied to estimate their functions in crustaceans.

Multiple sequence alignment (Figure 1), domain organization (Figure 4), and phylogenetic analyses (Figure 2) suggested that PajCHS

is a class 1 CHS. PajCHS was more closely related to insect CHS1 than to CHS2. Both CHS1 and CHS2 exist in insect genomes as a result of gene duplication from the ancestral CHS gene [12]. These two copies are also found in the cladoceran *D. pulex*, suggesting that the presence of two copies of these genes may be common in arthropods [8,11,13]. A homologous sequence of PajCHS from *Litopenaeus vannamei* (LvCHS ~300 bp, FJ229468) reported in a previous study [27] was too small to compare to our results. However, PajCHS showed a substantially different tissue expression profile than those of CHS1s in insect species. In insects, CHS1s are expressed in epidermis-related ectodermal tissues, whereas CHS2 is expressed predominantly in the gut PM [6,11,14,32]. In case of *M. sexta* CHS genes, MsCHS1 and MsCHS2 show differential expression patterns in tissues and among developmental stages [14]. One of the major differences compared to insect CHS1 is the hepatopancreatic expression of PajCHS (Figure 3). Although the insect fat body is thought to correspond to the crustacean hepatopancreas, CHS transcripts have not been detected in the fat body in insects [9]. This suggests that CHSs in decapod crustaceans may have additional functions compared to those in insect species. In addition, expression of PajCHS in the intestine suggests that a single CHS transcript may perform the functions of both CHS1 and CHS2 in insects. A partial LvCHS sequence from *L. vannamei* showed a similar expression pattern to PajCHS, but it was too small (~300 bp) to allow a comparison, and it is still unclear whether LvCHS is an ortholog of PajCHS. There is no evidence to support the presence of two copies of CHS genes in decapod crustaceans.

The PajCHS transcript was upregulated by both ESA and 20E injection. ESA is one of the best established ways to induce the production of 20E in hemolymph to accelerate ecdysis in crustaceans [33]. In insect species, ecdysteroids (molting hormones) play pivotal roles in the expression of CHS genes [34-36]. However, CHS1 and CHS2 respond differently to increased ecdysteroid levels. The expression of MsCHS1 in tracheal cells is highest at the beginning of the molt when the ecdysteroid level remains high. In contrast, the MsCHS2 gene in columnar cells is down regulated despite high ecdysteroid levels [34-36]. We found that PajCHS mRNA transcription levels were significantly upregulated in the epidermis and intestine after ESA, indicating a relationship between increased ecdysteroid level and CHS expression. Upregulation of the transcripts suggested that ecdysone activates CHS genes by activating nuclear receptor heterodimer consisting of the EcR and the *Drosophila* retinoid X receptor homolog of ultra spiracle protein (USP) [37]. Temporal differences in PajCHS transcript levels associated with ESA and direct 20E injection may be caused by the slow increase in ecdysteroids by ESA rather than 20E injection (Figure 6). As a consequence, PajCHS appears to be an ecdysteroid-response gene.

In conclusion, we isolated the full-length cDNA encoding PajCHS in a decapod crustacean, and described its molecular characteristics. Its tissue distribution and transcriptional response to 20E induction suggest that PajCHS is an ecdysteroid-response gene and that its manipulation may be useful in the decapod aquaculture industry.

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