PROPHENOLOXIDASE ACTIVATING FACTOR FROM THE BLUE CRAB, *CALLINECTES SAPIDUS*

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A Thesis Submitted to the
University of North Carolina at Wilmington in Partial Fulfillment
Of the Requirements for the Degree of
Master of Science

Department of Biological Sciences

University of North Carolina at Wilmington

2004

Approved by

Advisory Committee

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Dean, Graduate School

This thesis has been prepared in the style and format

Consistent with the journal

Comparative Biochemistry and Physiology, Part B

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ABSTRACT

Crustaceans are encased in a hard exoskeleton, making it necessary for them to shed this cuticle in order to grow. Once the old exoskeleton is shed, the newly synthesized cuticle can begin to harden. Tanning, or sclerotization, initially hardens the cuticle by crosslinking cuticular proteins attached to the chitin-fiber matrix. This process is catalyzed by an enzyme, phenoloxidase, whose activation is controlled by a serine protease cascade. The cDNA of a prophenoloxidase activating factor (PPAF) was cloned and sequenced from the blue crab, *Callinectes sapidus*. The expression pattern was determined by quantitative PCR and Northern blotting in both calcified and arthrodial tissue before, during, and after molting. High signal intensity was found in premolt RNA from the hypodermis of both cuticle types. This implies that PPAF gene is being transcribed in premolt tissue for translation and use of the protein in the tanning process, which occurs within a few hours after ecdysis. An increase in arthrodial signal at three to four hours post-molt may signify that arthrodial cuticle laid down in post-molt stages is being tanned. This pattern is not seen in calcified cuticle, as biomineralization of the endocuticle occurs simultaneously with deposition.

KEYWORDS: *Callinectes sapidus*, tanning, sclerotization, prophenoloxidase activating factor, phenoloxidase activation, clip domain, trypsin-like serine protease domain

ACKNOWLEDGEMENTS

I would like to thank all the people that helped me with my research. Thanks to my advisor, Dr. Shafer for all his help and cooperation. It was a long process, but I finally made it! To my committee members Drs. Roer, Kinsey, and McCartney—thank you for all your helpful suggestions and comments. This project wouldn't have come together if it weren't for you.

Thanks to all of my lab mates, especially Francie, Mark, and Anna. Francie Coblentz for always being willing to help. To Mark Gay who always had an answer if I had a question. Special thanks to Anna Wynn for her help troubleshooting. And goodness, knows there was a lot of troubleshooting!

Murry Bridges at Endurance Seafood in Kill Devil Hills, NC deserves particular thanks for putting up with us on our annual crab trip.

Thanks also to Dr. David Towle of the Mount Desert Island Biological Laboratory for processing mass amounts of quantitative PCR samples. And for his helpful suggestions about my research.

To Darryl, my family and friends, thank you for your support.

Support for this research was made possible by the National Science Foundation (Grant IBN-0114597 to R.D. Roer, R.M. Dillaman, T.H. Shafer, and M.A. McCartney).

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INTRODUCTION

Decapod crustaceans such as the blue crab, *Callinectes sapidus*, are covered and protected from their environment and potential predators by a hard exoskeleton or cuticle. This necessitates that the crab molt in order to grow. Molting is controlled by the underlying epithelial cells of the hypodermis, which regulate the timing of the molt cycle as well as the hardening of the new cuticle through the translation, release, and activation of cuticular proteins.

The mature crustacean cuticle is comprised of several layers, which are made up of a chitin-protein matrix. The epicuticle is the outermost layer, and is in contact with the outside media. Directly underlying this layer is the exocuticle, endocuticle, and the uncalcified membranous layer. The hypodermis is the epithelium responsible for the formation of the new cuticle layers both before and after molting, and is in direct contact with the membranous layer. The hypodermal cells form a single layer, the shape of which can range from squamous to columnar depending on the function of the cells at any particular stage of the molt cycle.

The molt cycle consists of five stages. The period before the molt, or pre-molt (stage D), consists of sub-stages D_0, D_1, D_2, D_3, and D_4. The beginning of pre-molt, at D_0, is characterized by apolysis. A hormone signal induces the hypodermal cells to separate from the existing cuticle (Roer and Dillaman, 1993). The outer epithelial cells of the hypodermis begin to grow more complex and columnar in preparation for the synthesis of a new cuticle. Deposition of the new cuticle begins in pre-molt. The epi- and exocuticle are fully formed but uncalcified before ecdysis, termed stage E.

Roer and Dillaman (1993) found that the initiation of mineralization of these pre-exuvial layers begins approximately four hours post-ecdysis, during post-molt stage A_1. The mineral is transported to the epicuticle and the outermost regions of the exocuticle via the pore canals, which draw sequestered calcium up from the hypodermis into the new exoskeleton. Once the calcium has reached the outer portions, the layers are calcified in an inward progression (Roer and Dillaman, 1984). The pattern of exocuticle mineralization has been recently described more completely by Hequembourg et al. (2004). During post-molt stages A_2, B_1, and B_2 the endocuticle is deposited and calcified simultaneously. Formation of the new membranous layer signals the beginning of intermolt, stage C.

Immediately after ecdysis, the crab cuticle consists solely of a soft, uncalcified epi- and exocuticle. At this time, the animal swells via the absorption of water across the gills and digestive epithelium causing the size increase indicative of molting. The soft cuticle is not sufficiently rigid to promote muscle function, however, the absorption of water facilitates the establishment of a hydrostatic skeleton that provides support for muscle contraction before hardening of the cuticle begins (Taylor and Kier, 2003).

The presence of a calcified exoskeleton affords crustaceans protection, support, and a site for muscle attachment (Felgenhauer, 1992). However, a stiff cuticle resists movement and the joints of the crab must be flexible. Uncalcified cuticle, known as arthrodial membrane, is located at the joints. It is similar in thickness and appearance to the adjacent hard cuticle. Though it is morphologically similar and is deposited by immediately adjacent hypodermal cells, the arthrodial membrane has been shown histologically to have distinctly different carbohydrate and protein composition from

calcified cuticle (Williams et al., 2003). Uncalcified cuticle also exists as a much thinner layer coating the inner surface of the branchial chamber, the gills and portions of the gut.

Anderson (1999) proposed that there are important molecular differences between flexible and stiff cuticles before any hardening of the exoskeleton occurs. These differences include the percentage of water in the tissues and the composition of proteins attached to the chitin fibers. This model indicates that flexible cuticles such as the arthrodial membrane have higher water content and more hydrophilic proteins. High water content promotes free movement of the hydrophilic proteins, and a more plastic membrane. Conversely, inflexible cuticles contain mostly hydrophobic proteins and little water. Aggregation among the hydrophobic proteins is common and forms tightly packed structures that resist deformation.

An anecdotal observation has been made that the cuticle of an ecdysial crab is easily deformed. However, as early as half an hour after molting the cuticle takes on a leathery texture, indicating that some initial hardening is occurring in the early post-molt stages. Because calcification is not initiated until approximately four hours post-molt another process must be in play to lend the cuticle some rigidity before mineralization occurs. Tanning, or sclerotization, is thought to accomplish this early hardening of the cuticle that precedes mineralization. A series of enzyme reactions crosslinks the cuticular proteins that are attached to chitin. This strengthens the cuticle and provides the crab with a structured environment for the nucleation of calcium carbonate. Tanning begins with the oxidation of tyrosine. The enzyme phenoloxidase (PO) catalyzes the formation of dihydroxyphenylalanine (DOPA) from tyrosine. DOPA is converted into dopamine by a decarboxylase. N-acetyl-CoA-acetyltransferase converts dopamine into N-

acetyldopamine (NADA), which acts as a substrate for the conversion of phenols to o-quinones. The highly reactive o-quinones produced by PO react with certain protein residues, crosslinking them to other cuticular proteins.

Sclerotization is under strict control. A stepwise cascade involves sequential activation of several enzymes that results in the activation of PO. Under normal conditions, PO exists in the hemolymph in an inactive form called prophenoloxidase (PPO) (Kwon et al. 2000, Satoh et al. 1999, Aspan et al. 1995, Hall et al. 1995, Chosa et al. 1997). The final enzyme that converts PPO into the active form, PO, is a prophenoloxidase activating factor (PPAF). This enzyme works by proteolytic cleavage and has been shown to have specificity for cleavage after arginine residues. Following cleavage by PPAF, PO is able to oxidize tyrosine into DOPA.

PPAF enzymes and the gene sequences coding for them have been identified in many insect species including *Drosophila melanogaster* (Fujimoto et al. 1995, Chosa et al. 1997), *Manduca sexta* (Hall et al., 1995), *Bombyx mori* (Satoh et al., 1999), *Holotrichia diomphalia* (Kwon et al. 2000, Lee et al. 1998), and *Tenebrio molitor* (Kwon et al., 2000). PPAF enzymes have also been identified in the crayfish, *Pacifastacus leniusculus* (Aspan et al., 1995). Identified PPAFs contain a carboxyl-terminal serine protease domain and an amino-terminal clip domain. The clip domain has been implicated in regulating the function of the enzyme through its interaction with other proteins (Jiang et al., 1998).

The combination of tanning and calcification provides crustaceans with a reinforced exoskeleton that protects them from predation. The mode of calcification has been investigated in numerous studies. However, tanning is less well explored in

crustaceans. The timing of this event is unclear and differences between tanning in the arthrodial cuticle and the calcified cuticle are unknown. The enzymology is poorly understood, including the synthesis and regulation of PO and PPAF in these two tissue types. This work contributes to understanding the molecular basis for tanning in arthrodial and calcified cuticle by reporting the cloning and sequencing of a cDNA encoding PPAF from the blue crab, *Callinectes sapidus*, and the analysis of the mRNA expression patterns in the two tissues during the critical periods of late pre-molt, ecdysis, and early pre-molt.

MATERIALS AND METHODS

Organisms and tissue

Adult blue crabs, *Callinectes sapidus*, were obtained from a commercial shedding operation in Kill Devil Hills, NC. Late pre-molt (stage D_2, D_3 or D_4), ecdysial (stage E) and post-molt crabs at 0.5 h, 1 h, 2 h, 3 h, 4 h, 8 h, and 24 h after ecdysis were sacrificed and hypodermal tissues obtained by scraping from the cuticle. Tissue was flash frozen in liquid nitrogen to preserve RNA integrity. The arthrodial membrane hypodermis was obtained at the carpus joints of the chelipeds, where a relatively large flat portion of the cuticle remains uncalcified at intermolt. Hypodermis depositing cuticle destined to calcify was obtained from a section of mid-dorsal carapace directly over the cardiac chamber. The dorsal-most part of the pericardium was peeled from the inner surface before scraping the hypodermis. This location was chosen because histological examination revealed that the epithelium obtained was free of muscle attachment sites and other types of tissue.

RNA extraction

Total RNA was extracted using the spin-column RNeasy Protect Mini Kit (Qiagen). Tissue was transferred from -80° C to RNAlater-Ice (Ambion) at -20° C for 16 hours. Mid-dorsal hypodermis from a single crab was homogenized in 1 ml buffer RLT (Qiagen) containing a 1:100 concentration of β-mercaptoethanol and then bound to the column with 50% ethanol. Arthrodial hypodermis from the two chelipeds of a single crab was homogenized in 1 ml Trizol (Invitrogen) and bound to the column with 70%

ethanol. Differences in extraction technique were deemed to be necessary to obtain maximum RNA yields. In both cases, RNA was eluted with 30 μl nuclease-free water. The same eluate was passed through the column a second time to completely elute the RNA. Total RNA was quantified by its absorbance at 260 nm using a microplate reader and KCJr software. The quality of the RNA was assessed by complexing with ethidium bromide and fractionating on a 1.0% agarose gel containing 2% formaldehyde as a denaturing agent. Only RNA with sharp ribosomal bands and a minimum of apparent degradation was used.

Reverse transcription and RACE-PCR

One to five μg of total RNA was used for first strand cDNA synthesis by reverse transcription. The RT reaction was primed with oligo(dT) containing an additional adapter sequence. This universal 3' primer site was used for 3' Rapid Amplification of cDNA Ends (RACE) using Gibco 3' RACE kit. 3' RACE-PCR was performed using Degenerate 3' RACE primer (refer to Table 1) as the gene-specific forward primer and the abridged universal adapter primer (AUAP) from the 3' RACE kit that is complementary to the adapter sequence added in cDNA synthesis. PCR conditions began with a 92° C denaturating step for 5 minutes followed by the addition of 50 nM REDTaq DNA polymerase (Sigma) during a 55° C hold. A total of 30 cycles were performed consisting of 92° C for 1 minute, 55° C for 1 minute, and 72° for 2 minutes. Ethidium bromide-stained PCR products were visualized on a 1.4% agarose gel.

Table 1. Oligonucleotide primer sequences for 3' RACE, 5' RACE, and PCR of cuticle PPAF. Location is indicated by nucleotide number from the 5' end of PPAF (see Fig. 1). Forward primers indicated by 'f'. Reverse primers indicated by 'r'.

Primer	Application	Sequence	Location
Degenerate 3' RACE	3' RACE	5' -GCCT(C/G)TACGAGCC(C/T)TACGACCAG-3'	603-626
PPAF1r	5' RACE	5'-CTACGAGCCTTACGACCAGG-3'	627-607
PPAF2r	5' RACE	5'-AAATCCTCAACCCGCAGC-3'	783-765
PPAF3f	Q-PCR	5'-ATCGACCTTCCCTACGTTCC-3'	857-878
PPAF4r	Q-PCR	5'-CACTGAAGCTGACAAGCTGC-3'	1152-1132
PPAF5f	PCR	5'-GCTGCGGCAACAGGAACTAC-3'	351-371
PPAF6r	PCR	5'-CACCAGGCTCGGCAAGTTCT-3'	927-907
PPAF7f	Probe	5'-GACGGCAGGAGTACAGAAGG-3'	11-31
PPAF8r	Probe	5'-CACTGAAGCTGACAAGCTGC-3'	986-966

5’ RACE was performed using the FirstChoice™ RLM-RACE system (Ambion). This system targets the 5’-cap for the addition of a universal adapter primer site that is copied during cDNA synthesis, thus insuring that amplification is only from full-length mRNAs. Furthermore, it employs a nested PCR strategy to increase specificity. 5’ RACE was performed using PPAF1r as the outer and PPAF2r as the inner gene specific primers. PCR conditions were similar to above except that Super Taq Plus (Ambion) was used.

When necessary to obtain overlapping products for verification of continuity of the 3’- and 5’ RACE products, two gene-specific primers were designed and traditional RT-PCR was performed.

Cloning and Sequencing

PCR products containing a single band were purified using a PCR purification kit (Qiagen) to remove excess primers and dNTPs. Alternately, bands were excised from gels and the DNA was extracted and purified using the Gel purification kit (Qiagen). In both cases, DNA was eluted from the column with 30 µl of nuclease-free water.

Ligation into pGEM®-T vector and transformation of JM109 high efficiency competent cells was performed using the pGEM®-T Easy Vector kit (Promega). Individual colonies grown on LB agar plates were used as template in a standard PCR reaction with Sp6 and T7 primers to amplify target DNA. The sizes of the inserts were assessed on a 1.4% agarose gel.

Products from colony PCR were purified as above. One µl of the isolated DNA was used in a cycle sequencing reaction containing fluorescent ddNTPs (ABI Big Dye

Terminators) and either forward or reverse vector primers, Sp6 or T7, respectively. Reaction conditions were 94° C for 45 seconds, 50° C for 45 seconds, and 60° C for 4 minutes for 25 cycles. The products were centrifuged through G-50 sephadex columns at 2,000xg for 1 minute before being analyzed on a capillary DNA sequencer (ABI 3100). Forward and reverse sequences were checked and assembled after trimming vector bases using Chromas (version 2.13) software.

Quantitative PCR

cDNAs were produced from known volumes of total RNA. Then accurate measures of the concentration of each RNA sample were determined using an Aligent 2100 Bioanalyzer so that all samples could be normalized to compare RNA concentrations. Quantitative real-time PCR using these cDNA's as templates and the PPAF3f /PPAF4r primer set was completed by the Marine DNA Sequencing Center at the Mount Desert Island Biological Laboratory. A MX4000 Multiplex Quantitative PCR system (Stratagene) was employed using SYBR Green fluorescent dye. A single cDNA sample was diluted through three orders of magnitude to check for linearity between the log concentration and critical threshold cycle number. Transcript concentration in all other samples are reported relative to this one.

Northern Blots

One μg of RNA from each time period and tissue type was complexed with ethidium bromide and fractionated on a 1.0% agarose gel with 2% formaldehyde as a denaturant. The RNA was allowed to migrate at 35 volts for 4-5 hours. The gel was

visualized under UV light and blotted to a Millipore Immobilon-NY+ membrane. The primer set PPAF7f and PPAF8r was designed to the PPAF gene sequence, yielding a product of 975 base pairs that was then cloned into the pGEM®-T vector (see above). Plasmids were purified with PerfectPrep Plasmid Mini Isolation Kit (Eppendorf) and cut with SPE 1 restriction enzyme to linearize. Riboprobes were transcribed from the T7 promotor and labeled with dioxygenin (DIG) using DIG RNA labeling kit (Roche). The blots were hybridized, washed, complexed with alkaline phosphatase-labeled anti-DIG, washed again, and exposed to x-ray film according to the Millipore Northern Blotting Protocol for Immobilon™-Ny+ membrane and Detection of DIG-labeled probe. 20X SSC (3M Sodium chloride, 0.3M Sodium citrate, pH 7.0) was used as transfer buffer. Bands were visualized by the addition of a chemiluminescent substrate CSPD (Roche) that is dephosphorylated by the alkaline phosphatase to produce the emission of light. The blots were exposed to x-ray film (Kodak, X-Omat AR) for 50 minutes and developed.

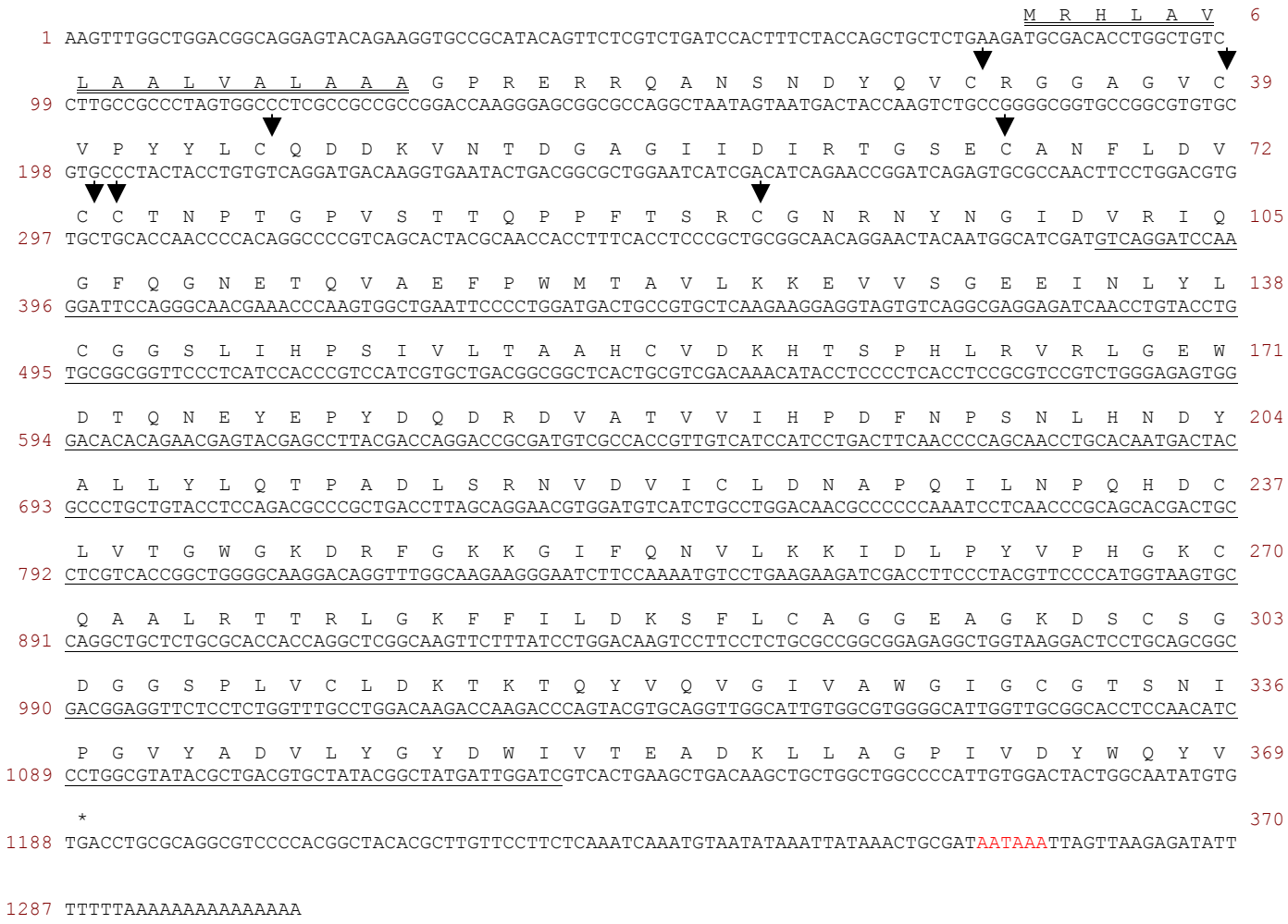
RESULTS

Cloning and Sequencing of PPAF cDNA

The BLAST search of the sequence isolated from 3' RACE yielded initial evidence for a *Callinectes sapidus* prophenoloxidase activating factor. The 3' RACE sequence showed high sequence homology with PPAFs from two insect species, *Tenebrio molitor* and *Holotrichia diomphalia*. The cDNA template used in the RACE procedure was produced using mRNA extracted from pre-molt, mid-dorsal hypodermis. This result was serendipitous since the original degenerate primer (Degenerate 3' RACE, Table 1) used in 3' RACE was actually designed based on a short amino acid sequence from a different cuticle protein (Tweedie et al. 2004). The 680 base pair long 3' RACE product contained one long open reading frame beginning at its 5' end. The deduced 194-amino acid sequence showed high homology to a conserved domain (NCBI: cd00190) for a trypsin-like serine protease, a universal C-terminal feature of PPAFs. Downstream from the stop codon was a 101 base-pair 3' UTR containing a typical polyadenylation site.

Because this putative blue crab PPAF fragment contained an incomplete coding sequence, nested 5' RACE was performed using two sequence-specific primers designed to the 3' end of PPAF (PPAF1r and PPAF2r). 5' RACE yielded a product of 599 base pairs containing an 80 base-pair 5' UTR. A set of primers (PPAF5f and PPAF6r) was made to verify the overlap of both sequences and to produce a contig. The complete sequence (Fig. 1) was 1283 base pairs and contained an open reading frame beginning at nucleotide 81 and ending at nucleotide 1191 (as submitted to GenBank, accession

Figure 1. Nucleotide sequence of cloned PPAF. cDNA (lower) and deduced amino acid sequence of the open reading frame (above). The amino acid sequence is numbered on the right from the initiation metionine residue to the first stop codon. The serine protease conserved domain is underlined. Arrows indicate the cysteine residues forming the clip domain. The signal peptide is denoted by a double underline. The polyadenylation site is highlighted in red.


M R H L A V 6
1 AAGTTTGGCTGGACGGCAGGAGTACAGAAGGTGCCGCATACAGTTCTCGTCTGATCCACTTTCTACCAGCTGCTCTGAAGATGCGACACCTGGCTGTC
L A A L V A L A A A G P R E R R Q A N S N D Y Q V C R G G A G V C 39
99 CTTGCCGCCCTAGTGGCCCTCGCCGCCGCCGGACCAAGGGAGCGGCGCCAGGCTAATAGTAATGACTACCAAGTCTGCCGGGGCGGTGCCGGCGTGTGC
V P Y Y L C Q D D K V N T D G A G I I D I R T G S E C A N F L D V 72
198 GTGCCCTACTACCTGTGTCAGGATGACAAGGTGAATACTGACGGCGCTGGAATCATCGACATCAGAACCGGATCAGAGTGCGCCAACTTCCTGGACGTG
C C T N P T G P V S T T Q P P F T S R C G N R N Y N G I D V R I Q 105
297 TGCTGCACCAACCCCACAGGCCCCGTCAGCACTACGCAACCACCTTTCACCTCCCGCTGCGGCAACAGGAACTACAATGGCATCGATGTCAGGATCCAA
G F Q G N E T Q V A E F P W M T A V L K K E V V S G E E I N L Y L 138
396 GGATTCCAGGGCAACGAAACCCAAGTGGCTGAATTCCCCTGGATGACTGCCGTGCTCAAGAAGGAGGTAGTGTCAGGCGAGGAGATCAACCTGTACCTG
C G G S L I H P S I V L T A A H C V D K H T S P H L R V R L G E W 171
495 TGCGGCGGTTCCCTCATCCACCCGTCCATCGTGCTGACGGCGGCTCACTGCGTCGACAAACATACCTCCCCTCACCTCCGCGTCCGTCTGGGAGAGTGG
D T Q N E Y E P Y D Q D R D V A T V V I H P D F N P S N L H N D Y 204
594 GACACACAGAACGAGTACGAGCCTTACGACCAGGACCGCGATGTCGCCACCGTTGTCATCCATCCTGACTTCAACCCCAGCAACCTGCACAATGACTAC
A L L Y L Q T P A D L S R N V D V I C L D N A P Q I L N P Q H D C 237
693 GCCCTGCTGTACCTCCAGACGCCCGCTGACCTTAGCAGGAACGTGGATGTCATCTGCCTGGACAACGCCCCCCAAATCCTCAACCCGCAGCACGACTGC
L V T G W G K D R F G K K G I F Q N V L K K I D L P Y V P H G K C 270
792 CTCGTCACCGGCTGGGGCAAGGACAGGTTTGGCAAGAAGGGAATCTTCCAAAATGTCCTGAAGAAGATCGACCTTCCCTACGTTCCCCATGGTAAGTGC
Q A A L R T T R L G K F F I L D K S F L C A G G E A G K D S C S G 303
891 CAGGCTGCTCTGCGCACCACCAGGCTCGGCAAGTTCTTTATCCTGGACAAGTCCTTCCTCTGCGCCGGCGGAGAGGCTGGTAAGGACTCCTGCAGCGGC
D G G S P L V C L D K T K T Q Y V Q V G I V A W G I G C G T S N I 336
990 GACGGAGGTTCTCCTCTGGTTTGCCTGGACAAGACCAAGACCCAGTACGTGCAGGTTGGCATTGTGGCGTGGGGCATTGGTTGCGGCACCTCCAACATC
P G V Y A D V L Y G Y D W I V T E A D K L L A G P I V D Y W Q Y V 369
1089 CCTGGCGTATACGCTGACGTGCTATACGGCTATGATTGGATCGTCACTGAAGCTGACAAGCTGCTGGCTGGCCCCATTGTGGACTACTGGCAATATGTG
* 370
1188 TGACCTGCGCAGGCGTCCCCACGGCTACACGCTTGTTCCTTCTCAAATCAAATGTAATATAAATTATAAACTGCGATAATAAATTAGTTAAGAGATATT
1287 TTTTTAAAAAAAAAAAAAAA

number AY555734). The open reading frame yielded a deduced amino acid sequence of 370 amino acids. A signal sequence was predicted by SignalP software that encompassed the first 16 amino acids. The complete serine protease domain encompasses 248 amino acids, 102 through 350 (Fig. 1).

Quantitative PCR

RNA was extracted from various time periods and tissues and cDNAs were made from exactly one µl of RNA. A time course of hypodermal samples included pre-molt, ecdysis and post-ecdysical times. RNA was extracted from both calcified and arthrodial hypodermis. RNA and cDNA samples were sent to the Marine DNA Sequencing Center at the Mount Desert Island Biological Laboratory for real-time, quantitative PCR analysis. A primer set (PPAF3f and PPAF4r) designed to the PPAF sequence to yield a 295 base pair product within the conserved domain. PPAF transcripts were detected in all RNAs sampled (Fig 2). The level of PPAF expression in mid-dorsal pre-molt (D stage) material was appreciably higher relative to either ecdysial or post-molt tissue. The intensity of signal decreased at ecdysis and remained low throughout the rest of the molt cycle. Arthrodial tissue showed a similar pattern; pre-molt tissue had a considerably higher signal than ecdysial or early post-molt tissue (Fig. 2). An increase in signal intensity was seen in the later post-molt tissue, namely at four, eight, and twenty-four hours (Fig. 3).

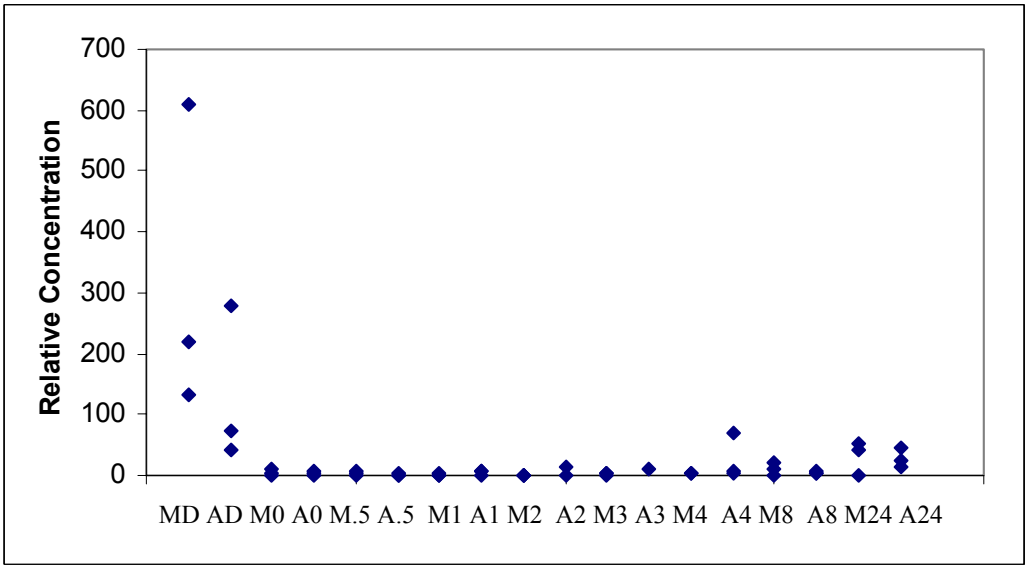
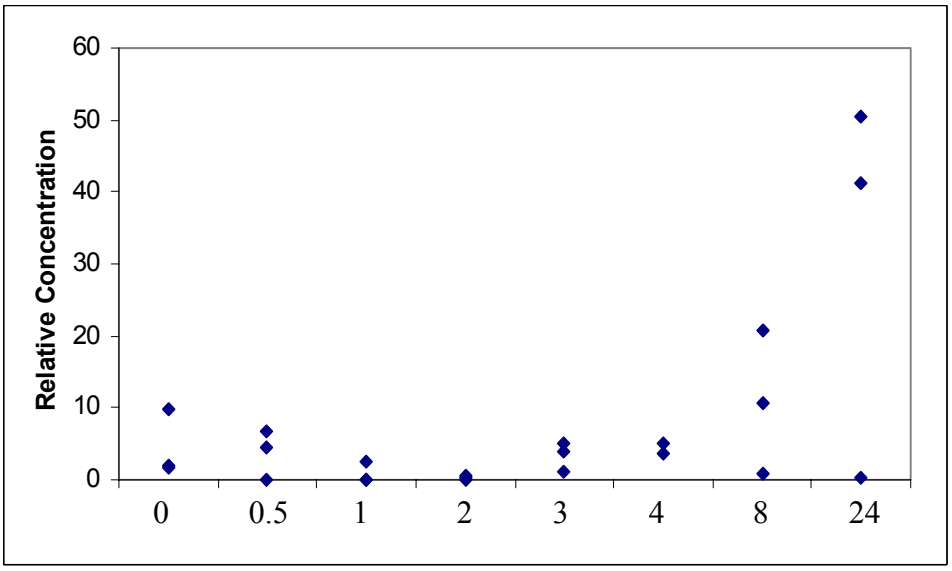


Figure 2. Quantitative, real-time PCR. cDNAs made from known concentrations of RNA was run on a MX4000 Multiplex Quantitative PCR system. Relative concentration was adjusted by multiplying all samples by 100 to best display the data. M indicates mid-dorsal hypodermis. A indicates arthrodial hypodermis. D signifies premolt tissue, and subsequent samples are labeled with the hour after ecdysis. Samples are arranged premolt to postmolt, alternating mid-dorsal and arthrodial.

a.



b.

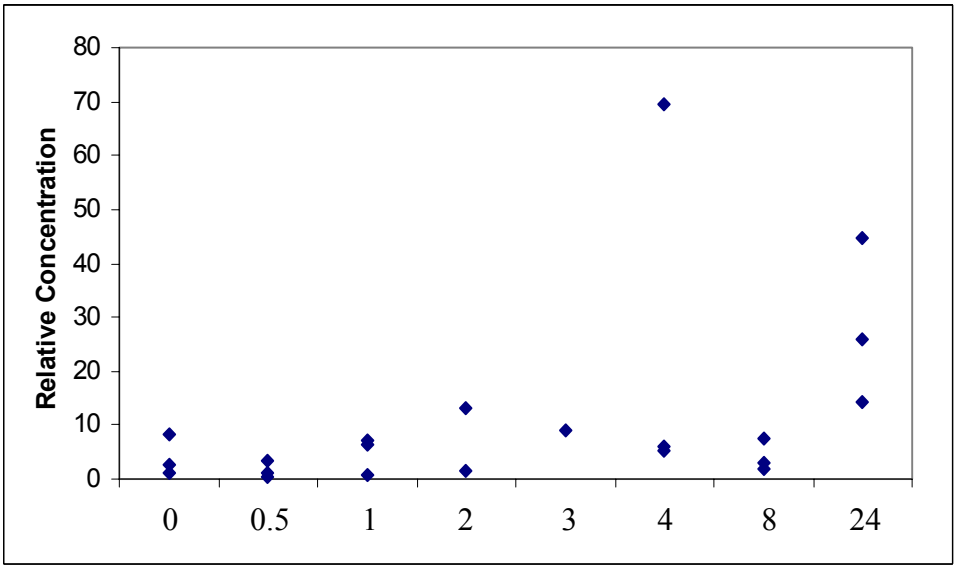


Figure 3. Quantitative, real-time PCR results for mid-dorsal (a) and arthrodial (b) hypodermis. Premolt samples were excluded to emphasize the increase transcript number in the postmolt times sampled. Relative concentration was adjusted by multiplying all samples by 100. Samples are labeled with the hour after ecdysis.

Northern Blots

Northern blot analysis was performed to verify Q-PCR data (Fig 4). A transcript of 1.5 kilobases was detected in all RNA sampled. D stage hypodermis from both calcified and arthrodial cuticle exhibited a high signal intensity, which decreased abruptly at ecdysis. Signal intensity remained relatively low in mid-dorsal tissue through four hours and an increase was seen at 24 hours post-molt. Arthrodial, but not mid-dorsal, hypodermis showed a subsequent increase in intensity in three-hour tissue with heightened signal in four and 24 hours post-molt.

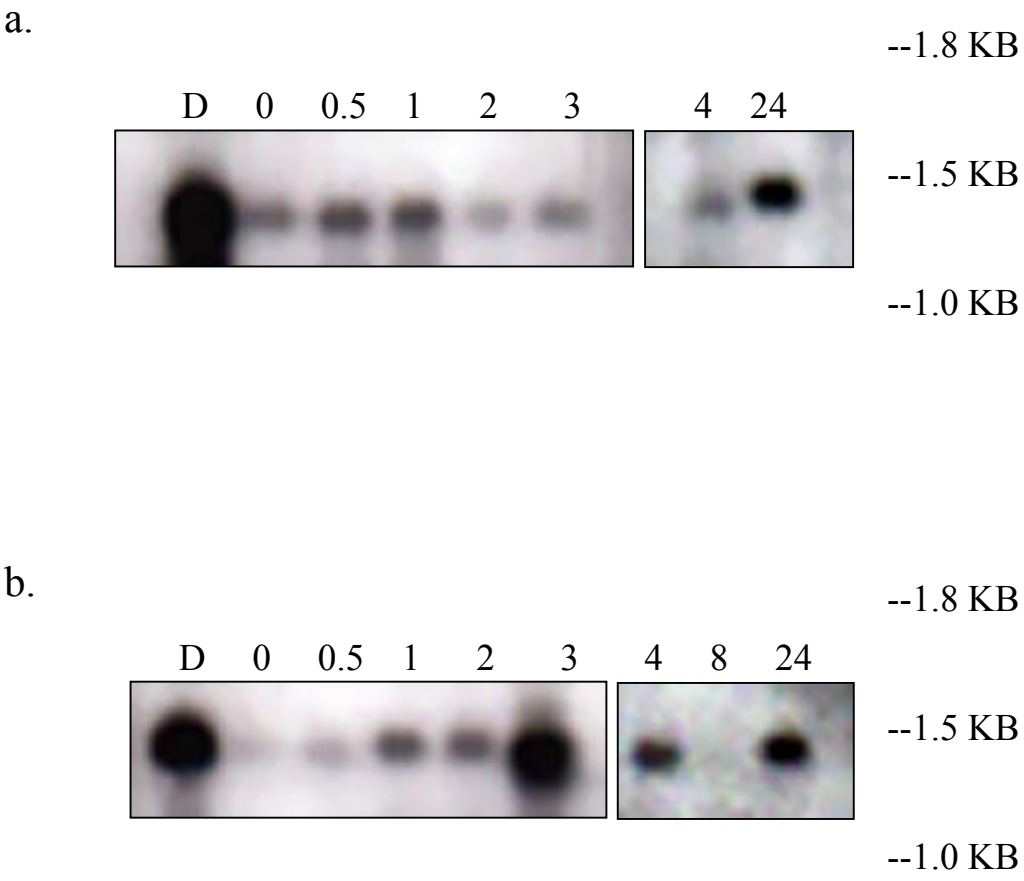


Figure 4. Northern Blot analysis of (a) mid-dorsal hypodermis and (b) arthrodial hypodermis RNA. RNA (1µg) was separated on a 1% denaturing agarose gel, transferred to Immobilon-NY+, and hybridized with a DIG-labeled, *C. sapidus* PPAF probe. Time course is indicated at the top of each figure. D signifies premolt, and subsequent numbers represent hours after ecdysis. Positions of RNA molecular weight marker are indicated to the right.

DISCUSSION

Temporal regulation of cuticle tanning and calcification is under strict control in *C. sapidus*. After ecdysis the crab is supported by a hydrostatic skeleton. In a matter of hours the cuticle is tanned and calcification begins. The serine protease cascade plays a crucial role in the timing of tanning in both calcified and uncalcified tissue. Specifically, prophenoloxidase activating factor (PPAF) facilitates in the activation of phenoloxidase (PO). The activation and subsequent enzymatic activity of PO allows for sclerotization to take place in the cuticle. However, PPAF is not believed to be the controlling enzyme in the tanning process. Roer and Dillaman (1993) hypothesized the control of tanning to occur at two possible places, either the introduction of PO into the cuticle or in the introduction of phenolic compounds. The expression pattern of PPAF indicates that the protein is involved in the process of tanning in pre-exuvial cuticle and possibly post-exuvial, arthrodial cuticle (Figs 2, 3, 4).

In *C. sapidus*, the PPAF gene codes for a protein of 370 amino acids containing a serine protease conserved domain at the carboxy-terminal end (Fig. 1). The PPAF probe used for Northern blotting detected a transcript of 1.5 kilobases, which is approximately 200 nucleotides larger than the full-length sequence as determined by 3'- and 5' RACE (Fig. 1). The discrepancy in size may be due to incomplete sequence data in the UTR regions, possibly the Poly(A) tail. The presence of an initiation methionine, a signal sequence, and polyadenylation site implies that the deduced amino acid sequence is full-length.

An amino acid alignment of *C. sapidus* PPAF and homologous insect BLAST hits was created using VectorNTI software (Fig. 5). The catalytic serine protease domain contains three residues, His154, Asp203, and Ser307, which exhibit complete identity with the active triad sites described by previous studies (Lee et al. 1998, Jiang et al. 1998, Jiang et al. 2003, Kwon et al. 2000, Satoh et al. 1999, Wang et al. 2001). This triad forms the active site of the PPAF protein. Proteolytic activity of *C. sapidus* PPAF can be implied due to the complete conservation of these residues. Homologous PPAF sequences include a single cleavage site between a lysine and isoleucine residue. The *C. sapidus* sequence contains a Lys-Ile grouping at amino acids 124-125 that corresponds to cleavage sites in the *H. diomphalia* and *B. mori* sequences.

The amino-terminal portion of the protein contains six conserved cysteine residues (Fig. 6). The positions of these residues are consistent with the clip domain described by Muta et al. (1993). The cysteine residues of clip domains are hypothesized to form three disulfide bridges that fold this part of the protein in a characteristic way (Jiang and Kanost, 2000). A seventh cysteine forms a disulfide bridge that links the clip-domain and the serine protease domain together after proteolytic activation of the proenzyme. In the *C. sapidus* PPAF sequence, the pairing of cysteine residues is hypothesized to be Cys32—Cys73, Cys39—Cys66, and Cys45—Cys74. The cysteine, at position 92, is speculated to form an interdomain link with a cysteine inside the serine protease conserved domain. The function of the clip-domain in unknown but may be a recognition site for an activating protease (Muta et al., 1990).

Quantitative PCR and Northern blots were performed to determine the expression pattern of PPAF in blue crab cuticle hypodermis. Expression data indicates that PPAF is

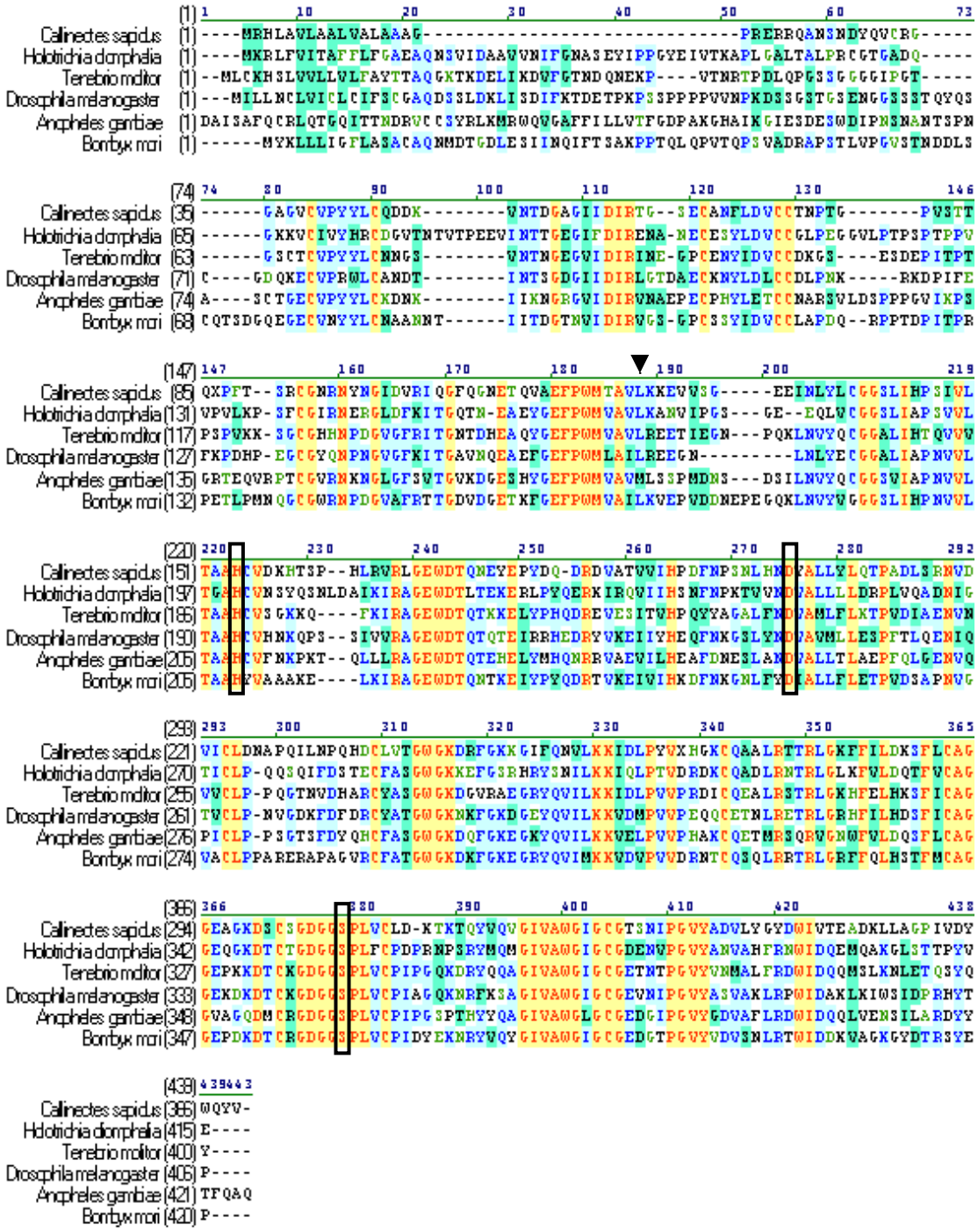


Figure 5. Multiple amino acid alignment of PPAF from *Callinectes sapidus* (Accession number AY555734), *Holotrichia diomphalia* (CAC12665), *Tenebrio molitor* (CAC12696), *Drosophila melanogaster* (AAF52904), *Anopheles gambiae* (EAA00427), and *Bombyx mori* (AAN77090). Yellow denotes identity for all species. Blue denotes identity in most species. Green indicates amino acid similarity. The active triad of the serine protease domain, as identified by the above accessions, is boxed. An arrowhead denotes a putative cleavage site.

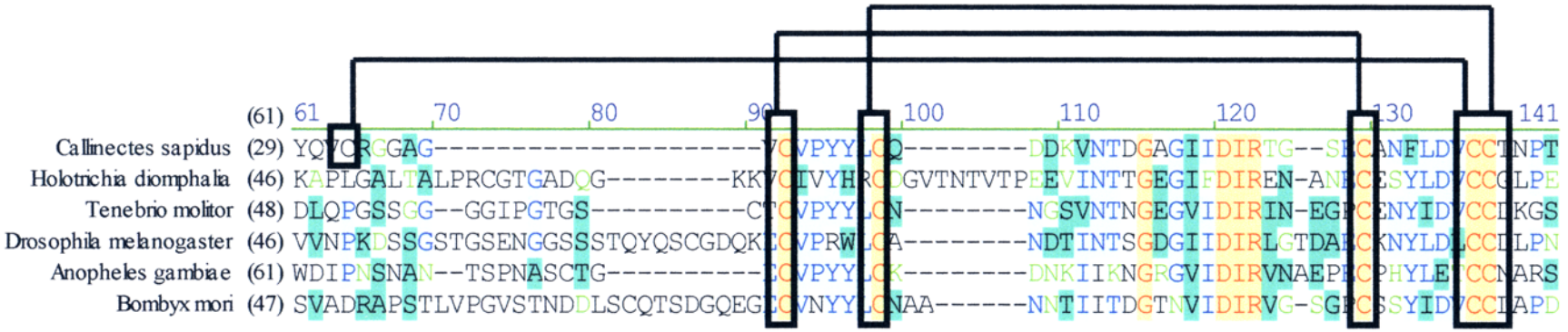


Figure 6. Alignment of sequences with a clip-like domain. Amino acids are numbered from the initiation methionine. Yellow denotes identity for all species. Blue denotes identity in most species and green indicates amino acid similarity. Conserved cysteine residues are boxed. Lines indicate cystine residues that form disulfide bridges.

most highly expressed in pre-molt (D stage) RNA. I hypothesize that the PPAF protein is being translated in the hypodermis and subsequently transported into the non-cellular cuticle. The presence of a signal sequence in the deduced peptide sequence supports this supposition. Because PPAF is not thought to be the controlling factor in the tanning process, the protein can be introduced into the cuticle without activating the cascade. Pre-molt expression is observed before tanning is thought to occur in the epi- and exocuticle. This suggests that PPAF is being transcribed before the protein is needed to participate in the cascade and is subsequently available for involvement in the tanning process.

The relative expression of PPAF decreases noticeably after pre-molt in hypodermis from calcified cuticle. The initial peak of mRNA production supports the supposition that PPAF plays a crucial role in the tanning process and afterward transcription is down regulated as calcification occurs. Down regulation is indicated by the dramatic decrease in signal intensity after pre-molt. This evidence leads to the conclusion that the onset of tanning occurs soon after ecdysis and as a precursor to calcification in the epi- and exo-cuticle. Endocuticle deposition, and simultaneous calcification, begins approximately three to eight hours post-molt. Travis (1955) established that the endocuticle of the spiny lobster, *Panulirus argus* is not tanned, but only calcified. The low signal intensity observed in *C. sapidus* post-molt hypodermis supports this conclusion. If the endocuticle of calcified tissue were tanned, an increase in PPAF transcription might be expected before this layer is deposited. In most crustaceans, the partial reabsorption of the endocuticle before ecdysis provides a source of calcium for subsequent mineralization of the new cuticle. Tanned cuticle, such as the epi- and exo-

cuticle is not available for reabsorption because of the covalent bonds between cuticle proteins. Our data suggests that the calcified endocuticle is not tanned, supporting the reabsorption of this layer from calcified tissue.

Arthrodial membrane shows a high PPAF signal level in D-stage hypodermis, comparable to that seen in calcified tissue. This suggests that both calcified and arthrodial cuticle undergo tanning of the epi- and exocuticle. During ecdysis and up to one hour post-molt, the arthrodial signal intensity is decreased and remains low, signaling a lapse in PPAF transcription. The signal begins to increase in the latter post-molt stages. There is a considerable increase in intensity at three to four hours, which is maintained through 24 hours post-molt. Eight-hour arthrodial hypodermis showed low expression levels in both the Northern blots and quantitative PCR. However, the data at these time periods show some variation that can be attributed to individual crab deviation. There is a trend of increasing signal intensity in the post-molt arthrodial hypodermis at three to four hours that is not observed in mid-dorsal. Williams et al. (2003) investigated the timing of *C. sapidus* cuticle deposition before hardening. They found that arthrodial membrane and calcified tissue are deposited concurrently. Unlike other types of uncalcified cuticle, the majority of arthrodial membrane is laid down in the post-molt stages. The increase of PPAF transcript at three to four hours post-molt may suggest that arthrodial endocuticle, laid down post-ecdysially, is being tanned. The absence of any mineralization in the arthrodial membrane could necessitate the need for additional hardening that could be accomplished by tanning of the endocuticle. However, because of the variation between individual samples and the increase in transcript expression in mid-dorsal hypodermis at 24 hours, this conclusion is only supposition. The reabsorption

of the endocuticle found in calcified cuticle may not be a factor in arthrodial membrane as these sections of the cuticle are relatively small, and contain no calcium.

Twenty-four hour hypodermis shows an increase transcription level in both mid-dorsal and arthrodial hypodermis. I have hypothesized that PPAF is being expressed, and the protein transported to the cuticle to await activation. However, an increase of expression is not expected at this time period. The expression of PPAF at 24 hours when the protein is not needed for tanning suggests that the protease is available to react with proPO even after tanning has occurred in the cuticle.

In insects, the serine protease cascade functions not only in sclerotization, but also in wound healing and immune response (Aspan et al. 1995, Chosa et al. 1996, Jiang et al. 1998, Kwon et al. 2000, Lee et al. 1998, Satoh et al. 1999). Foreign microorganisms are sequestered by the production of melanin. PO catalyzes the reaction of diphenols to o-quinines, which then produce melanin and encapsulate invading pathogens. Sclerotization is also necessary when an insect receives a wound that punctures the exoskeleton. The activation of PO by an activating factor facilitates the sealing of the cuticle to prevent infection. Expression at 24 hours post-molt may indicate the stockpiling of PPAF in the cuticle. Because PPAF is not hypothesized to be a controlling factor in the cascade, it may be amassed in the cuticle in the event an immune response is necessary. In light of the activity of the serine protease cascade in the immune response of insects, it can be hypothesized that PPAF may function in a similar way in crustacean cuticle. More work is necessary to determine PPAF's role, if any, in the immune response of *C. sapidus*.

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