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Cuticular chitin synthase and chitinase mRNA of whiteleg shrimp *Litopenaeus vannamei* during the molting cycle

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ABSTRACT

Chitin metabolism is of high relevance for shrimp growth because it has to be synthesized and cleaved in each molt. We studied chitin synthase and chitinase mRNAs from whiteleg shrimp *Litopenaeus vannamei*. For this, cDNA coding for cuticular chitin synthase (*LvChS*) and chitinase isoenzymes (*LvChi1*, *LvChi2* and *LvChi3*) was amplified, sequenced and identified. In a qualitative analysis, *LvChi1* and *LvChi3* were detected only in the hepatopancreas and are probably involved in digestion of food chitin. *LvChi2* transcript was found in pleopods, uropods, gills, eyestalk, and digestive tube; *LvChi2* is likely to be involved in the hydrolysis of chitin from the exoskeleton and peritrophic membrane, but not in food chitin digestion. *LvChS* was found widely distributed in the organism, including the hepatopancreas. Thus, it seems to be involved in synthesis of chitin to build the exoskeleton and also the peritrophic membrane. Relative expression of *LvChS* and *LvChi2* genes was evaluated by quantitative RT-PCR in the integument. These transcripts had a varying pattern of abundance during the molt cycle, based on the need of shrimp to synthesize or hydrolyze chitin from exoskeleton.

1. Introduction

Chitin is a linear homopolymer of β-linked N-acetylglucosamine (NAcGlc) that occurs widely in nature. World annual bioproduction of chitin was estimated at 100×10^9 t, being an important source of carbon for heterotrophic bacteria (Cauchie, 2002; Tharanathan and Kittur, 2003). Chitinous structures are found in several taxa as well as the cell wall of fungi (Roncero, 2002), egg shells of nematodes (Brydon et al., 1987) and exoskeleton and peritrophic matrix of insects (Merzendorfer and Zimoch, 2003) and crustaceans (Roer and Dillaman, 1984). Thus, the study of chitin metabolism is of biotechnological, ecological, and physiological relevance.

The exoskeleton of crustaceans is a four-layered matrix that confers support, rigidity, and impermeability to the body. Chitin is present in the inner three layers, forming a sclerotized complex with structural proteins and calcium salts (O'Brien et al., 1991). For crustaceans to grow, the rigid exoskeleton has to be discarded by hydrolysis of cuticular components and a new exoskeleton has to be synthesized to match the new body size (Promwikorn et al., 2004). The process of synthesizing a new exoskeleton and discarding the old one (ecdysis or molting) is frequently repeated during the life cycle of shrimp. The molt cycle comprises the time between one molt and another, including five stages: post-molt which includes stages A (subdivided in A_1 and A_2) and B, intermolt stage C, pre-molt stage D (which is subdivided in D_0 through D_4), and molting or ecdysis (stage E), as described by Smith and Dall (1985). These stages are accompanied by a number of processes induced by hormonal signals, including gene expression, enzyme activity, and physiological and behavioral changes (Mikami, 2005). Chitin is also present in the peritrophic matrix that is secreted along the length of the digestive tract and serves as an impermeable coat for the food bolus, thereby protecting the lumen from digestive enzymes and attack by pathogens (Ceccaldi, 1989). The study of synthesis and hydrolysis of chitin during the molting cycle will contribute to understanding its physiology.

Chitin synthase (ChS; E.C. 2.4.1.16, UDP-N-acetyl-D-glucosamine: chitin 4-β-N-acetylglucosaminyl transferase) is the enzyme that assembles monomers of NAcGlc into chitin polymers. ChS is a cell membrane-bound enzyme composed of two identical subunits and has been found in the membrane or in cytoplasmic microsomes in insects (Merzendorfer, 2006). After ChS polymerizes chitin, the growing polymer is deposited in the extracellular space (Cohen, 1987). Several sequences of insect ChS cDNA have been reported, including ChS from the sheep fly *Lucilia cuprina* (Tellam et al., 2000), tobacco hornworm *Manduca sexta* (Zimoch et al., 2005), mosquito *Aedes aegypti* (Ibrahim et al., 2000), fruit fly *Drosophila melanogaster* (Gagou et al., 2002), and red flour beetle *Tribolium castaneum* (Arakane et al., 2004). Compared to abundant studies of insect ChS, there are no studies about activity or immuno-localization of crustacean ChS, and only one cDNA sequence of

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lobster ChS (*Humarus americanus*) is available in the GenBank (Accession GQ169704.2).

Chitinases (Chi; E.C. 3.2.1.14, poly [1,4-(N-acetyl-β-D-glucosamine)] glycanohydrolase) hydrolyze chitin at intermediate regions to produce oligomers of NAcGlc. Chitinases have been studied in several crustaceans; chitinase activity is induced prior to molting in the integument of Antarctic krill *Euphasia superba* (Buchholz, 1989) and the common prawn *Palaemon serratus* (Spindler-Barth et al., 1990). Three cDNA sequences encoding chitinase isoenzymes, known as Chi1, Chi2, and Chi3, were first described in the Kuruma prawn *Marsupenaeus japonicus*. Chi1 and Chi3 are found in the hepatopancreas and are involved in food chitin digestion (Watanabe et al., 1996; 1998) and Chi2 is expressed in the integumental tissue and has a putative role in molting (Watanabe and Kono, 1997). Isoenzymes of chitinase are described in other shrimp, such as *Fenneropenaeus chinensis*, *Penaeus monodon*, and *Litopenaeus vannamei* (Boone, 1931; synonymous *Penaeus vannamei*). Hepatopancreatic Chi1 from the giant tiger prawn *P. monodon* (*PmChi1*) is regulated during the molting cycle (Tan et al., 2000). A prior study revealed at least 6 chitinase isoenzyme mRNAs in the whiteleg shrimp and their sequences are available in the GenBank (Huang et al., 2010). However, despite the economic importance of this species in Latin America and Asia, the role of chitinase isoforms in the molting cycle has not been studied in detail; also, very few studies exist concerning chitin synthesis in cuticular tissues of this shrimp. The opposite function of chitin synthases and chitinases suggests that their activities should be highly coordinated in the integument during the molting cycle.

In this study, we performed a qualitative analysis of transcripts of a novel chitin synthase (*LvChS*), and the chitinases *LvChi1*, *LvChi2* and *LvChi3* (Accession EU883591, FJ888479.1 and AF315689, respectively) in different organs of whiteleg shrimp. Also, we quantified the mRNAs for cuticular *ChS* and *Chi2* at seven molt stages in whiteleg shrimp.

2. Materials and methods

2.1. Specimens and sample preparation

At our facilities, 100 adult whiteleg shrimp *L. vannamei* (30 ± 2 g) were kept in plastic tanks containing seawater at 28 °C, salinity of 34 ppt, and O_2 at 6 mg l^{-1}. Feces, dead shrimp, and exuvia were removed once a day from the bottom of each tank. For qualitative analysis (Section 2.3), shrimps in intermolt stage were killed and we dissected pleopods, uropods, gills, eyestalk, hepatopancreas, muscle, hemocytes, digestive tube and nervous system. For quantitative analysis (Section 2.4) shrimp were starved for 48 h, then selected according to setogenesis in the uropods at molting stages A, B, C, D_0, D_1, D_2, and D_3 ($n = 4$ each) as described by Dall et al. (1990). Animals were killed and we dissected uropods. Samples for both experiments were kept at −80 °C in TRIzol™ reagent (Invitrogen) until used.

2.2. Total RNA isolation and cDNA synthesis

Total RNA was isolated from the stored samples in TRIzol™ as described by Chomczynski and Sacchi (1987). Integrity of RNA samples was verified by electrophoresis in denaturing formaldehyde agarose gels (Sambrook and Russell, 2001). Contaminating genomic DNA was removed from 10 μg total RNA using 4 units of DNAse I (Sigma, #D5319) following the manufacturer's protocol. Then 4 μg DNA-free RNA were used for complementary DNA (cDNA) synthesis using reverse transcriptase III (Invitrogen, #18080-051) and oligo-dT for priming, according to the manufacturer's instructions. For the quantitative analysis (Section 2.4), the reverse transcription step was performed in duplicates for each organism.

2.3. Detection of LvChS and LvChi isoenzyme transcripts in shrimp organs

PCR amplifications of *ChS* and three *Chi* isoenzymes were conducted with the cDNA from pleopods, uropods, gills, eyestalk, hepatopancreas, muscle, hemocytes, digestive tube and nervous system as template. We also obtained PCR products of the ribosomal protein *L8* as the control gene (GenBank Accession DQ316258). Oligonucleotides used for PCR priming are listed in Table 1. PCR reactions were carried out with DNA polymerase (Gotaq Green Master Mix, Promega, #M7122), including a denaturation step prior to amplification: 95 °C for 3 min, 35 cycles of 95 °C for 30 s, 57 °C for 30 s, 68 °C for 1 min, and a final extension at 68 °C for 10 min. Electrophoresis in 1% agarose gel was carried out in order to detect the PCR products. Each product was sequenced (Macrogen, Seoul, South Korea) to confirm its identity.

2.4. Relative quantification of LvChS and LvChi2 transcripts at different molting stages

Quantification of *LvChS* and *LvChi2* mRNAs from shrimp at different molt stages was carried out by reverse transcription, followed by quantitative PCR (qRT-PCR; iQ5 Multicolor Real-Time PCR Detection System and iQ SYBR Green Supermix, Bio-Rad). The thermal cycler program was 95 °C for 5 min, 40 cycles of 95 °C for 1 min, 57 °C for 1 min, and 72 °C for 1 min. Duplicates of cDNA from uropods of 4 shrimps in each molt stage were used as the template in triplicate qPCR reactions of 20 μl, using 1 μM of either *L8*, *LvChS*, or *LvChi2* forward and reverse oligonucleotides (numbered 1, 2, and 4 in Table 1). Efficiency of the qPCR reactions was determined with software (iQ5, Biorad), as well as the Ct value of each PCR reaction, which was used to calculate the relative expression of each gene by the $2^{-(\Delta\Delta Ct)}$ method, where: $\Delta\Delta C_T = [(C_{T,\ ChS/Chi2} - C_{T,\ L8})_{\text{molt stage}}] - [(C_{T,\ ChS/Chi2} - C_{T,\ L8})_{\text{intermolt stage}}]$ (Livak and Schmittgen, 2001). Amplification of the ribosomal protein *L8* cDNA was included in the analysis as an internal control for normalization of gene expression. Results are expressed as the fold-change relative to intermolt stage C.

2.5. Statistics

Expression data obtained from different molt stages were analyzed by median comparison, since normality and variance homogeneity were absent. The nonparametric Kruskal–Wallis ANOVA-by-ranks was performed, and differences ($P<0.05$) were located by Mann–Whitney test with the statistical software OriginPro 8 (OriginLab Corporation).

3. Results

3.1. LvChS and LvChi isoenzymes transcript detection in shrimp organs

PCR products corresponding to the cDNA of one chitin synthase and three chitinase isoenzymes were obtained by RT-PCR. *LvChi1*

Table 1
Oligonucleotides sequence used for PCR amplification of ChS, Chi1, Chi2, and Chi3.

Primer number and name	Direction of primer	Sequence (3′–5′)	Product size (bp)	GenBank accession number
1. *L8*	Reverse	TCC TGA AGG AAG CTT TAC ACG	167	DQ316258
	Forward	GGA AGA TCG ATG GCT GTG C		
2. *LvChS*	Reverse	AAG GCA CCC ACC AAC ATA AG	291	FJ229468[a]
	Forward	AAG TAT CCC GAC ATG AAG AC		
3. *LvChi1*	Reverse	CYA CCG TGT CGA AGG CCT C	259	EU883591
	Forward	CAG TCG CCT TGT GAA CAA C		
4. *LvChi2*	Reverse	TCA GGG TGG ACA TAC ATA GG	375	EU861222[a]
	Forward	AGT TCG ACG TGG AGG ACA TC		
5. *LvChi3*	Reverse	TTC AAG TTG GCA TTC TGC TG	178	AF315689

Underlined oligonucleotides were used during real-time PCR amplification.
[a] Indicates sequences obtained in this study.

and *LvChi3* were amplified from hepatopancreas cDNA, were of the expected size, and their nucleotides sequence confirmed their identity. Analysis using the BLAST algorithm (Zhang et al., 2000) against nucleotide sequences in GenBank (Accessions EU883591 and AF315689) resulted in 100% identity to the reported *LvChi1* and *LvChi3*, respectively. The cuticular chitinase Lv*chi2* deposited in the GenBank (Accession EU861222) and BLAST analysis showed that it is 89% similar to the cuticular chitinase from the Kuruma prawn *M. japonicus* (*MpChi2*); more recently, another partial cDNA for Chi2 from *L. vannamei* was deposited in GenBank (FJ888479.1), being 100% similar to *LvChi2* reported in this study. *LvChS* was not previously reported in a gene database. The partial sequence of this novel mRNA was reported in GenBank (Accession FJ229468). BLAST analysis of *LvChS* showed that this 340-bp nucleotide sequence had 82% similar to the *ChS* from lobster *H. americanus* (Accession GQ169704.2).

Fig. 1 shows the qualitative analysis detecting the mRNAs of *ChS* and isoforms of *Chi* in shrimp organs. The ribosomal protein *L8* mRNA showed bands of similar intensity in all organs, indicating constant abundance of transcripts detected in all cases. In contrast, the presence of the transcripts coding for *ChS* and *Chi* isoforms was cell-type specific. Chitin synthase *LvChS* was detected in the integument of pleopods and uropods, gills, hepatopancreas, and the digestive tube. *LvChi1* and *LvChi3* were exclusively found in the hepatopancreas. Cuticular chitinase *LvChi2* was also detected in the integument of pleopods and uropods, gills, eyestalks, and the digestive tube. Arrows in Fig. 1 indicate detection of *LvChS* and *LvChi2* in the digestive tube, although it was not abundant. PCR amplification after 40 cycles confirmed the presence of these bands (not shown).

3.2. Relative quantification of LvChS and LvChi2 transcripts at different molt stages

Figs. 2 and 3 show the fold change in gene expression of *LvChS* and *LvChi2* at each molt stage resulting from analysis by the $2^{-(\Delta\Delta Ct)}$ method relative to the *L8* reference mRNA and intermolt stage (Livak and Schmittgen, 2001). The abundance of each transcript at intermolt was set as the baseline, thus the value of the amount of mRNA at stage C was set at one. Efficiency of the qPCR reactions ranged from 95% to 105% for all genes (104.4%, 101.1%, and 104% for *L8*, *LvChS*, and *LvChi2*, respectively). *LvChS* showed a clear pattern of high abundance at postmolt Stages A and B and maintenance of expression near the baseline thereafter, except for an increase at Stage D_2 ($P<0.05$; Fig. 2). Maximum expression occurred at Stage A, reaching a median

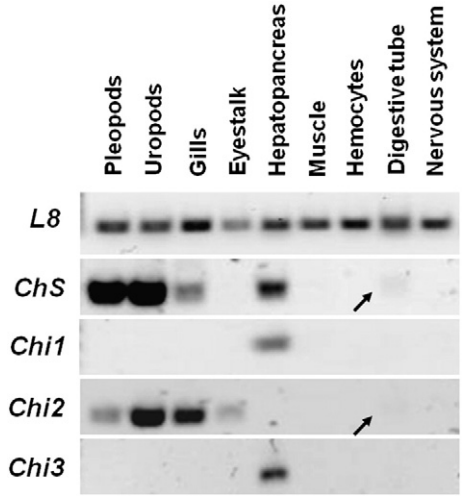

Fig. 1. Qualitative RT-PCR analysis of expression of *LvChS*, *LvChi1*, *LvChi2*, and *LvChi3* in organs of whiteleg shrimp *Litopenaeus vannamei* in the intermolt stage. Arrows show weak bands of *LvChS* and *LvChi2* in cDNA from the digestive tube.

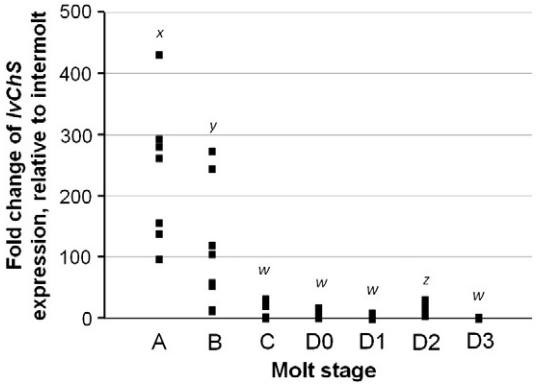

Fig. 2. Fold changes of the relative expression of *LvChS* during the molting cycle in whiteleg shrimp *Litopenaeus vannamei* measured in uropods. Values are expressed in x-fold changes relative to the intermolt Stage (C) and normalized with *L8*. Dots indicate average of triplicate PCR for each cDNA. Lower case letters indicate significant differences ($P<0.05$).

of 260-fold increase over the baseline. At Stage B, median expression of *LvChS* was 80-fold higher than the baseline; *LvChS* at stage D_2 was 10-fold higher than the baseline.

The amount of *LvChi2* mRNA also changed during the molting cycle ($P<0.05$), as shown in Fig. 3. At post-molt Stages A, *LvChi2* relative expression was 9-fold higher than the baseline ($P<0.05$). There was a significant 15-fold increase in the median of expression of *LvChi2* from the intermolt Stage C to early post-molt D_0 stages; at Stages D_1, D_2 and D_3 the amount of transcript was the same as the baseline ($P>0.05$).

4. Discussion

Prior to this study, Parvathy (1970) referred indirectly to chitin synthesis during the molting cycle of the sand crab *Emerita asiatica* by measuring sugars in hemolymph; also, a crustacean ChS was demonstrated in the brine shrimp *Artemia salina* as a microsomal enzyme capable of synthesizing chitin (Horst, 1981). No other studies on ChS from crustaceans have been published and besides *LvChS* that we report in this study (GenBank Accession FJ229468), only one ChS cDNA sequence from the *H. americanus* lobster has been recently deposited in GenBank (Accession GQ169704). We characterized tissue specificity and quantified the levels of expression during molting of *LvChS*. We also report a partial cDNA sequence encoding a cuticular chitinase from the species *L. vannamei LvChi2*, which was submitted to GenBank (Accession EU861222). Later, another partial cDNA corresponding to

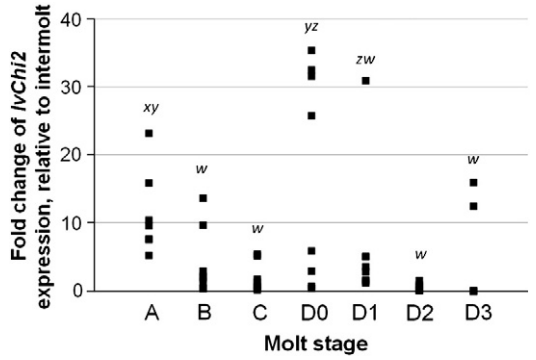

Fig. 3. Fold changes of the relative expression of *LvChi2* during the molting cycle in the white shrimp *Litopenaeus vannamei* measured in uropods. Values are expressed in x-fold changes relative to the intermolt Stage (C) and normalized with *L8*. Dots indicate average of triplicate PCR for each cDNA. Lower case letters indicate significant differences ($P<0.05$).

whiteleg shrimp Chi2 was uploaded in the GenBank database (Accession FJ888479.1) (Huang et al., 2010). As the enzymes codified by these mRNAs are closely involved in shrimp growth and development, the sequences and the data generated could be the starting point for future work to understand these complex processes.

As shown in Fig. 1, the ribosomal protein *L8*, considered a housekeeping gene in some gene expression studies (Gómez-Anduro et al., 2006; Sánchez-Paz et al., 2008) was expressed in all shrimp organs included in our qualitative analyses. *LvChS* was detected in pleopods, uropods, gills, hepatopancreas, and digestive tube. Even though we clearly detected a single band of the same size in these organs, we did not discard the possible presence of *ChS* isoforms among shrimp cells. The absence of *LvChS* mRNA in muscle and hemocytes supports our assumption that integumental cells were responsible for synthesis of the transcript when we tested its expression in cDNA from pleopods and uropods.

Tissue-specific isoforms of *ChS* have been described in some insect species, such as the red flour beetle *T. castaneum* chitin synthases (Arakane et al., 2005). Although the only known biochemical function of *ChS* is the synthesis of chitin, this polymer is not only used by shrimp as a structural component of the exoskeleton, it is also present in the peritrophic membrane, which is secreted along the gut and is also discarded during molting and has to be re-synthesized after ecdysis (Ceccaldi, 1989; Merzendorfer and Zimoch, 2003). This explains the wide presence of *LvChS* in shrimp organs, including integumental tissue, gills, and the digestive system.

We also assayed for the presence of Chi isoenzymes 1, 2 and 3 mRNA in shrimp organs of *L. vannamei*. *LvChi1* and *LvChi3* were only detected in hepatopancreas cDNA samples, which indicate that these chitinase isoenzymes are involved in food chitin digestion. Huang et al. (2010) also reported that *Chi1* and *Chi3* mRNAs are exclusively found in the hepatopancreas of the whiteleg shrimp. However, chitinases are secreted into the extracellular space after translation (Arakane et al., 2003), so the protein product of *Chi1* and *Chi3* could be found in hepatopancreas and other organs. Immunological detection of these proteins in other shrimp organs would be required to study other possible functions of these isoenzymes.

Cuticular chitinase *LvChi2* was also present in organs of diverse functions. We detected *LvChi2* mRNA in pleopods, uropods, gills, eyestalks, and the midgut. The absence of cuticular chitinase in the hepatopancreas may indicate that the enzyme encoded by this mRNA is not likely to be involved in food chitin digestion and may have a role in hydrolyzing chitin from the exoskeleton prior to molting. A very light band of *LvChi2* was detected in the digestive tube, suggesting that cuticular chitinase could be involved in peritrophic matrix hydrolysis. Some previous findings disagree with this suggestion. Digestive isoenzymes of chitinase from the hepatopancreas of *M. japonicus* (Chi1 and Chi3) are thought to be involved in peritrophic matrix hydrolysis (Watanabe et al., 1996, 1998). In insects, different isoenzymes of chitinases hydrolyze chitin from the exoskeleton and peritrophic matrix (Arakane et al., 2004). Huang et al. (2010), only found Cht2 mRNA in gills and eyestalk; in that study, shrimps used were only 9–12 g of weight (versus 30 g weight of the shrimps in this study), but the reason of these contrasting results is unknown.

Analysis of the quantitative data from *LvChS* and *LvChi2* gene expression during the molting cycle included normalization, using the constitutive protein mRNA *L8* and the endogenous basal condition of the intermolt (Stage C). In this stage, no molt-related activities occur because the exoskeleton is fully formed and pre-molt processes have not yet started (Kuballa and Elizur, 2007). When compared to variations of *LvChS* and *LvChi2*, *L8* mRNA changes were negligible during the molting cycle (not shown), so this transcript is a suitable internal control gene, which accords with studies by Gómez-Anduro et al. (2006) and Sánchez-Paz et al. (2008).

The detected variations of *ChS* mRNA can be explained as molt-related processes. Higher values of *LvChS* were detected at post-molt (Stage A), afterwards it declined at Stage B. Strong up-regulation occurred from Stages D_3 to A, indicating that expression of *LvChS* is induced after ecdysis to synthesize the chitin that will form the exoskeleton that is completed at Stage C (O'Brien, et al., 1991; Skinner, 1962). High up-regulation should be accompanied by greater availability of the substrate for *ChS*, UDP-NAcGlc. In juvenile whiteleg shrimp, a high concentration of glucose was also detected prior to ecdysis (Chan et al., 1988). During intermolt (Stage C), expression of *ChS* was very low, suggesting the absence of chitin synthesis activity when the exoskeleton is fully formed (Kuballa and Elizur, 2007). A 10-fold increase in *LvChS* at Stage D_2 coincides with the beginning of synthesis of a new epicuticle and exocuticle during early pre-molt. Chitin is not present in the outer layer epicuticle, but it is present in the exocuticle (O'Brien et al., 1991), which explains why this mRNA was not up-regulated until the late pre-molt Stage D_2, when this layer is synthesized.

During the molting cycle, the amount of mRNA of *LvChi2* changed in a way that cannot be fully explained by molt-related processes; also, a high level of individual-specific variations was detected. However, our results agree with previous investigations of chitinase activity or gene expression during the molting cycle (Buchholz, 1989; Tan et al., 2000). At post-molt Stages A and B, *LvChi2* expression was higher than the baseline; the maximum median expression was found at early pre-molt Stage D_0 and the minimum at Stage D_3, when ecdysis is about to start. The increasing expression of chitinase at Stages A and B does not match the needs of shrimp when synthesizing a new cuticle, but a similar pattern of chitinase activity was detected in the Antarctic krill *Euphausia superba* at post-molt (Buchholz, 1989) and an increase in chitinase transcripts was detected in the tiger prawn *P. monodon* (Tan et al., 2000) during post-molt. These authors did not discuss this observation. The maximum rise of *LvChi2* (15-fold) occurred at Stage D_0, but we did not observe this induction of expression in all individuals. Up-regulation of chitinase expression in the integument suggests that pre-molt has started and that shrimp requires the enzymes that hydrolyze the exoskeleton and to absorb its components, including NAcGlc, the building blocks of chitin. Similarly, Watanabe and Kono (1997) found that expression of *MjChi2* was higher at the pre-molt stage than at intermolt stage.

Ecdysis related mechanisms start with strong and coordinated regulation of several molecules. However, comparing our results with the literature, we found that this regulation can be observed in any sub-stage of pre-molt, such as D_0, D_1, D_2, or D_3. Down-regulation of molt inhibiting hormone was observed at Stages D_2 and D_3 (Chen et al., 2007) and a rise in ecdysone titers was found at Stage D_1 in juvenile whiteleg shrimp (Chan et al., 1988). Up-regulation of blood sugars (Parvathy, 1970) increases the activity of chitinases, N-acetylglucosaminidases, and proteases (Buchholz, 1989) and over-expression of *Chit1* was detected at Stage D_2 (Tan et al., 2000).We suggest that the levels of stress in each case we cite could explain the different stages in which pre-molt related processes are detected.

Originally, we hypothesized that expression of *LvChS* and *LvChi2* was coordinated because of their opposite biochemical and physiological function during the molting cycle. We found that *LvChS* has a high expression at Stages A and B, and *LvChi2* is up-regulated mainly during pre-molt (Stage D_0) suggesting certain level of coordination. Temporal coordination of ChS and Chi mRNA was previously described during the larval and pupal stages of the fall armyworm *S. frugiperda* (Bolognesi et al., 2005). However, whole patterns of *ChS* and *Chi2* expression are not temporally exclusive during the molt cycle of *L. vannamei*. Temporal expression of other isoenzymes, as well as localization of the functional enzymes could bring more detailed information about coordination of these physiological functions.

This study is the first work where a direct relationship between chitin synthesis and hydrolysis is determined in a crustacean. Moreover, we quantified cDNAs from integumenal cells in the whiteleg shrimp, from which further investigations on integument metabolism and catalytic properties of the enzymes encoded by these cDNAs would be appropriate.

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