DISSERTATION

REGULATION OF MUSCLE ATROPHY AND LIMB REGENERATION DURING MOLTING IN CRUSTACEANS: CHARACTERIZATION OF A MUSCLE-SPECIFIC CALPAIN AND A LIMB AUTOTOMY FACTOR

Submitted by

Xiaoli Yu

Graduate Degree Program

in Cell and Molecular Biology

In partial fulfillment of the requirements

for the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Spring 2002

COLORADO STATE UNIVERSITY

March 21, 2002

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISON BY XIAOLI YU ENTITLED REGULATION OF MUSCLE ATROPHY AND LIMB REGENERATION DURING MOLTING IN CRUSTACEANS: CHARACTERIZATION OF A MUSCLE-SPECIFIC CALPAIN AND A LIMB AUTOTOMY FACTOR BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work

Donald L. Mykles

Adviser

Department Head

ABSTRACT OF DISSERTATION

REGULATION OF MUSCLE ATROPHY AND LIMB REGENERATION DURING MOLTING IN CRUSTACEANS: CHARACTERIZATION OF A MUSCLE-SPECIFIC CALPAIN AND A LIMB AUTOTOMY FACTOR

Molting in crustaceans requires the precise coordination of virtually all-major organ systems. During molting, regeneration of lost appendages must be coordinated with various other physiological processes to ensure successful exuviation. If a limb regenerate (limb bud) is removed before a critical period during premolt, growth of any remaining primary (1°) limb buds (LBs) stops to allow regeneration of a secondary (2°) LBs. I show that 2° LBs contain a factor, termed limb autotomy factor-proecdysis (LAFpro), that blocks molting when injected into premolt animals. Secondary LB extracts inhibited the growth rate of 1° LBs about 68% during the first week of injection, while 1° LB extract had no effect. LAFpro was stable when boiled for 15 min in deionized water, but was inactivated when boiled in 0.1 M acetic acid and with the incubation of Proteinase K. Limb bud autotomy reduced the ecdysteroid levels in the hemolymph of premolt animals. These data suggest that LAFpro is a molt-inhibiting hormone-like polypeptide that suppresses ecdysteroid synthesis and secretion by the Y-organs.

Crustacean muscles contain four calcium-dependent cysteine proteinases (CDPs or calpains) which are involved in the degradation of myofibrillar proteins during molting. Using nested PCR and inverse PCR, a full-length (1977 bp) lobster calpain gene (Ha-CalpM) was cloned. The deduced amino acid sequence of the polypeptide (575 aa, 66.3 kDa) has high sequence identity to other calpains. Ha-CalpM contains three domains with domain IV absent. Gene expression analysis revealed that Ha-CalpM is highly expressed in skeletal muscle, with little or no expression in other tissues. Real-time PCR showed that it is

up regulated during the premolt stage in claw muscles undergoing atrophy. A polyclonal antibody raised against a unique amino acid sequence in domain I was used to determine the relative amounts and the location of Ha-CalpM protein expressed in lobster tissues. It detected 62-kDa and 68-kDa proteins in western blots. Immunocytochmistry indicated that Ha-CalpM is localized in both the cytoplasm and nuclei of muscle fibers.

Xiaoli Yu
Graduate Degree Program in Cell and Molecular Biology
Colorado State University
Fort Collins, CO 80523
Spring 2002

ACKNOWLEDGMENTS

I thank Dr. Donald Mykles for your mentoring, patience, support and understanding in directing me through the journey of my graduate study. Your constant encouragement and tireless guidance have made my graduate study a pleasant experience. I am grateful for the friendship we've developed. Thanks to Drs. A.S.N. Reddy, Randal Voss and Tomas Wilson for being my committee members, and Dr. Bill Wright for serving part of the committee. Thank you for your time, advice and efforts.

When I started my graduate work, I was along in the lab, but I did not feel helpless because I've got tremendous help from the friends in Dr. Reddy's lab. Especially, I would like to thank Dr. Maxium Golovkin for your countless help. You have put so many hours in teaching me the basics and in that I think you deserve an honor committee member position. Friends and families have helped me through the difficult times. Thanks to all of you for your love and faith in me. Last, I like to express my gratitude and appreciation to my husband Alan Polonsky, with love and unconditional support you've made this possible.

TABLE OF CONTENTS

Chapter One: Introduction

I. Molting

Like other arthropods, crustaceans must periodically shed their exoskeleton through the process of molting (ecdysis) for continued organismal growth. Molting is hormonally controlled by steroid hormone ecdysteroids, the molting hormones of all arthropods (Hoffmann and Porchet, 1984), and the molt-inhibiting hormone (MIH). It is now generally accepted that molting processes are initiated and sustained by increased ecdysteroid synthesis by the Y-organs, resulting in increased hemolymph ecdysteroid (20-hydroxyecdysone, which is a metabolite of ecdysone) titers. This process is thought to be suppressed during intermolt by MIH produced by a neurosecretory center in the eyestalk. These centers are composed of the X-organ sinus gland complex. When they reduce their output of MIH, the Y-organs are no longer repressed, molting hormone is secreted in great abundance, and a whole series of interrelated molting events are initiated, coordinated, and sustained (Skinner, 1985; Fingerman, 1987; Watson *et al.*, 1989; Lachaise *et al.*, 1993)

Y-organs, the source of molting hormone, are paired ectodermal derivatives that lie just beneath the epidermis within the cephalothorax. Y-organs were cited as a homolog of the insect prothoracic glands (Karlson and Skinner, 1960). Most data support the role of Y-organs as a primary source of ecdysteroids, under the negative control of MIH, but the occurrence of secondary sources seems highly likely (Skinner, 1985).

The effects of steroids involve long-term change in the cell and they act intracellularly. By virtue of their hydrophobic nature, they traverse the plasma membrane and exert their effects within the cell (actually within the nucleus) where they control the activities of specific genes (Tsai *et al.*, 1994). Ecdysteroid is no exception. The regulatory effects of ecdysteroid occur at the level of transcription of steroid-responsive genes. Ecdysteroids act by binding in the cytosol to specific receptor-ecdysone receptor (EcR)

proteins, which dimerize under the influence of the hormone. Binding in the cytosol is followed by movement of the hormone-receptor complex into the nucleus, where the complex interacts with specific DNA sites-hormone responsive elements (HREs) (Leid *et al.*, 1992; Schmitt and Stunnenberg, 1993).

Ecdysteroid action is mediated mainly by the intracellular ecdysteroid receptor, a member of the nuclear receptor family (Klaus-Dieter *et al.*, 2001). The ecdysteroid receptor is composed of several functional domains that are responsible for transactivation, DNA binding, ligand binding, dimerization with other nuclear receptors, and interaction with additional proteins (e.g. comodulators). Receptor function is modulated by intramolecular interactions between receptor domains and intermolecular allosteric effects of various dimerization partners. Tissue-, developmental stage-, and gene-specific diversification of the hormonal signal is necessary to coordinate the activity of more than 100 ecdysteroid dependent genes. The ecdysone receptor gene has been identified in the fiddler crab *Uca pugilator* regenerating limbs (Durica and Hopkins, 1996). The ecdysone receptor in *U. pugilator* has similar nucleic acid and deduced amino acid sequences to those of ecdysone receptors in insects, and it is a member of the large family of nuclear receptors (Chung *et al.*, 1998a).

The regulation of hormone biosynthesis by steroidogenic cells is exerted at three different levels: (1) the availability of cholesterol (the ecdysteroid precursor) and its access to enzymes through short-lived carrier proteins; (2) the activity of steroidogenic enzymes; and (3) the availability of enzyme cofactor, mainly NADPH for cytochrome P450 monooxygenases (Baghdassarian *et al.*, 1996). As mentioned earlier, the main control mechanism of crustacean molting glands is through MIH. Occupancy by MIH of its receptor in the Y-organ cell membrane activates a signaling system that directs inhibition of cellular uptake of cholesterol, protein synthesis and the expression of steroidogenic enzymes (Webster, 1993). Steroidogenic cells display the following three general features:

(1) protein synthesis is increased in active glands (Mattson, 1986; Dauphin-Villemant *et al.*, 1995; Lachaise *et al.*, 1996) (2) ecdysteroid biosynthesis requires the continuous synthesis of short-lived proteins, as it is strongly reduced by proteins synthesis inhibitor (Mattson and Spaziani, 1986; Dauphin-Villemant *et al.*, 1995), and (3) cyclic nucleotides are involved in the transduction mechanism of MIH (Mattson and Spaziani, 1985). In the review on signaling pathway for ecdysteroidogenesis in crustacean Y-organ, Spanziani *et al.* (2001) summarized that ecdysteroidogenesis in crustacean is cAMP (or cGMP)-driven and does not involve phospholipase (PLC) nor inositol triphosphate (IP3). The cellular level of cyclic nucleotide is inversely related to the degree of steroid output. The sole established hormonal controller of the Y-organ MIH regulates negatively by raising cyclic nucleotide concentrations (Spaziani *et al.*, 2001).

The existence of a molt-inhibiting hormone was established by the observations that eyestalk ablation results in a significant acceleration of the molting cycle and that injection of extracts of the eyestalk neurosecretory X-organ-sinus gland complex delays the molting of eyestalk-ablated animals (Kleinholz, 1975; Skinner, 1985). When it was demonstrated that incubation of Y-organs *in vitro* leads to the biosynthesis of the pro-molting hormone ecdysone (Willig and Keller, 1973) (Chang and O'connor, 1977), a plausible rationalization was that MIH acts by inhibiting this ecdysteroidogenesis in the Y-organ. A decrease in the biosynthesis of ecdysone by the Y-organ would reduce the blood concentration of the active hormone, 20-hydroxyecdysone. It has been proposed to define MIH as a factor that can delay the onset of ecdysis and lengthen the interval between successive molts. However, MIH could also act by increasing the rates of metabolism or excretion of ecdysteroids or by antagonizing 20-hydroxyecdysone at the epidermis (Naya *et al.*, 1989).

Attempts to isolate MIH have involved the use of either *in vivo* or *in vitro* bioassays. *In vivo* assays were employed in the isolation of an 8.7-kDa peptide from the sinus glands of *Homarus americanus* (Chang *et al.*, 1987). It has been suggested that an initial small

release of ecdysteroid triggers the transition from intermolt to premolt gene expression in the crayfish *Astacus leptodactylus* (Traub *et al.*, 1987), thus initiating the proecdysial period. The first release is then followed by a second surge to higher ecdysteroid concentrations. Furthermore, precocious molting observed in Y-organless animals after multiple limb autotomy suggested that a mechanism may exist for the storage of ecdysteroids outside the Y-organ (Connat *et al.*, 1986). MIH in *Carcinu maenas* is mostly found in the sinus glands, and it is a heat stable, trypsin sensitive peptide with a molecular weight of greater than 6,000 (Webster, 1986). Webster and Keller (1986) have shown that in addition to MIH, CHH (crustacean hyperglycemic hormone) is also active in repressing ecdysteroid synthesis *in vitro*. MIH was shown to have no immunochemical cross-reactivity to CHH and was not hyperglycemic, suggesting major structural differences. This may preclude the possibility that the activity of CHH in repressing ecdysteroid synthesis is due to receptor cross-reactivity.

Recent molecular cloning has made great progress in understanding the structure of the MIH genes and peptides. Klein et al. (1993) cloned a cDNA encoding MIH of the shore crab, *Carcinus maenas*. MIH cDNA from the blue crab, *Callinectes sapidus* and the Dungeness crab, *Cancer magister* were also isolated (Lee *et al.*, 1995; Umphrey *et al.*, 1998; 2000) reported that the *Cancer pagurus* genome contains at least two copies of the MIH gene. For the MIH genes cloned, they all share high sequence homology. Brachyuran MIH genes encode a 113-amino acid prohormone composed of a 35-residue signal peptide and 78-residue mature MIH (Watson *et al.*, 2001). The MIH genes of *Cancer feriatus* and *C. pagurus* consist of three exons and two introns, and span approximately 4.3 kb on the genome. The 5' flanking regions showed several potential binding sites for known transcription factors such as cAMP response element binding protein, putative CF1/USP, and Broad Complex Z2 transcription factor elements (Chan *et al.*, 1998) (Lu *et al.*, 2000).

The MIH transcript is detectable in eyestalk neural ganglia throughout the molt cycle of the blue crab, *Callinectes sapidus*. Stage-specific changes in the abundance of MIH mRNA in *C. sapidus* eyestalks are generally consistent with the hypothesis that MIH negatively regulates ecdysteroid production during the molt cycle. Recombinant MIH (rMIH) was produced using prokaryotic and eukaryotic expression systems. Recombinant MIH produced in *E. coli* was of the predicted size, MIH immunoreactive, and it did not have MIH bioactivity. Polyclonal antiserra raised against the prokaryotic-expressed rMIH bound specifically to neurosecretory cells in the X-organ, their associated axons, and axon terminals in the sinus gland. Recombinant MIH expressed using the baculovirus system was of the predicted size, was MIH immunoractive, and inhibited ecdysteroid production by Y-organs *in vitro* (review in Watson *et al.*, 2001).

Molting in crustaceans requires the precise coordination of virtually all-major organ systems. Molt includes the degradation of the old exoskeleton and synthesis of several layers of a new exoskeleton, formation of gastroliths in some species, atrophy of somatic muscle in the chelae that is replaced following ecdysis, and regeneration of missing pereopods (Skinners, 1985). The crustacean molt cycle is divided into five major stages (stage A to E) by Drach (1939). Holland and Skiner (1976) have described the temporal relationship between the molt cycle and limb regeneration in the land crab, *Gecarcinus lateralis*. Stage A immediately follows ecdysis (E), and it is very brief (1 to 2 days). In stage B which lasts 2 to 5 days, synthesis of endocuticle layers begins and continues into Stage C, during which the exoskeleton hardens further by calcification. The completion of the membranous layer, the innermost layer of the exoskeleton, concludes Stage C_3; Stage C_4, or intermolt period, is the longest part of the cycle, 10 to 11 months in duration, while the premolt period, Stage D, lasts 1 to 2 months. Regeneration of missing limbs occurs only during the premolt period (Bliss, 1956; Skinner, 1962). Skinner (1962) has divided the molting cycle into five stages on a cellular level by showing the remarkable sequential structural changes in the epidermis of the crab. Briefly, the structure of the epidermis is

indistinguishable in anecdysis (stage C, intermolt) and in early proecdysis. The first sign of proecdysis (stage D, premolt) in integumentary tissues is apolysis, the separation of epidermis from the old exoskeleton. Apolysis marks the initiation of stage D_1. Epidermal cells enlarge, and in stage D_2, secrete a new epicuticle and exocuticle. Degradation of the old exoskeleton continues up to ecdysis (stage E, molt). Epidermal cells start to shrink just prior to ecdysis and continue to decrease until they reach a size equivalent to that of anecysisal animals. The stage following ecdysis has been termed metecdysis (stage A, B, postmolt) (Carlisle and Dohrn, 1953).

II. Molting and Regeneration

Decapod crustaceans readily autotomize their walking legs and claws in response to injury or predation; lost appendages are regenerated before the next molt (Skinner, 1985; Hopkins, 1993). Thus, limb regeneration and molting are obligatorily linked. Regeneration is a specific type of growth in many crustaceans. Regardless of where along the length of the limb an injury occurs, the autotomy response insures that the limb is cast off at a predetermined point at the base of the leg between the coxa and the basiischium. Loss at the predetermined point insures the most efficient and fastest regeneration (Hopkins, 2001). Limb regeneration is divided into two distinct phases: (a) basal growth or regeneration, which can occur at any time during the intermolt cycle, involves primarily cell division and differentiation and results in the formation of basal papillae; (b) proecdysial growth, which is restricted to the proecdysial period (mostly D_0), involves primarily protein synthesis and water uptake and is under hormonal control (Hopkins, 1993). In the fiddler crab, exposure to 20-HE is essential for the proecdysial growth phase (Hopkins, 1989). Regenerating limbs provide an external (and therefore noninvasive) measure, defined as the regeneration index (R index = length of limb regenerate x 100/carapace width (Bliss *et al.*, 1966), of the progress of proecdysial events; in the land crab, the R index increases from 0 to a maximum of 22-24 just before ecdysis (Holland and Skinner, 1976).

The interdependence of regeneration and molting in arthropods has long been known. Arthropods that continue to molt and grow as adults (primitive insects and crustaceans), continue to regenerate as adults. Needham (1965) noted that regeneration was dependent upon the presence of an active molt hormone-secreting organ, and Bliss (1956) reported that the regenerating limb bud was an excellent criterion for the crustacean molt cycle stages. Multiple regeneration (the loss of more than one limb) interrupts a normal molting cycle in the crabs *Gecarcinus lateralis* and *Uca pugilator* and the shrimp *Palaemonetes kadiakensis* (Skinner and Graham, 1972) (Fingerman and Fingerman, 1974; Stoffel and Hubschman, 1974; Hopkins, 1989). The injection or perfusion of high concentrations of exogenous ecdysteroids into the land crab during this time inhibited the basal growth (Hopkins *et al.*, 1979). Thus, while ecdysteroids may be required for basal growth, apparently the circulating levels of ecdysteroids must remain low in order for basal growth to occur (Hopkins, 2001). The transition from anecdysial (C_4) to proecdysial (D_0) growth requires a transitory pulse of ecdysteroids. Circulating levels of ecdysteroids, however, return to anecdysial levels, because early proecdysial growth of the regenerating limb also requires low levels of circulating ecdysteroids. A second transitory pulse coincides with the end of proecdysial regeneration and the beginning of terminal plateau. During terminal plateau there are a series of large pulses of circulating ecdysteroids which appear to be necessary for successful ecdysis of the entire exoskeleton (Hopkins, 2001).

Skinner and Graham (1970, 1972) have shown that the loss of more than four appendages is an adequate stimulus to molting preparations in intermolt specimens of *Gecarcinus*. Holland and Skinner (1976) reported that loss of one or more limbs or partially regenerated limbs (primary regenerates or primary limb buds, 1° LBs) from proecdysial animals before a critical period (probable stage D_0) suspends proecdysis until new secondary regenerates (secondary limb buds, 2° LBs) appear and grow to approximately the same size as those primary regenerates that remain. The magnitude of the inhibitory effect depends on the stage of the animal in its molting preparations. Prior to a

certain critical time (late D_0 or early D_1), the growth of the remaining primary regenerates is slowed (at R index = 7-10) or stopped (at R index = 10-17) while the secondary regenerates "catch up". After the critical time (late D_1 to ecdysis, R index >17) autotomy of partial regenerate does not influence the remaining regenerates or the duration of the molting period.

The suspension of proecdysis while regeneration of secondary regenerates was initiated resulted in a 2-5 week increase in the duration of proecdysis. The inhibition of proecdysis by the suspension of growth of limb buds occurs in animals with or without eyestalks. Limb autotomy factor, proecdysial (LAFpro) was proposed which inhibits the growth of primary limb buds. Growth inhibition by LAFpro was also demonstrated by following DNA synthesis in autotomized limb buds incubated *in vitro* with ^{3}[H]thymidine (Holland and Skinner, 1976). The interaction between the growth of primary regenerates and secondary regenerates is diagrammed in Fig. 1-1.

III. Claw Muscle Atrophy

As much as 60% of the claw muscle atrophies during premolt stage. Atrophy facilitates the withdrawal at ecdysis of the very large mass of muscle contained in the propodus through the narrow aperture of the old exoskeleton at the basi-ischial joint (Mykles and Skinner, 1990). In the weeks prior to ecdysis, the chela closer muscle undergoes a 30-60% reduction in mass (Skinner, 1966). This results from the loss of protein fibers and not from water. The ratio of protein to DNA decreases ~32% during proecdysis. The decrease in this ratio results from a uniform reduction of protein from the entire population of fibers of the chela muscle rather than a selective elimination of fibers. This atrophy is highly specific, it occurs in the chelae but not in the walking legs of land crabs (Mykles and Skinner, 1982). Mykles and Skinner (1981) have shown that atrophy is associated with a decrease in myofibrillar cross-sectional area. Thus, there is a loss of myofilaments so that the numbers of myofibrils and muscle fibers remain relatively constant

throughout the molting process (Mykles, 1999). During atrophy there is a decrease in the thin-to-thick myofilament ratio from approximately 9:1 to 6:1, resulting in a dramatic increase in myofilament packing density (Mykles and Skinner, 1981). After ecdysis, protein is re-synthesized and the muscle is restored and grows larger to fill the enlarged space of the new exoskeleton (Skinner, 1966).

Claw muscle atrophy requires accelerated protein degradation while retaining the structure of the contractile apparatus. There is no apparent loss of fibers since the reduction in fiber diameter is proportional to the reduction in myofibrillar cross-section areas (Mykles and Skinner, 1981; Ismail and Mykles, 1992). In claw muscles undergoing atrophy, protein synthesis actually increases, necessitating an even larger increase in protein degradation to effect a reduction in mass (Skinner, 1965; El Haj and Houlihan, 1987).

Calcium-dependent proteinases (CDPs or calpains) constitute a large and diverse group of cytosolic cysteine proteinases that require Ca^{2+} for optimal activity (Mellgren and Murachi, 1990; Mykles, 1998; Hatzizisis *et al.*, 2000). These enzymes are inhibited by sulfhydral reagents, Ca^{2-} chelators, peptide aldehydes, and epoxysuccinyl depeptides (Mykles, 1998). Four CDPs (CDP I, IIa, IIb, and III) have been identified biochemically in crustacean muscles (Mykles and Skinner, 1986a). Native masses vary from 310 kDa for CDP I to 59 kDa for CDP III. Lobster muscle CDP IIa and CDP IIb appear to be homodimers of 60-kDa and 95 kDa subunits, respectively (Mykles and Skinner, 1986a; Beyette *et al.*, 1993; Beyette *et al.*, 1997; Beyette and Mykles, 1997). Given its small size (59 kDa), CDP III is likely to be a single polypeptide (Mykles, 1999). All four CDPs require millimolar Ca^{2+} for maximal substrate degradation, but display different preferences for myofibrillar protein substrates (Mattson and Mykles, 1993). Lobster CDP IIb undergoes a Ca^{2+}-dependent autolysis resulting in sequential cleavage of the N-terminus of the 95 kDa polypeptide (Beyette *et al.*, 1993; Beyette and Mykles, 1997). The four CDPs play a role in the breakdown of myofibrillar proteins in crustacean muscle. They degrade all

the myofibrillar proteins to acid-soluble products (Mykles and Skinner, 1983, 1986a; Mattson and Mykles, 1993). In the land crab, CDP activity is more than two-fold greater in chela muscles from late proecdysial animals than in muscles from anecdysial animals (Mykles and Skinner, 1982). Organ culture experiments confirm the importance of Ca^{2+}-dependent proteolysis in claw closer muscle. Raising intracellular Ca^{2+} stimulates myofibrillar protein breakdown and preferentially hydrolyzes the Z line, which is inhibited by cysteine protease inhibitors EST, Ep-475, or leupeptin (Mykles, 1990). The preferential degradation of the Z line would facilitate removal of myofilaments from myofibrils, which is required for subsequent proteolysis by cytosolic enzymes (Mykles and Skinner, 1983).

Immunological analysis has revealed the relationships between arthropod and mammalian CDPs (Beyette *et al.*, 1997), suggesting that the lobster muscle CDP IIb is the crustacean homolog of a CDP (Dm-calpain or CalpA) cloned from *Drosophila* (Emori and Saigo, 1994). Polyclonal antibodies raised against CDP IIb and Dm-calpain cross-react. These same antibodies do not react with lobster CDP IIa. An antibody raised against a highly conserved peptide sequence from the active region of mammalian calpains (Grynspan *et al.*, 1997) reacts strongly with CDP IIa, but not with CDP IIb (Beyette *et al.*, 1997). These data suggest that CDPs IIa and IIb are products of distinct calpain-like genes. Antibodies to mammalian μ- and m-calpain bind to several proteins in lobster muscle extracts (Kuo *et al.*, 1995; Kuo *et al.*, 1996), but their relationship to the lobster CDPs' is uncertain, since anti-mammalian calpain antibodies do not react with purified CDP IIa or IIb (Beyette *et al.*, 1997).

IV. Calpains

Calpain (EC 3.4.22.17) is a Ca^{2+}-activated intracellular non-lysosomal cysteine proteinase distributed widely in tissues of various organisms. They are active at neutral pHs, and their activity is regulated by calcium concentration and by specific inhibitors and

activators (Johnson and Hammer, 1990; Croall and Demartino, 1991; Goll *et al.*, 1992; Suzuki *et al.*, 1992b; Sorimachi *et al.*, 1994; Jekely and Friedrich, 1999a). Members of the calpain superfamily are important biomodulators in physiological as well as pathological cell functions. Through the limited proteolysis of its key cellular components, calpain has been implicated in a wide variety of cellular processes such as cell division (Yamaguchi *et al.*, 1994) and differentiation (Patel and Lane, 1999), signal transduction (Sato and Kawashima, 2001), apoptosis (Debiasi *et al.*, 1999), muscle homeostasis (Richard *et al.*, 1995), and muscle degradation (Huang and Forsberg, 1998). Several pathological conditions have also been linked to calpains including myelin protein degradation in autoimmune demyelinating disease, cataract formation and neuronal degeneration in ischemia and Parkinson's and Alzhemer's diseases (Saito *et al.*, 1993; Azuma *et al.*, 1995; Bartus *et al.*, 1995b; Tsuji *et al.*, 1998; Shields and Banik, 1999). Skeletal muscle specific calpain p94 (CAPN3) is responsible for limb girdle muscular dystrophy types 2A (Richard *et al.*, 1995). Calpain 10 is linked to type-2 diabetes mellitus (Horikawa *et al.*, 2000). The CAPN4 gene knockout mouse, which lacks the common small subunit for μ- and *m*-calpains, results in embryonic lethality (Arthur *et al.*, 2000). Therefore, indispensable cellular functions are clearly indicated for the calpain superfamily members.

Calpains are regulated by a variety of factors, including a 30-kDa regulatory subunit (Sorimachi *et al.*, 1994), calcium and phopholipids (Suzuki *et al.*, 1992a; Saido *et al.*, 1994; Sorimachi *et al.*, 1994), and calpastatin, a widely distributed calpain-specific inhibitor (Takano *et al.*, 1995). Calpastatin possesses four inhibitory domains that contain a consensus inhibitory sequence (TIPPEYR), which in itself, is able to inhibit calpain specifically (Emori *et al.*, 1988; Maki *et al.*, 1989; Croall and Mcgrody, 1994). Potential protein substrates of vertebrate calpains are cytoskeletal and membrane proteins such as spectrin, fodrin, lamins and microtubule-associated proteins i.e. proteins known to control various functional processes in response to extracellular stimuli. Protein kinase C has been

reported to be down regulated by calpain in some cell lines (Ohno *et al.*, 1990; Eto *et al.*, 1995), and the tumor suppressor p53 has been described to be sensitively proteolyzed and regulated by calpain (Gonen *et al.*, 1997). Other *in vivo* substrates are the plasma membrane Ca^{2+}-ATPase pump (Salamino *et al.*, 1997), several transcription factors (Hirai *et al.*, 1991) and some adhesion-related molecules.

Many calpain isoforms in vertebrates have been identified including the ubiquitous μ- (CAPN1) and m-calpains (CAPN2), and the tissue-specific calpains. Typical calpains (μ-calpain, m-calpain) are heterodimers of an isoform-specific large catalytic subunit (80-kDa), and an invariant 30-kDa small regulatory subunit (CAPN4). The catalytic subunit of typical calpain is composed of four domains. Domain I the N-terminal regulatory domain, is a single α-helix composed of ten-odd amino acid residues. The helix is very important for the stability and activation of some calpain, but several other members have unique sequences instead at their N-termini including Zn-fingers, trans-membrane sequences, etc. Most significantly, the N-termini of *Trypanosoma brucei* calpain-like proteases show similarity to calpastatin, although the identities are quite low (around 15%). The N-terminal domains of some nematode calpain-like proteases contain glycine clusters resembling domain V of 30K (the small subunit). These members are interesting considering the process of evolutional conservation of calpain (Sorimachi and Suzuki, 2001).

Domain II is a cysteine protease domain homologous to other cysteine proteases such as papain and cathepsins B, H, and L. The conserved cysteine proteinase domain contains cysteine, histidine, and asparagine residues that form the catalytic triad characteristic of cysteine proteinases (Berti and Storer, 1995). This domain is involved in recognition and cleavage of substrate proteins. The three-dimensional structure of m-calpain has shown that domain II is divided into two sub-domains, IIa and IIb, which contain the active site Cys-105 and His-262/Asn-286, respectively. A recombinant domain II of m-calpain has been shown to have Ca^{2+}-dependent proteolytic activity (Hata *et al.*, 2001).

Domain III shows no significant amino acid sequence similarity to any other sequences in the database. The 3D structure of m-calpain has revealed that this domain consists of 8 β-strands, forming a β-sandwich structure with a topology similar to tumor necrosis factor α and the C2-domain found in several Ca^{2+}-regulated proteins such as protein kinase C and synaptotagmins (Rizo and Sudhof, 1998){{Duncan, 2000 #9905}. In the structure of m-calpain, this domain is characterized by an acidic loop between the 2nd and 3rd β-strands (Strobl *et al.*, 2000). Domain III is in close contact with domain II, and is considered to be very important in the regulation of activity (Hosfield *et al.*, 1999a; Strobl *et al.*, 2000). Although no primary sequence similarity can be found between calpain domain III and any of the C2-domains, the overall structure similarity strongly suggests that this domain is responsible for the Ca^{2+}-dependent translocation of calpain to the membrane (Sorimachi and Suzuki, 2001). The study of purified recombinant domain III regions of rat μ-, m and Dm-CalpB indicated that domain III-C2 has the capacity for Ca^{2+} and lipid binding in a mutually cooperative manner. Domain III might be the primary lipid binding site of calpain and may play a decisive role in orchestrating Ca^{2+} - and lipid activation of the enzyme (Strobl *et al.*, 2000; Tompa *et al.*, 2001).

Domain IV is highly similar to domain VI in 30K small subunit (consists of domain V and VI), and has 5 EF-hand motifs in one domain. Domain IV interacts with the small subunit (Crawford *et al.*, 1993) and with endogenous inhibitor calpastatin (Ma *et al.*, 1994). The 5th EF-hand motif of domains IV and VI interact with one another to form the heterodimer. Each EF-hand motif shows slight similarity to that of calmodulin. EF-hand containing proteins can be divided into 66 classes. (Nakayama, 1995). The 5EF-hand-containing proteins, the FEF or PEF (penta-EF-hand) family represented by calpain, are unique not only in that they contain an odd number of EF-hand motifs, but also in that the primary sequences are significantly distinct from those of calmodulin (Maki *et al.*, 1997;

Sorimachi *et al.*, 1997). Furthermore, FEF proteins are known to form homo- and/or hetero-dimers, and Ca^{2+}-binding causes only slight changes to the structure (Jia *et al.*, 2000).

The atypical calpains (e.g., CAPN5 and CAPN6) comprise domains II and III similar to the typical calpains, a divergent domain I, and lack of domain IV, the calmodulin-like Ca^{2+} binding domain (Barnes and Hodgkin, 1996; Dear *et al.*, 1997; Dear *et al.*, 2000). The proteolytic activity of most of these calpains (the calmodulin-deficient human hTra-3 and the C-terminally truncated form of nCL-2, nCL-2', essentially consisting of the catalytic domain alone; (Sorimachi *et al.*, 1997) depends on the presence of free calcium, while some other calpains (CAPN3) (Sorimachi *et al.*, 1993), CAPN 5 and 7(Dear and Boehm, 1999; Dear *et al.*, 1999) do not seem to require calcium for their activity. There are a number of calpains that exhibit some degree of tissue-specificity: CAPN3 (in skeletal muscle), Lp82 (an alternative splice product of p94 found in lenses; (Ma *et al.*, 1998; Herasse *et al.*, 1999), CAPN 6 (in placenta), CAPN 8 (possibly smooth muscle), CAPN 9 (stomach and small intestine), and CAPN 11 (testis) (Dear *et al.*, 1999) and CAPN 12 in hair follicles (Dear *et al.*, 2000).

CAPN3 is expressed almost exclusively in skeletal muscle, at least in the adult (Kinbara *et al.*, 1998b; Richard *et al.*, 1999). It has three unique insert regions: a novel sequence (NS) at the N-terminus, insert region No. 1 (IS1) in the protease domain, and insert region No. 2 (IS2) specifically binds CAPN3 to connectin (titin) in muscle (Sorimachi *et al.*, 1997). The muscle specific CAPN3 appears to consist of only a 94-kDa catalytic subunit without a small subunit, requires little or no Ca^{2+} for activity, and is characterized by rapid and complete autolysis, with a half-life of <10 min (Sorimachi *et al.*, 1993; Kinbara *et al.*, 1998a). Mutations in the CAPN3 gene are responsible for limb girdle muscular dystrophy type 2A (LGMD2A) in human (Richard *et al.*, 1995), possiblely through the disruption of a signaling pathway involving CAPN3.

Many calpain gene structures have been studied. The genomic organization of CAPN3, the skeletal muscle specific calpain of human was revealed by Richard *et al.* in 1995. The gene extends over 40 kb with 24 exons ranging in length from 12 bp to 309 bp, with a mean size of 100 bp. The size of introns ranges from 86 bp to about 10-16 kb. The intron-extron boundaries exhibit close adherence to 5' and 3' splice site consensus sequences. When CAPN3 sequences of mouse, rat, and human are compared, very high conservation region is observed. Sequence comparison revealed several potential binding sites for ubiquitous and skeletal muscle specific transcription factors. Among them, the existence of some conserved E-boxes in the upstream regions of mouse and rat CAPN3 genes strongly suggest that CAPN3 expression is under the control of MyoD.

In invertebrates, calpain homologs have only been identified from *Caenorhabditis elegance* (*Tra-3* gene for sex determination) (Barnes and Hodgkin, 1996), *Shistosoma mansoni* (Karcz *et al.*, 1991; Scott and Mcmanus, 2000) and *Drosophila melonogaster* (Dm-CalpA and CalpB) (Emori and Saigo, 1994; Theopold *et al.*, 1995; Jekely and Friedrich, 1999a). Dm-CalpA is specifically expressed in a small set of nerve, midgut and blood cells (Theopold *et al.*, 1995). *Drosophila* CalpA (Emori *et al.* 1994) is involved in the dynamic changes in the embryonic cytoskeleton, especially actin-related structures, during early embryogenesis prior to cellularization. The protease domains of the invertebrate calpains show significant similarities to domain II of vertebrate calpain large subunit. However, no small subunit has been found suggesting the lack of the small subunit in protostomes (Jekely and Friedrich, 1999b).

By studying the expressed proteins of Dm-CalpA and CalpB, Jekely et al. (1999) have clarified the controversial regulatory mechanism of calpains. The widely accepted models state that calpains become active through N-terminal autolysis (Suzuki *et al.*, 1981; Baki *et al.*, 1996), while the alternative view holds that intact calpain is the active form (Molinari *et al.*, 1994; Elce *et al.*, 1997). Jekely et al. stated that the parallel nature of autolysis and activation in Dm-CalpB and the lack of an activation phase in the fully

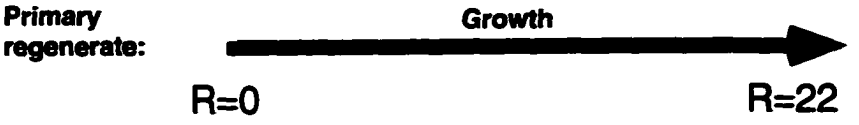
autolyzed enzyme argue for the necessity of autolysis in *Drosophila* calpain activation *in vitro*. The authors further indicated that the near identical position of the self-cleavage site in vertebrate and the fruit fly calpains reflects conserved structural features in the unrelated N-terminal regions.

Through the overexpression of dominant-negative m-calpain and overexpression of calpastatin inhibitory domain, Huang and Forsberg (1998) reported the role of calpain in skeletal-muscle protein degradation. They found that under conditions of accelerated degradation (serum withdrawal), inhibition of m-calpain reduced protein degradation by 30%, whereas calpastatin inhibitory domain expression reduced degradation by 63%. Inhibition of calpain also stabilized nebulin. Their observation indicated that calpains play key roles in the disassembly of sarcomeric proteins. For therapeutic purposes, calpain inhibitors have been used for preventing certain cytoskeletal and neuron degradation conditions. These cell-permeable calpain inhibitors are calpeptin, E-64d, MDL-28170, AK275, and AK295 (Lee *et al.*, 1990; Mehdi, 1991; Bartus *et al.*, 1995a).

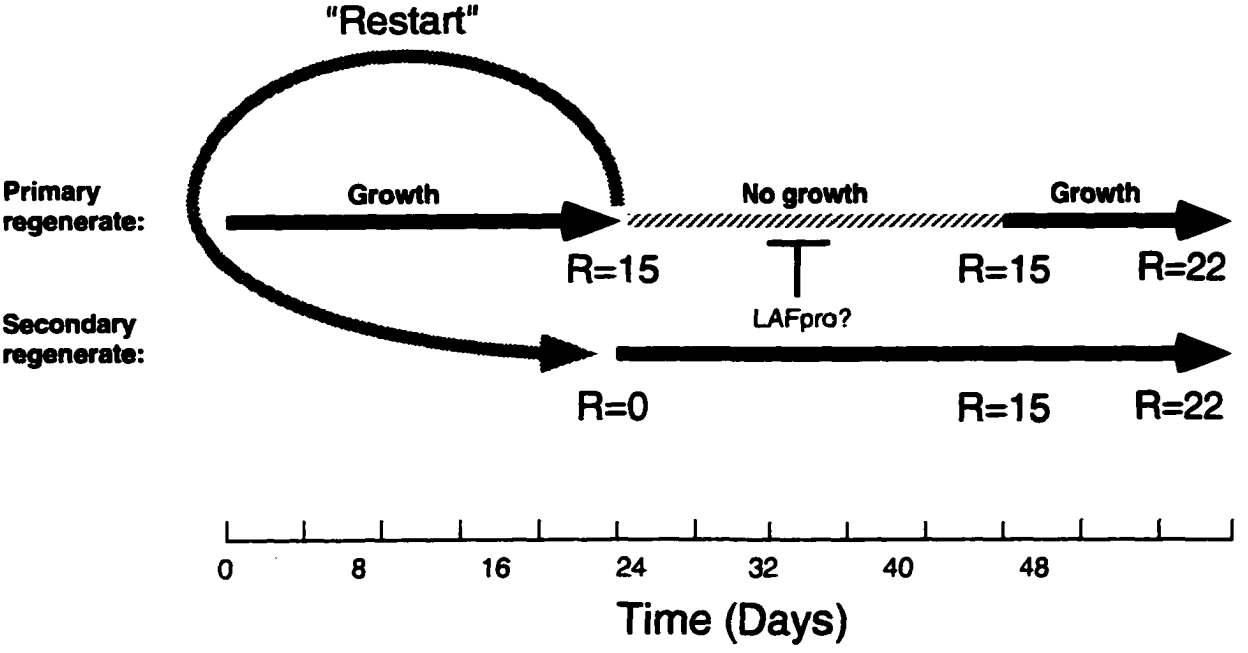
Regeneration, muscle atrophy, and molting are closely related. My graduate work has focused on molting and regeneration, and characterizing a skeletal muscle specific calpain, which is up-regulated in molting muscle. In chapter two I discuss the results of the characterization of a limb regeneration factor LAFpro in the land crab, *Gecarcinus lateralis*. Chapter three details the identification and gene expression study of lobster muscle specific calpain. I also include an appendix describing the cloning of a full-length lobster α-actin cDNA and the gene expression of ubiquitin and actin in molting muscles.

Fig. 1-1. Limb regeneration in the land crab, *Gecarcinus lateralis*. (A). If 8 walking legs are autotomized without subsequent limb bud autotomy (LBA), 1° LBs grow continuously without interruption. Ecdysis occurs 7-10 days after LBs are fully formed (R index = 22). **(B).** If, for example, one 1° LB is autotomized at R index = 15, regeneration "restart" at that limb position to form a 2° LB. Between R indexes 0 and about 15, the 2° LB secrets LAFpro, which inhibits growth of the 1° LBs. At about R index of 15, growth of 1° LBs resumes so that regeneration of both 1° and 2° LBs is completed at about the same time. The overall effect of LBA is delaying ecdysis about 3 weeks, which is the time needed for the 2° LB to reach R index ≈ 15. Based on data from Holland and Skinner (1976) (Mykles, 2001).

A. Legs removed, no limb buds autotomized



B. Legs removed, one limb bud autotomized at R=15



Chapter Two

Molting and Regeneration: Characterization of a Limb Autotomy Factor-Proecdysis (LAFpro)

A manuscript based on data in this chapter has been accepted for publication in The Biological Bulletin.

ABSTRACT

During molting, regeneration of lost appendages must be coordinated with various other physiological processes to ensure successful exuviation. If a limb regenerate (limb bud) is removed before a critical period during premolt, growth of any remaining primary (1°) limb buds (LBs) stops to allow regeneration of a secondary (2°) LBs. I show that 2° LBs contain a factor, termed limb autotomy factor-proecdysis (LAFpro), that blocks molting when injected into premolt animals. Animals regenerating 1° LBs (regeneration index 13 to 17) were injected three times at 2-day intervals with water-soluble extracts of either 1° or 2° LBs (about 2 LB equivalents per injection). Secondary LB extracts inhibited the growth rate of 1° LBs about 68% during the first week of injection, while 1° LB extract had no effect. LAFpro was stable when boiled for 15 min in deionized water, but was inactivated when boiled in 0.1 M acetic acid. The factor is also inactivated by incubation with Proteinase K. Limb bud autotomy reduced the ecdysteroid levels in the hemolymph of premolt animals. These data suggest that LAFpro is a molt-inhibiting hormone-like polypeptide that suppresses ecdysteroid synthesis and secretion by the Y-organs.

INTRODUCTION

Molting is controlled by X-organs/sinus glands, located in the eyestalks, which secrete molt-inhibiting hormone (MIH). MIH inhibits the synthesis and the secretion of ecdysone by the Y-organs. MIH acts by inhibiting steroidogenesis through a high-affinity receptor in the Y-organ cell membrane (Webster, 1993) coupled to a second-messenger cascade involving cAMP and cGMP (Sedlmeier and Fenrich, 1993; Spaziani *et al.*, 1999). Permissive physiological and environmental cues result in a decline in circulating MIH. In response, Y-organs secrets ecdysone, which is converted to 20-hydroxyecdysone (20-HE), the active molting hormone at the peripheral tissues. 20-HE acts on target tissues to initiate proecdysial processes in preparation for molting (ecdysis). It is thought that timing of proecdysial events is regulated by circulating titers of ecdysteroid, which increases during proecdysis. Previous studies, however, indicate that control of these events is more complex (review: Skinners, 1985).

Molting can be induced by eyestalk ablation or autotomy of at least 5 walking legs. Adult land crabs molt 6-8 weeks after multiple leg autotomy (Skinner and Graham, 1970; 1972). Autotomy of one or more of the 1° regenerates (limb bud autotomy or LBA) before a critical period interrupts proecdysis until 2° limb buds re-regenerate and grow to the approximate size of those lost (Holland and Skinner, 1976). Based on these observations, Skinner (1985) proposed that limb buds produce two factors that control proecdysial events. Limb Autotomy Factor anecdysis (LAFan), produced by 1° limb buds when at least five legs are autotomized, stimulates anecdysial animals to enter proecdysis (Fig. 1). Limb Autotomy Factor proecdysis (LAFpro), produced by 2° limb buds in proecdysial animals when at least one 1° limb bud is autotomized, inhibits proecdysial processes (Fig. 2-1). Here I report the initial characterization of a factor from 2° LBs, the putative LAFpro that

suspends molting in the land crab. The effects of LBA on 1° LB growth and hemolymph hormone levels were determined. The stability of the molt inhibitory activity in 2° LB extracts to boiling, acetic acid, and proteinase K was characterized. The results suggest that LAFpro is a MIH-like peptide.

MATERIALS AND METHODS

Animals

Adult land crabs, *Gecarcinus lateralis*, were collected in Bermuda or Puerto Rico and maintained as described (Koenders *et al.*, 2002). All 8 walking legs were autotomized (multiple limb autotomy or MLA) to induce limb regeneration and molting (Skinner, 1970 1972). The molt stage was monitored by measuring the growth of the limb bud at the location of the second walking leg on the right side (R2). The regeneration (R) index (the ratio of the LB length to carapace width expressed as a percentage) varies from zero after autotomy to 22-24 by late proecdysis (Holland and Skinner, 1976). Limb regenerates that formed after MLA are referred to as 1° LBs, whereas those formed after autotomy of one or more 1°LBs (limb bud autotomy or LBA) are designated as 2°LBs.

Preparation of limb bud extracts

Seven 1° LBs were autotomized from each animal at R index 13-15. The R2 regenerate was left for determining the effect of LBA on 1° LB growth. Secondary LBs were autotomized at R index 8 -10 while growth of the R2 1° LB was still inhibited. Limb buds were frozen immediately in liquid nitrogen and stored at –80 °C.

Six or seven LBs from each animal were homogenized in 2 ml of ice-cold deionized water with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) at medium speed until the tissue was completely homogenized (about 30 s). The R indices of the 1° LBs were between 13 and 15 (average weight: 38.85 mg/LB) and of the 2° LBs were between 8 and 10 (average weight: 7.43 mg/LB). The homogenates were centrifuged at

5,000 x g at 4 °C for 15 min. The supernatant fraction was divided between three 1.5-ml Eppendorf tubes, lyophilized with a Speed-Vac centrifuge, and stored at –20 °C.

To assess the temperature stability of LAF_{pro}, lyophilized pellets containing three 2° LB equivalents were dissolved in 200 µl deionized water or 0.1 N acetic acid (pH 2.9), incubated in a boiling water bath for 15 min, and centrifuged at 5,000 x g for 15 min at room temperature. Supernatant fractions were lyophilized and stored at –20 °C; they were dissolved in 120 µl deionized water just before use. To assess the stability of LAF_{pro} to proteolytic digestion, supernatant fractions from extracts boiled in deionized water were incubated with proteinase K (Boehringer Mannheim; 0.5 µg/µl) at 50 °C for 1.5 h, lyophilized, and stored at –20 °C; they were dissolved in 120 µl deionized water just before use. The rationale for heating the extract before proteinase K treatment was to remove contaminating proteins (LAF_{pro} was resistant to high temperatures) and therefore increase the effectiveness of the enzyme by reducing the amount of potential substrates. Secondary LB extracts from the same animal without the treatments served as positive controls.

Bioassay for inhibitory activity

The bioassay used proecdysial animals with eight 1° LBs (R index 13-16) that were growing at a constant rate (approximately one R index unit per day). Each animal received a series of three 40-µl injections of LB extract at 2-day intervals through the arthrodial membrane at the base of a walking leg. Initial experiments examined the effects of extracts from 1° or 2° LBs. Lyophilized pellets containing 6-7 LB equivalents were dissolved in 120 µl deionized water and used for an injection series. For the heat-, acetic acid-, and proteinase K-treated 2° LB extracts, three LB equivalents in 120 µl deionized water were used for an injection series, as it was found that one LB equivalent per injection was as effective in inhibiting 1° LB growth as two LB equivalents. The growth of the R2 1° LB was monitored before, during, and after the injection series at 2-day intervals.

Measurement of ecdysteroid by radioimmunoassay (RIA)

Samples (100 µl) of hemolymph were combined with 300 µl methanol and stored at –20 °C for later analysis. Suspensions were centrifuged at 10,000 x g for 3-5 min to remove precipitated protein. Supernatant fractions were measured for ecdysteroid by RIA as described (Chang and O'connor, 1979). Duplicate 100-µl samples were lyophilized and dissolved in 100 µl ^{3}H-ecdysone (8,000 cpm/100 µl 0.1 M sodium borate, pH 8.4). Ecdysone antiserum (Horn *et al.*, 1976) (100 µl 1:1000 dilution in 6% normal rabbit serum, 0.002% methiolate, and 0.1 M sodium borate, pH 8.4) was added to each tube and incubated at room temperature for 2-4 h. After incubation, antibody was precipitated with 200 µl saturated (100%) ammonium sulfate in 0.1 M sodium borate (pH 8.4) and centrifuged at 4,800 x g at 4 °C for 15 min. The pellets were washed with 400 µl 50% saturated ammonium sulfate, centrifuged at 4,800 x g at 4 °C for 15 min, dissolved in 25 µl deionized water, and mixed with 600 µl CytoScint™ scintillation cocktail (ICN Biomedicals, Inc.). Radioactivity was quantified with a Beckman LS7000 liquid scintillation counter.

Statistical analysis

Data are presented as mean ± 1 standard deviation (S.D.). The Student's t Test was used to determine the significance of the means between control and experimental treatments, except for the data presented in Figure 2-7, in which the Wilcoxon Matched-Pairs Signed-Ranks Test was used.

RESULTS

Effects of leg or limb bud autotomy on molting

Multiple leg autotomy (MLA) induced precocious molting (Fig. 2-2). The 1°LB at the second position on the right side (R2) was measured every other day after formation of basal papillae (R index 4-8). It took 30 to 45 days for animals to reach a R index of 15; the R index reached a maximum of 22-24 about 1 week before ecdysis (Fig. 2-2). There was considerable individual variation in LB growth. Some animals responded quickly, with rapid LB growth rates and subsequent ecdysis 5 to 6 weeks after MLA (Fig. 2-2, animal #578), while others responded more slowly and molted approximately 10 weeks after MLA (animal #641). Some animals had intermediate responses (animal #508).

As shown previously (Holland and Skinner, 1976), LBA before a critical period (R index <17) suspended LB growth and delayed molting (Fig. 2-3). The R2 1°LB stopped growing when the other seven 1° LBs were autotomized. Primary LB growth remained inhibited until the L2 2°LB reached a size similar to that of the autotomized 1° LB; subsequently, both 1° and 2° LBs grew continuously (Fig. 2-3). A consequence of LBA was that molting was delayed several weeks; for the data from the animal shown in Figure 2-3, molting was delayed about 30 days.

Autotomy of 2° LBs prolonged molting even further. In the experiment shown in Figure 2-4, seven 1° LBs were autotomized at R index 12.5 (twenty-five days after MLA). On Day 70, all seven 2° LBs were autotomized; tertiary (3°) LBs differentiated and grew. Growth of the 1°LB remained inhibited until the L2 3° LB attained the size of the 1° LB. It took longer for the 3° LB to grow to a similar size as the 2° LB that was autotomized at the same position.

Effects of LBA on hemolymph ecdysteroid

LBA reduced ecdysteroid levels in the hemolymph of animals induced to molt by MLA. In a control group in which molting was not interrupted by LBA, ecdysteroid levels increased continuously during proecdysis (Fig. 2-5, open circles). In another group of

animals, LBs were autotomized at R index 13-16 and hemolymph was sampled either during inhibition or after resumption of 1° LB growth (Fig. 2-5, filled and open triangles, respectively). The average ecdysteroid concentration in inhibited animals (130.3 ± 16.2 ng/ml; n = 24) was 45% that of the control animals (287.7 ± 28.3 ng/ml; n = 30) within the same range of R indices (between 14 and 18 in Fig. 2-5). However, the means were not significantly different due to the large amount of variation between individuals. The ecdysteroid levels of animals after resumption of 1° LB growth fell within those of the control group.

Since ecdysteroid titers varied between individuals, samples were taken from the same individuals on the day of LBA and the days following LBA. In Figure 2-6, hemolymph ecdysteroid concentrations and 1° LB growth (R index) from two animals (#805 and #771) are presented. LBA resulted in an immediate cessation of 1° LB growth and ecdysteroid levels decreased about 52% by one week post-LBA. Levels remained low until 1° LB growth resumed and the animals eventually molted. However, resumption of 1° LB growth did not always follow an increase in ecdysteroid concentration. For animal #805 (Fig. 2-6, upper panel), the 1° LB resumed growth by Day 30 post-LBA, even though ecdysteroid level did not increase until Day 36. For animal #771, however, hemolymph ecdysteroid began to increase by Day 14, but 1° LB growth did not resume until Day 19 (Fig. 2-6, lower panel). Ecdysteroid reached a maximum concentration of more than 1,000 ng/ml just before ecdysis. Data from a group of animals in which hemolymph samples were taken on the day of LBA and one week after LBA are summarized in Figure 2-7. The level was reduced 73% at one week post-LBA; the means are significantly different ($P < 0.043$, using the Wilcoxon Signed Rank Test).

Effects of primary and secondary limb bud extracts on molting

Proecdysial animals (R index 13-15) with continuously growing 1° LBs were selected for determining the effects of LB extracts on molting. Either 1° or 2° LB extracts were

injected three times at 2-day intervals (two LB equivalents/injection). Twelve proecdysial crabs were injected with 2° LB extracts. The data from three animals are shown in Figure 2-8 (open symbols) to represent the range of responses observed. Secondary LB extracts inhibited the growth of 1° LB during the injection series. This effect was usually sustained 2-4 days after the last injection before 1° LB growth resumed (Fig. 2-8, animals #124 and #386). In contrast, injection of 1° LB extracts had no effect on 1° LB growth. The data from one animal (Fig. 2-8; #331) is a representative of the results from ten proecdysial animals that were injected with 1° LB extracts.

To quantify the effects of LB extracts, the 1° LB growth rates were determined for the 1-week intervals before and after the first injection of the series (Fig. 2-9). Secondary LB extracts significantly inhibited 1° LB growth by 68%, from 1.08 R index/day before injection to 0.35 R index/day during injection ($P < 0.005$; $n = 12$). The 1° LB growth rates of animals injected with 1°LB extracts were the same before and during the injection series ($P < 0.86$; $n = 10$). For animals that were allowed to complete proecdysis and molt, injection of 2° LB extracts delayed ecdysis about 6 days. The interval between the first injection to ecdysis was 16 to 18 days for 3 animals injected with 1° LB extract and 22 to 24 days for 3 animals injected with 2° LB extract. The pattern of mean growth rate of both 2° LB and 1° LB injected animals are summarized in Fig. 2-10 and Fig. 2-11, respectively.

Characterization of LAF_{pro}

The reduction in hemolymph ecdysteroid concentration after LBA suggested that LAF_{pro}, like MIH inhibits ecdysteroid secretion by the Y-organs. Consequently, I determined whether LAF_{pro} shared physical and chemical properties with MIH. MIH is a neuropeptide that is resistant to boiling in deionized water or acids, but is inactivated by proteases (Ranga Rao, 1965; Freeman and Costlow, 1979; Webster, 1986; Webster and Keller, 1986; Chang *et al.*, 1990).

To determine the properties of LAF_{pro}, 2° LB extracts were either boiled in deionized water, in 0.1 N acetic acid, or incubated with proteinase K. An injection series consisted of 3 injections at 2-day intervals. Each injection contained one LB equivalent (Since I found that one LB equivalent was as effective as two, I used one LB equivalent to conserve material). The growth of the R2 1° LB was measured for the week before and the week after the first injection. The thermal stability of LAF_{pro} was determined by heating 2° LB extracts in a boiling water bath for 15 min. The denatured protein was removed by centrifugation and the supernatant fraction was injected into proecdysial animals. The boiled extract significantly inhibited 1° LB growth by 68%, indicating that the factor was heat-stable (Fig. 2-12, 2-15). In a second set of experiments, the injection of extracts boiled in 0.1 N acetic acid had no effect on 1° LB growth (Fig. 2-13, 2-15), while the positive control (2° LB extracts from the same animal boiled 15 min in deionized water) inhibited growth (data not shown). In a third set of experiments, incubation of 2° LB extracts with proteinase K also destroyed LAF_{pro} activity, as injection of treated extracts did not inhibit 1° LB growth. Positive controls, in which untreated 2° LB extracts from the same animals were injected, inhibited 1° LB growth (Fig. 2-14, 2-15).

DISCUSSION

Since LB growth in decapod crustaceans is restricted to the proecdysial stage, limb regeneration must be coordinated and integrated with other physiological processes in order for an animal to molt successfully. In various decapod species, multiple leg autotomy (MLA) acts as a potent inducer of precocious molting, probably due to the need to restore functional appendages as quickly as possible (Skinner and Graham, 1970; Holland and Skinner, 1976; Skinner, 1985). Limb bud autotomy (LBA) can delay molting so that animals will molt with a full complement of appendages (Holland and Skinner, 1976; Weis, 1976; Mcconaugha, 1991). In *G. lateralis*, Holland and Skinner (1976) showed that

autotomy of one or more LBs before a critical stage in proecdysis (R index <17) delays ecdysis several weeks so that 2° LBs are regenerated before the next molt. LBA causes slowing (autotomy at R index 7-10) or complete cessation (autotomy at R index 10-16) of growth of any remaining 1° LBs, until 2° LBs differentiate and grow to approximately the same size as the 1° LBs that remain (Holland and Skinner, 1976). I obtained similar results in this study (Fig. 2-2). Furthermore, I showed that proecdysis is prolonged further by autotomy of 2° LBs (Fig. 2-3). Growth of the 3° LBs is slower than that of the 2° LBs, which suggests that repetitive regeneration places increasing energetic demands on an animal. Such demands can limit the number or size of regenerates or reduce the size increment at ecdysis (Chittleborough, 1975; Kuris and Mager, 1975; Mcconaugha, 1991).

20-HE plays a central role in regulating molting. Thus, it is not surprising that LBA affects hemolymph ecdysteroid levels. The changes in ecdysteroid concentration over the molt cycle follow the same general pattern in all decapod species. Beginning at proecdysis, decreased secretion of MIH results in derepression of the Y-organs. Ecdysteroid levels increase during proecdysis to a maximum in stage D_3, drop precipitously at stage D_4, and remain at low levels through metecdysis and anecdysis (reviewed in Skinner, 1985). Circulating ecdysteroid drops in response to LBA and remains low during basal regeneration; ecdysteroid titers begin to increase during the subsequent growth of the 2° LB (Figs. 2-5, 2-6) (Mccarthy and Skinner, 1977). Primary LBs removed later in proecdysis (R index >17) are not regenerated and the animal molts without the full complement of appendages (Holland and Skinner, 1976). In this case hemolymph ecdysteroid levels remain elevated (Fig. 2-5). This may result from reduced release of LAF_{pro} from 2° LBs. Alternatively, if LAF_{pro} release is sustained at R index >17, then elevated ecdysteroid may result from reduced sensitivity of Y-organs to LAF_{pro}. This is analogous to the reduced sensitivity of Y-organs to MIH during late proecdysis when there is maximal ecdysone production and high hemolymph ecdysteroid levels (Blais *et al.*, 1994).

Primary and 2° LBs respond differently to the same concentration of ecdysteroid, suggesting that limb regeneration is not regulated simply by ecdysteroid levels. It probably involves interactions between ecdysteroid and peptide factors on tissues that may have different sensitivities to these factors. Low ecdysteroid allows formation of basal papillae, since injection of exogenous ecdysone inhibits LB differentiation (Hopkins *et al.*, 1979). Higher levels are required for sustained proecdysial LB growth (Hopkins, 1989). LBA causes a decline in ecdysteroid, but the levels are still higher than those of anecdysial and early proecdysial animals (Figs. 2-5). At these levels following LBA, 2° LBs differentiate and grow while 1° LBs stop growing. This differential sensitivity may be due to a higher expression of ecdysteroid receptors in 2° LBs, thus causing the 2° LBs to respond as if the ecdysteroid concentration was higher. In *U. pugilator*, ecdysteroid receptor mRNA levels increase in 1° LBs during the proecdysial growth phase, but receptor expression was not examined in 2° LBs (Chung *et al.*, 1998b). Furthermore, resumption of LB growth is not always preceded with increases in hemolymph ecdysteroid (Fig. 2-5), suggesting that other factors may involved. One possible factor is a limb growth-inhibiting factor (LGIF), which may be secreted by eyestalk neurosecretory centers to slow LB growth in *G. lateralis* (Hopkins *et al.*, 1979).

Secondary LBs contain a factor that inhibits proecdysial growth of limb buds and delays molting (Figs. 2-8, 2-9). I believe this factor is LAF_{pro}, the existence of which was originally proposed by Skinner (1985). Extracts of 2° LBs were injected into proecdysial animals before (R index 13-15) or near (R index 16-17) the critical period. Previous work by Holland and Skinner (1976) suggested that animals near the critical period would be less responsive to the extract. As expected, growth of 1° LBs slowed in the animals near the critical period (data not shown), but was completely blocked in animals at R index 13-15. Limb bud growth resumed 3-5 days after the final injection. Since injection of 1° LB extracts did not inhibit LB growth, it is unlikely that the cessation of LB growth by 2° LB extracts was a consequence of the injection procedure or a general toxic reaction.

Initial characterization of LAF_{pro} indicates that it is a novel molt-inhibitory factor. It is a small peptide, as it is not denatured when boiled in deionized water and it is degraded by proteinase K (Fig. 2-14, 2-15). However, LAF_{pro} is inactivated in 0.1 N acetic acid, whereas other inhibitory peptides, such as MIH and LGIF, are acid-stable (Ranga Rao, 1965; Freeman and Costlow, 1979; Hopkins *et al.*, 1979; Webster, 1986; Webster and Keller, 1986; Chang *et al.*, 1990). MIH is a neuropeptide of about 8.5 kDa (71-78 amino acid residues, depending on species) that inhibits synthesis and secretion of ecdysone by the Y-organs (reviewed in Chang, 1993; De Kleijn and Van Herp, 1995; Lacombe *et al.*, 1999; Chang, 2001). LGIF (limb growth inhibiting factor, M_r approximately 1 kDa) inhibits LB growth in *G. lateralis* at concentrations that have no effect on molting and thus appears to be distinct from MIH (Hopkins *et al.*, 1979).

While LAF_{pro} appears to be a small peptide related to MIH, its identity remains incomplete until the inhibitory factor is purified and sequenced. Those efforts are underway. The site of LAF_{pro} synthesis is also unknown, but the factor may be synthesized in neurons in the thoracic ganglion and/or nerve roots and transported via axonal processes to the 2° LBs. This is supported by the recent discovery of crustacean hyperglycemic hormone (CHH)/MIH-immunoreactive neurons in the thoracic second roots in lobster (Chang *et al.*, 1999). Cells containing CHH-like peptides are also found in the pericardial organ and gastrointestinal tract of *Carcinus maenas* (Chang *et al.*, 1999; Webster *et al.*, 2000; Dircksen *et al.*, 2001). In lobster, hemolymph levels of CHH/MIH levels drop after 20 days after eyestalk ablation, although significant amounts are detected as long as one year without eyestalks (Chang *et al.*, 1998). These data indicate that extra-eyestalk sites for neuropeptide synthesis and secretion are common and are biologically important. Perhaps the most compelling evidence for an extra-LB site of LAF_{pro} synthesis is that LBA has an immediate inhibition on 1° LB growth before any 2° LBs differentiate (Fig. 2-2; Holland and Skinner, 1976). The tissue target(s) of LAF_{pro} also needs to be identified. All the data are consistent with LAF_{pro} acting directly on the Y-organs by inhibiting ecdysone synthesis and secretion.

The neurosecretory centers in the eyestalks are not involved, since LBA inhibits 1° LB growth and lowers hemolymph ecdysteroid in eyestalk-ablated animals (Holland and Skinner, 1976; McCarthy and Skinner, 1977). The working model is that severing the thoracic nerve by LBA results in LAF_{pro} transport from neurosecretory cells in the central nervous system and release of LAF_{pro} into the hemolymph at the autotomy plane (reviewed in Mykles, 2001). LAF_{pro} suppresses ecdysone secretion by Y-organs, thus inhibiting 1° LB growth and other proecdysial processes that are ecdysteroid-dependent.

Fig. 2-1. Hormonal regulation of molting in land crab, *Gecarcinus lateralis*.

In intact animals, molting is controlled by X-organs/sinus glands, located in the eyestalks, that secrete molt-inhibiting hormone (MIH). MIH inhibits the synthesis and secretion of ecdysone by the Y-organs, located in the cephalothorax. Permissive physiological and environmental cues result in a decline in circulating MIH; in response, Y-organs secret ecdysone, which is converted to active hormone, 20-hydroxyecdysone (20-HE) by peripheral tissues. 20-HE acts on target tissues to initiate proecdysial processes in preparation for ecdysis. Molting can be induced by eyestalk ablation or by autotomy of at least 5 legs (multiple leg autotomy or MLA). Skinner (1985) proposed that the basal papillae of 1° limb buds (LBs) produce a factor, LAFan, which may act directly on the eyestalk neurosecretory centers. During early proecdysis (stage D_0, R index < 16), autotomy of a single of 1° LB delays molting; growth of remaining 1° LB stops until a 2° LB grows to the approximate size of the 1° LB. A putative factor produced by the 2° LB, LAFpro is thought to mediate this process (Skinner, 1985). The target(s) of this factor is unknown; it may act on the Y-organs and/or peripheral tissues (Mykles, 2001).

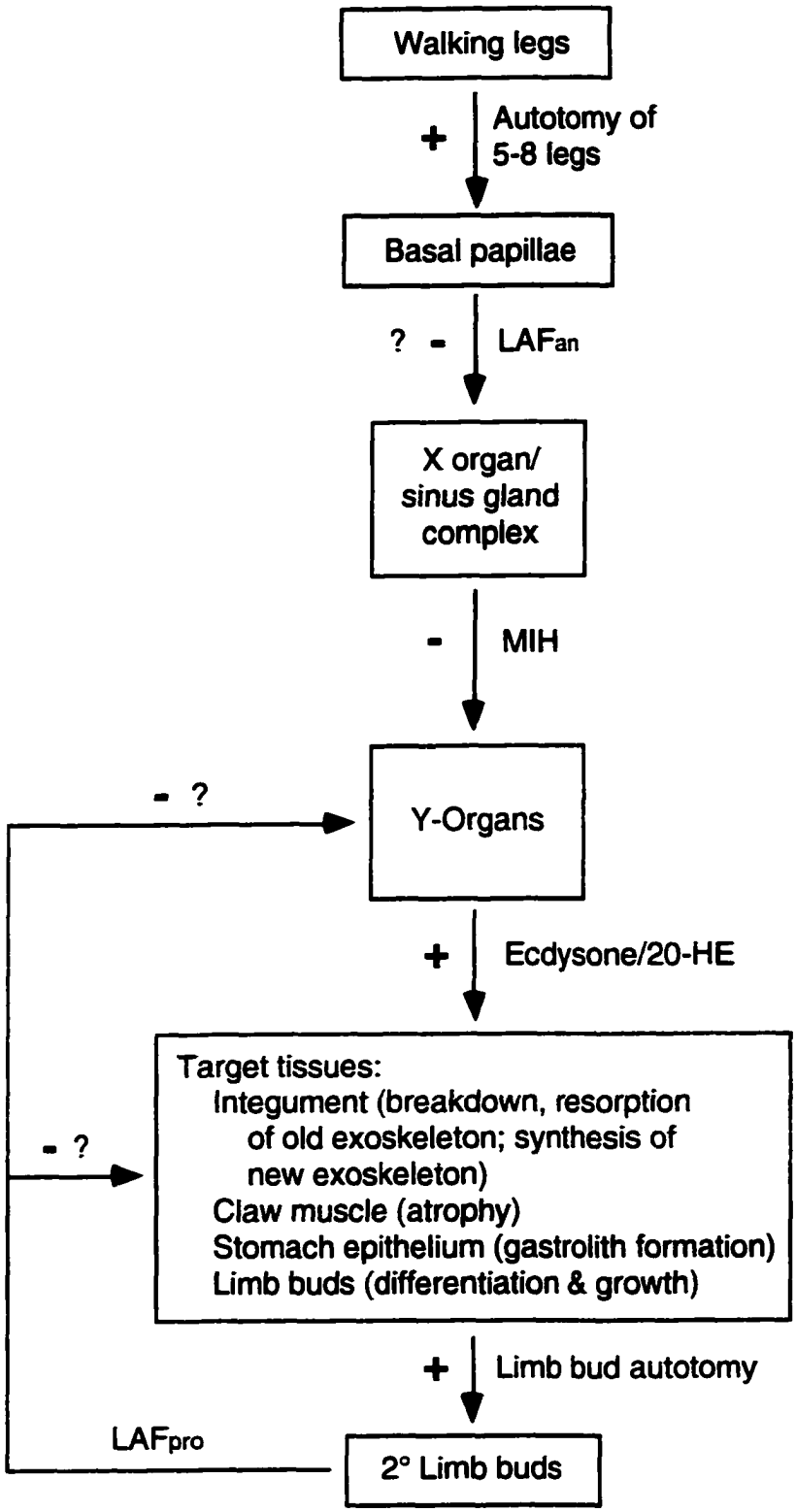


Fig. 2-2. Growth pattern of primary regenerates without interruption by limb bud autotomy. Animals were induced to autotomize eight walking legs on day 0. The length of the primary limb buds at the second position on the right side (R2) was measured every two days until molting; R indices were calculated as described in Materials and Methods. Three adult males (#508, # 578, and #641) were selected to represent the range of responses to MLA. In most cases it took 30-45 days for animals to reach an R index of 15 after MLA (#578 ●, #508 □). In some cases, this period could be as long as 60 days (#641 Δ). Crabs molted at an R index of 20-23.

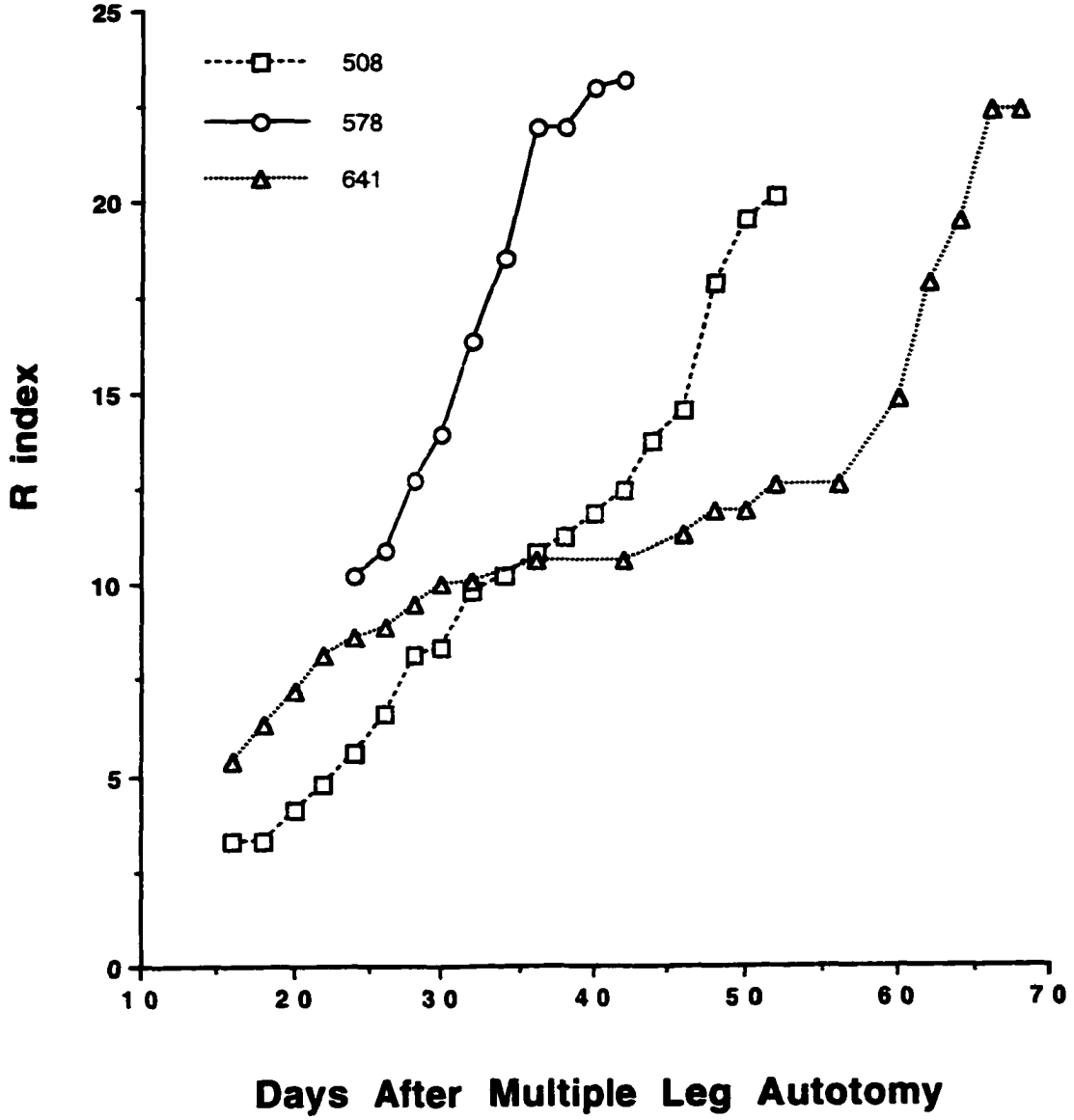


Fig. 2-3. Limb bud autotomy suspends molting. All eight walking legs were autotomized from an adult male at day 0, and the growth of the R2 1° LB was measured. At day 50 (R index = 15), seven primary limb buds were autotomized (●). LBA suspends proecdysis until the 2° LBs (Δ) appear and grow to approximately the same size as the R2 1° LB that remained.

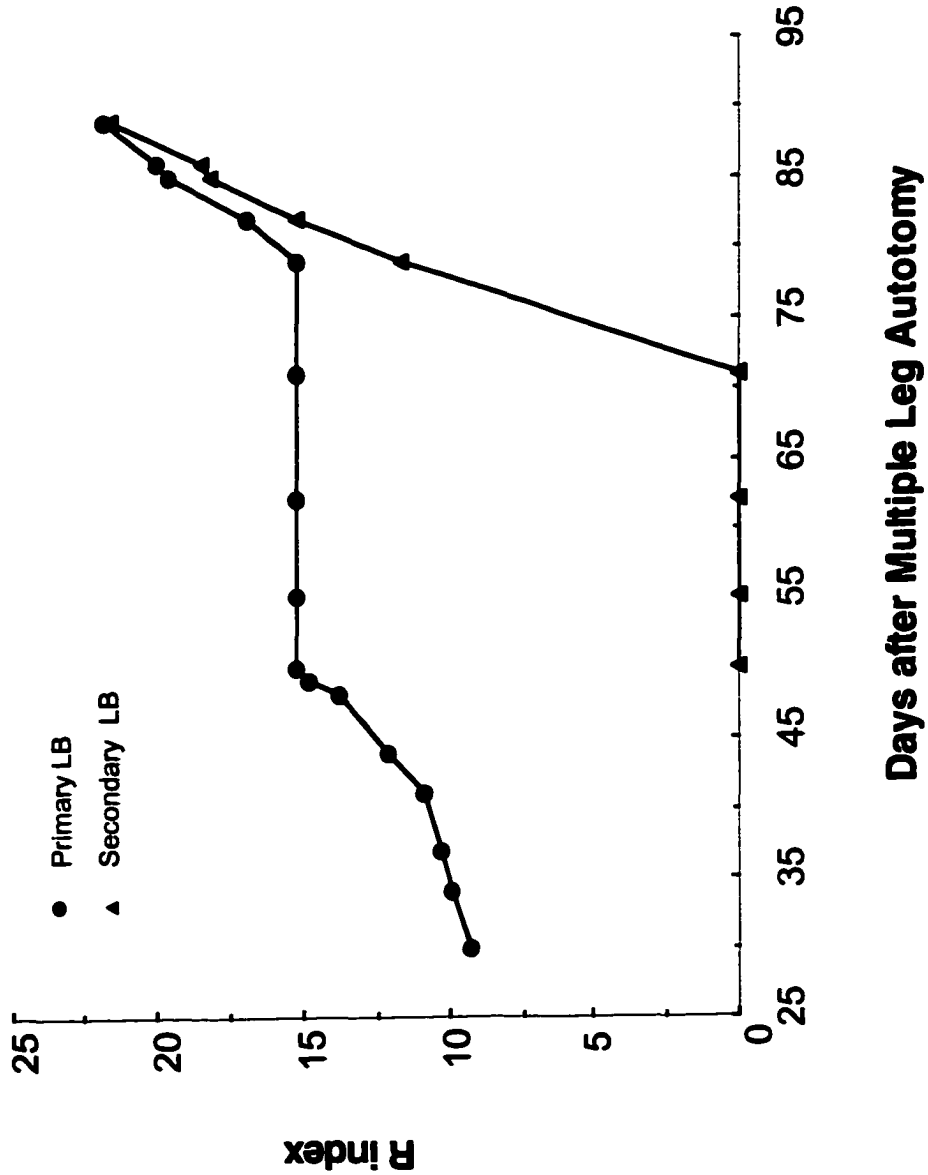


Fig. 2-4. Autotomy of secondary limb buds prolongs proecdysis. Seven primary limb buds were autotomized at R index of 12.5 (●). Growth of the R2 1° LB was suspended (▲) until the 3° LBs (♦) grew to the size of the 1° LB. The crab molted about 145 days after MLA.

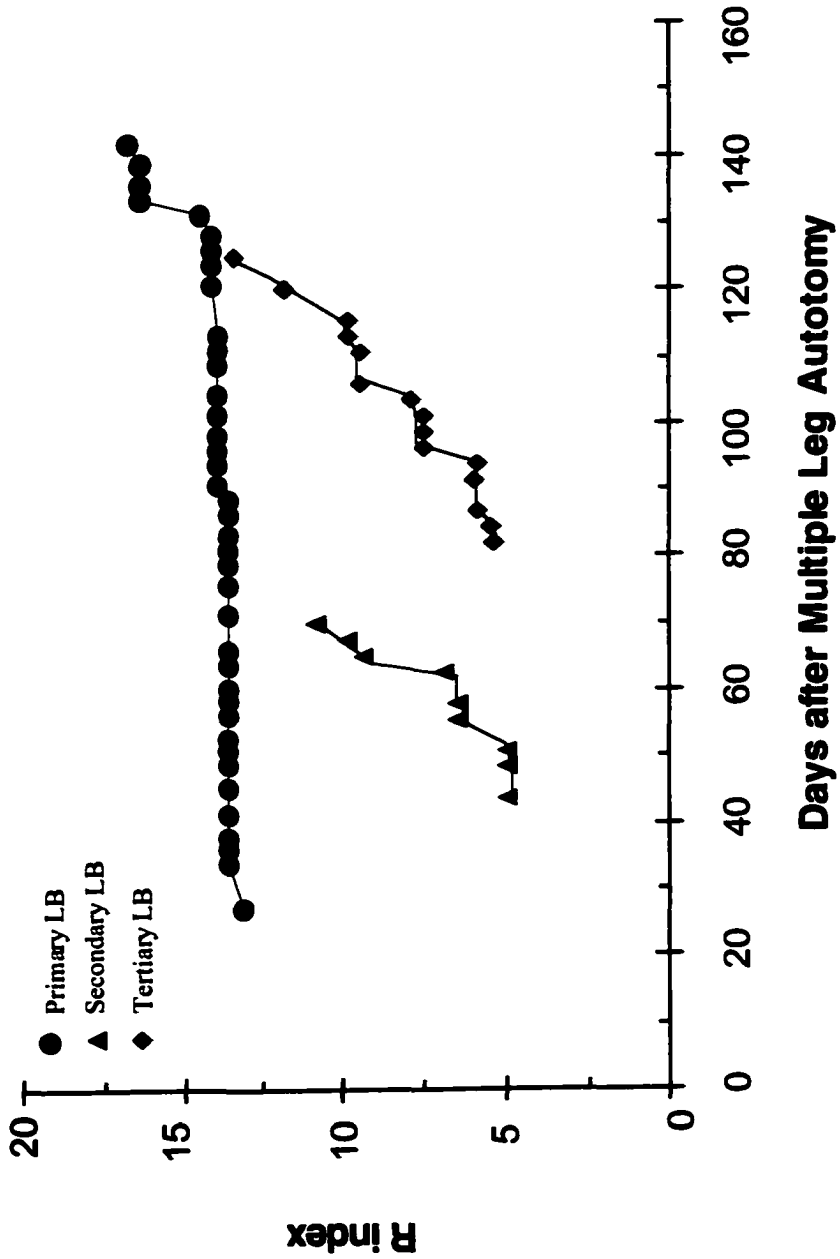


Fig. 2-5. Hemolymph ecdysteroid concentration during proecdysis. Molting was induced by autotomy of all eight walking legs and 1° LB growth monitored. Measurements were taken from three groups of animals. (1) Animals with 8 1° LBs with no limb bud autotomy (○); (2) animals subjected to LBA at R index 13-15 with 2° LBs (R index 8-10) inhibiting 1° LB growth (▲); and (3) animals subjected to LBA at R index 15-17 with 2° LBs (R index >16) no longer inhibiting 1° LB growth (Δ). In most cases, hemolymph ecdysteroid was lower in animals in which 2° LBs inhibited 1° LB growth.

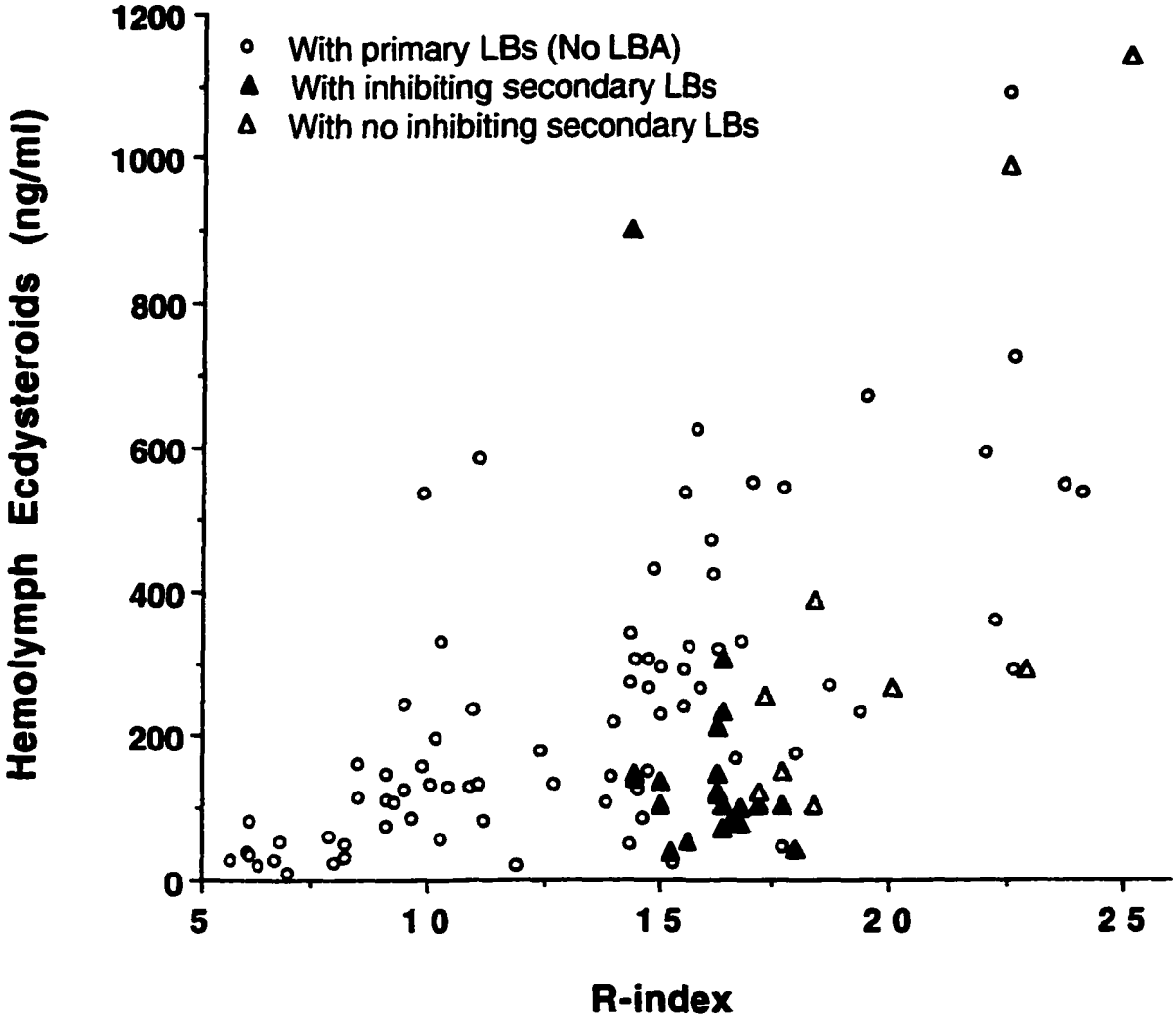


Fig. 2-6. Effect of limb bud autotomy on hemolymph ecdysteroid levels in two individual crabs during proecdysis. The ecdysteroid concentration dropped within one week after LBA and remained low until the 2° LB approached the size of the contralateral 1° LB. This occurred on Day14 Post-LBA for crab #771 (lower panel) and on Day 16 post-LBA for crab #805 (top panel). The animals molted on Day 38 (#771) and Day 49 (#805) post-LBA.

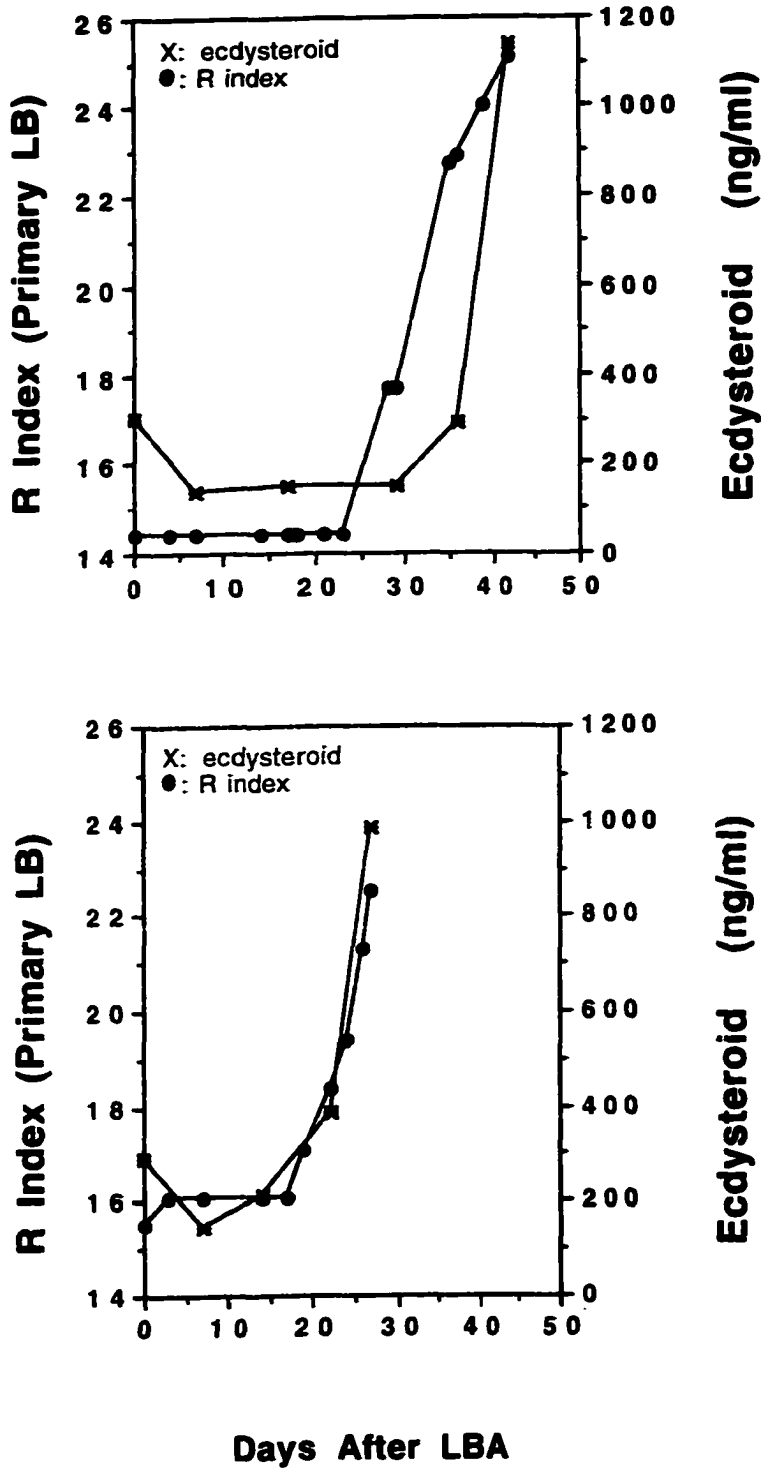


Fig. 2-7. Comparison of hemolymph ecdysteroid levels in animals at the day of limb LBA and the day after one week of LBA. LBA reduced the mean ecdysteroid level 73% by one week after LBA ($P<0.043$, $n = 5$). Error bars are standard deviations (± 1).

LAB Lowers Ecdysteroid Level

Fig. 2-8. Extracts of secondary limb buds inhibit molting. Four proecdysial animals (R index 13-15) were injected with extracts of either 1° LBs (●) or 2° LBs (○, , Δ) three times at 2-day intervals beginning at Day 0 (arrow). Each injection contained two LB equivalents. The effect of injections was determined by monitoring the growth of 1° LBs. The injection of 2° LB extracts stopped 1° LB growth for 2-7 days (#124 Δ, #386 □ and #341 ○). In contrast, 1° LB growth was not inhibited in the crab injected with 1° LB extract (#331 ●).

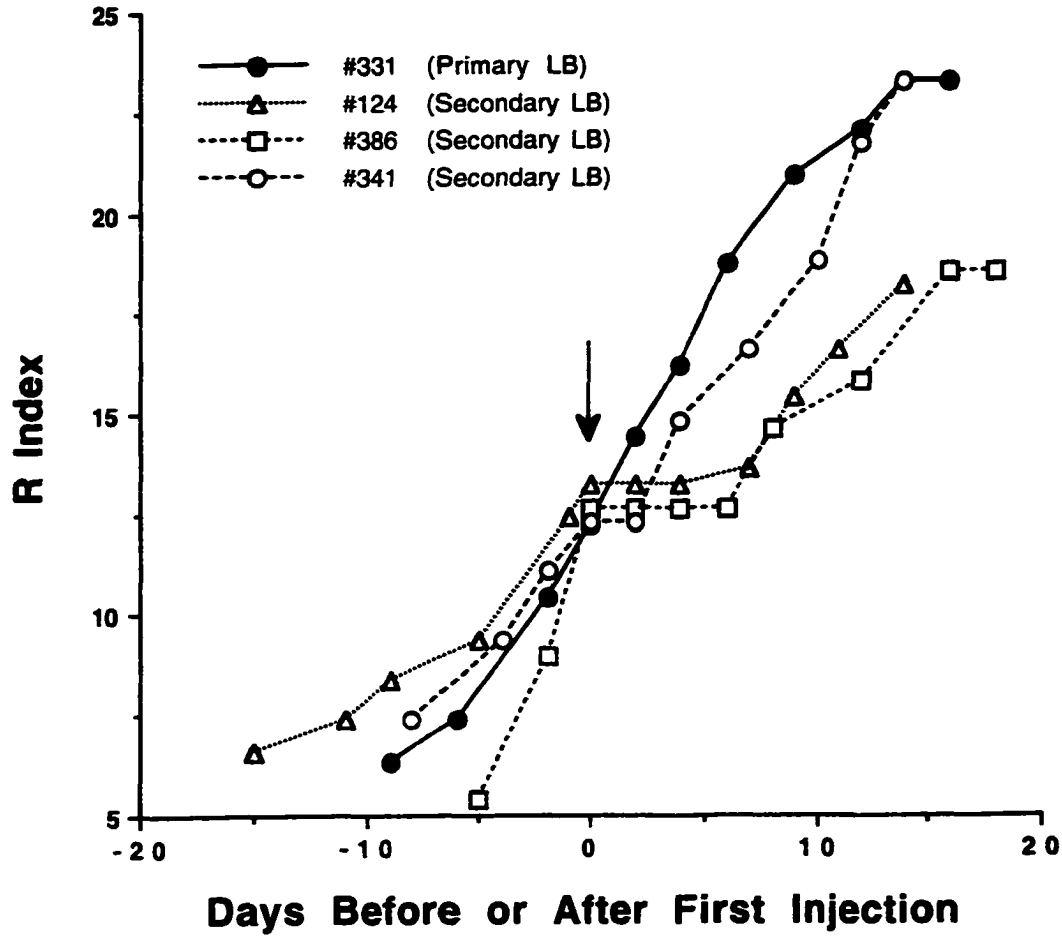


Fig. 2-9. Effects on 1° LB growth in proecdysial animals injected with extracts of 1° or 2° LBs. Data summarizes the mean growth rates one week before and one week after the first injection. Secondary LB extract significantly inhibited 1° LB growth (P-value < 0.05, n=10); whereas 1° LB extract did not have any significant effect on 1° LB growth (P-value: 0.86, n=6).

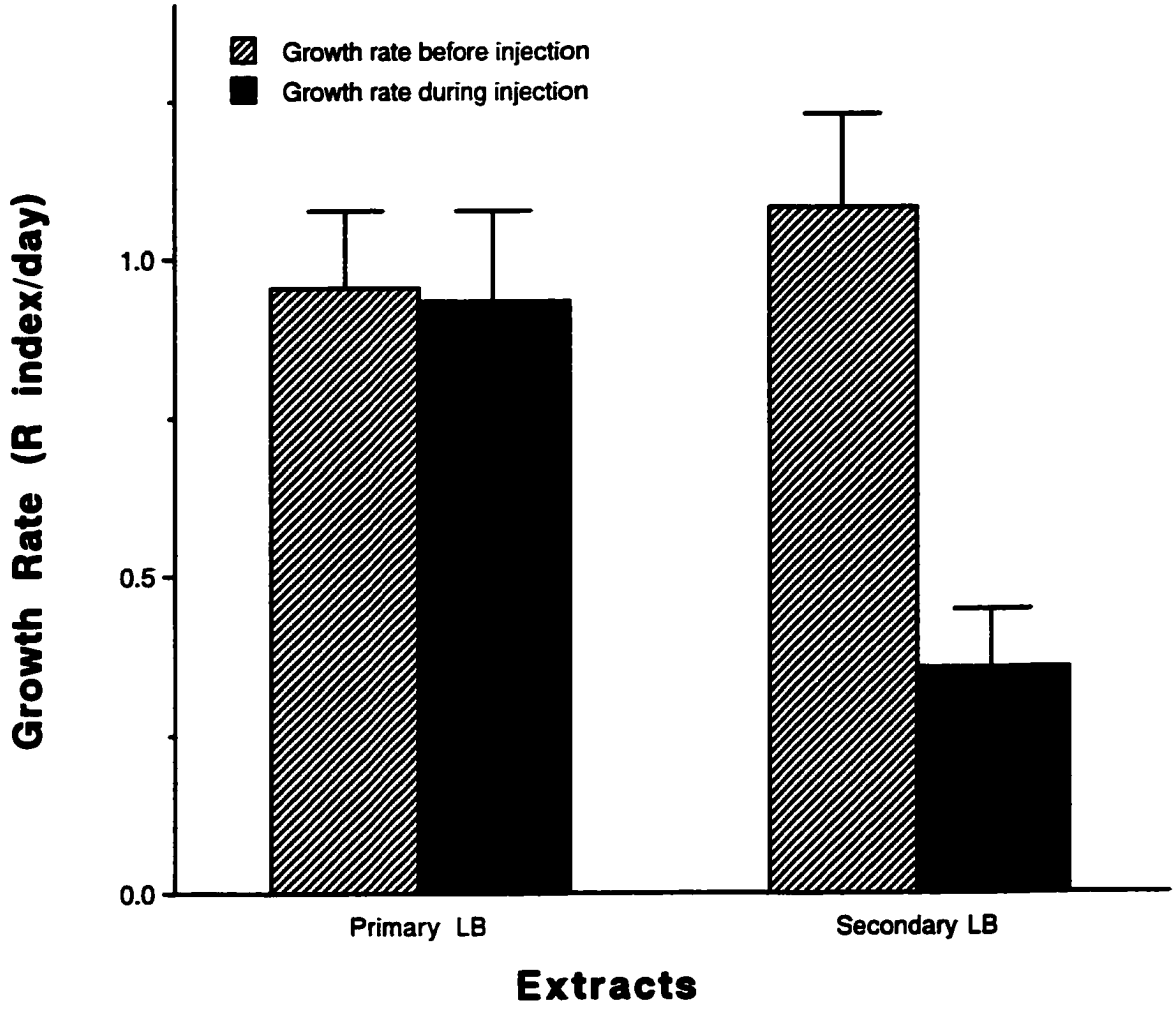


Fig. 2-10. Pattern of mean growth rate of secondary limb bud extract injected animals. A total of 10 crabs were injected and measured. The horizontal bars indicate the standard error of time (range of time). The vertical bars show the standard errors of the measurements. N represents the number of measurements. After the first injection of 2° LB extract (at day 0), there is a drop of the growth rate (from 1.1 to 0.2). The figure also shows that the growth rate is higher before R index reaches to R=15. Day 0 was the day of first injection.

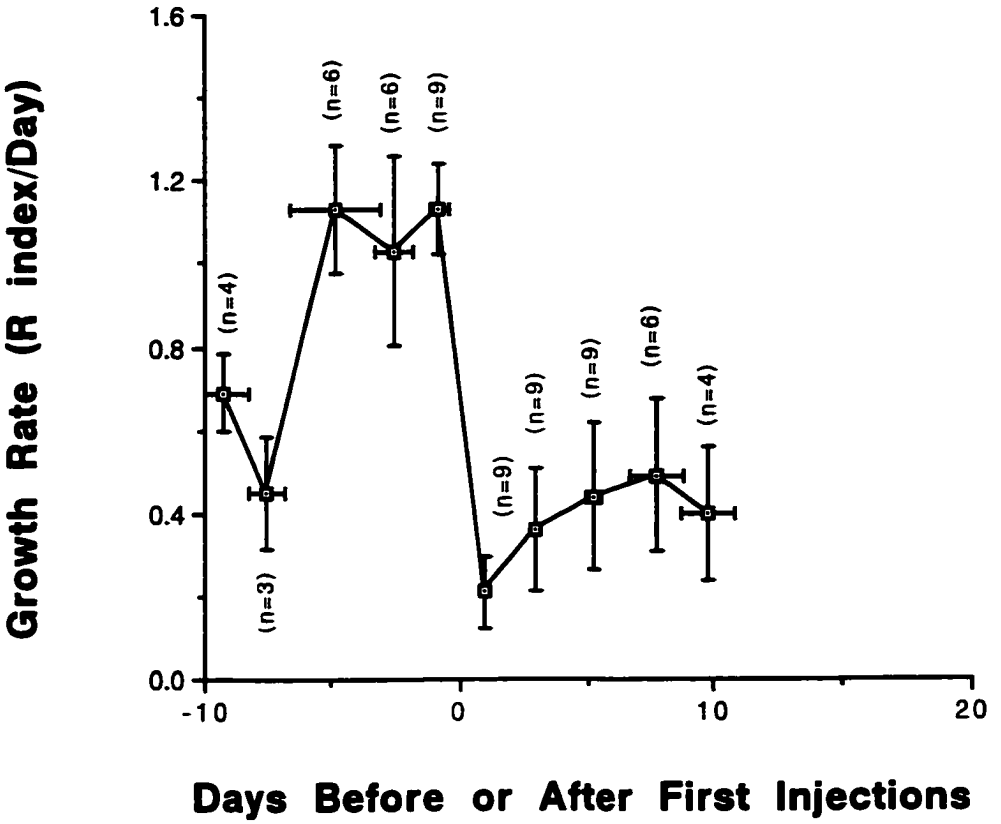


Fig. 2-11. Pattern of mean growth rate of primary limb bud extract injected animals. A total of 6 crabs were injected and measured. The horizontal bars indicate the standard error of time (range of time). The vertical bars show the standard errors of the measurements. N represents the number of measurements. Day 0 was the day of first injection.

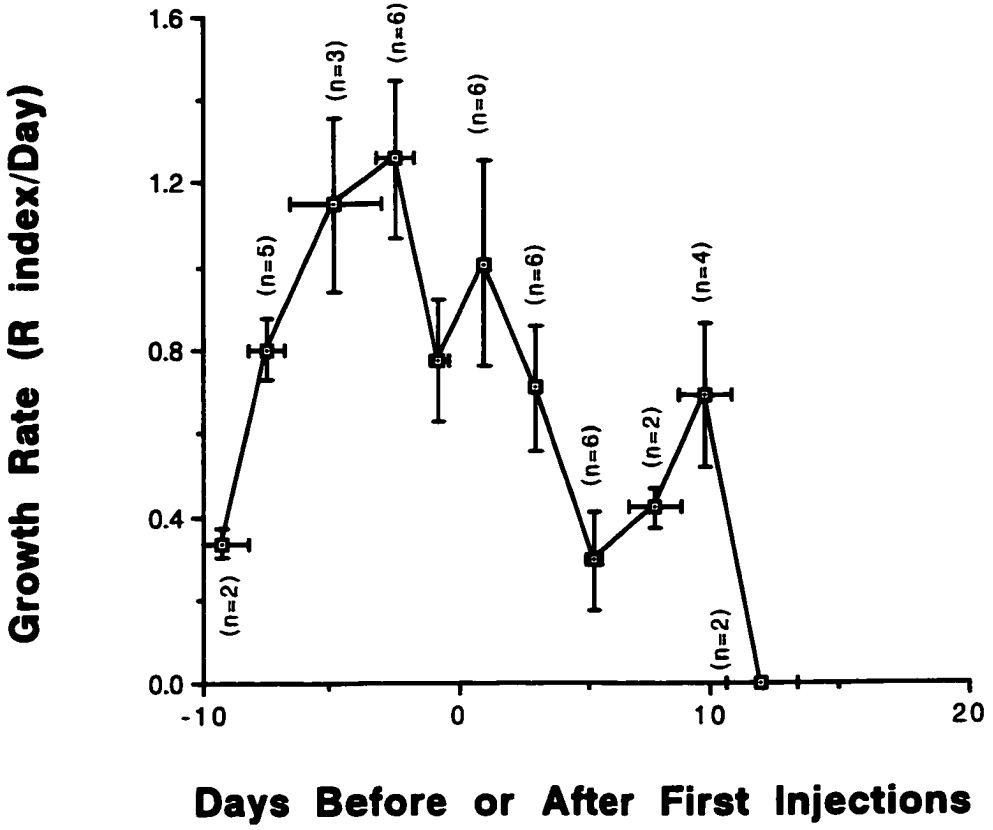


Fig. 2-12. LAFpro is heat-stable. The secondary limb bud extract (three limb bud equivalents) in 200 µl of dH_2O was incubated in boiling water for 15 min. After centrifugation at 10,000 rpm for 15 min, the supernatant was brought to 120 µl. Proecdysial animals (R index 13-15) were injected with 40 µl of the heated 2° LB extract for three times at 2-day intervals. Shown are four crabs with their R index plotted for the effect of the injection. Injection of heated 2° LB extract inhibit molting. The delay of premolt after the injection can last as long as 8 days. Arrow indicates the day of first injection.

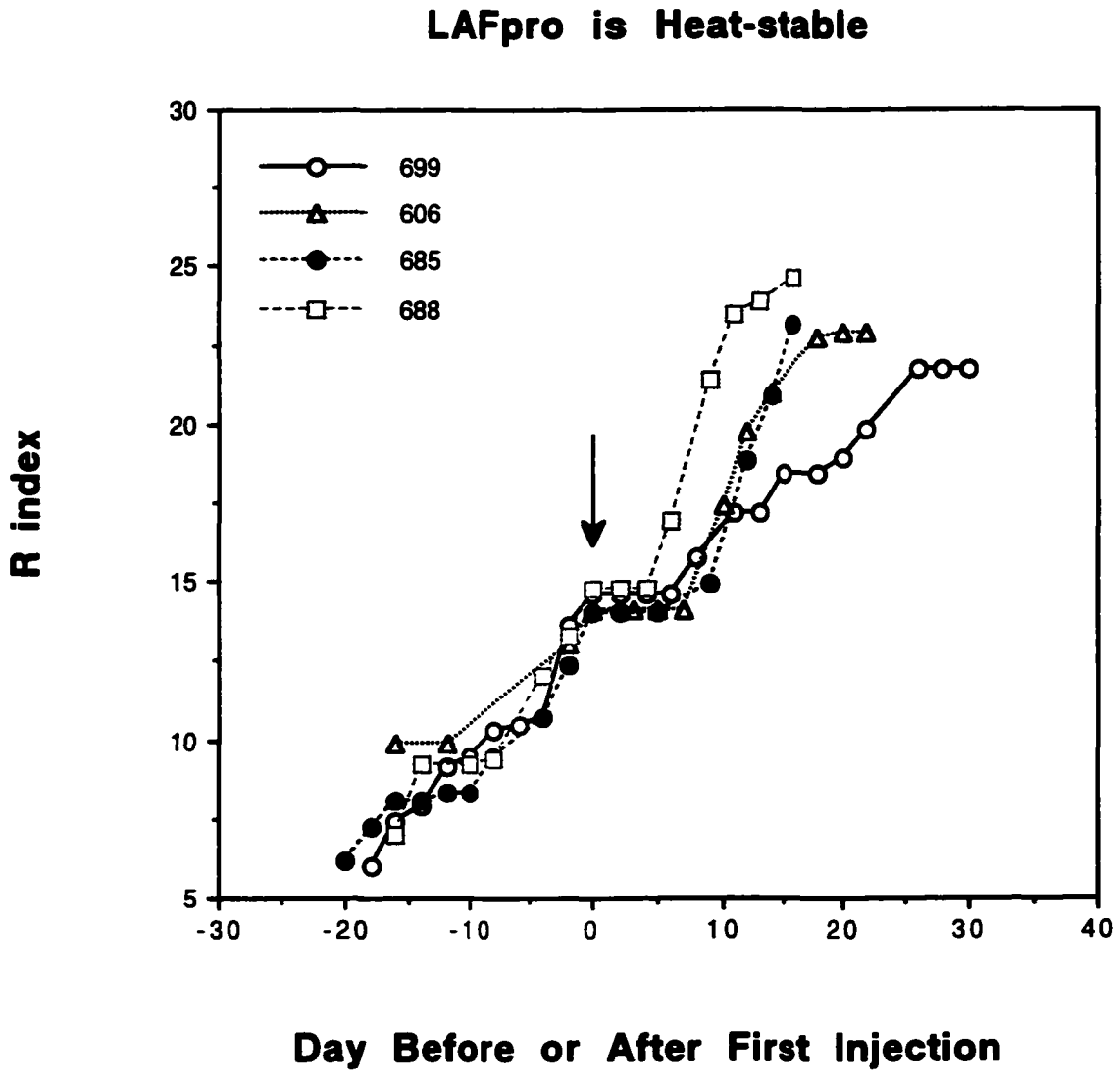


Fig. 2-13. LAFpro is acid-labile. The 2° LB extract (three limb bud equivalents) was incubated in 200 µl 0.1 M acetic acid in boiling water for 15 min. After centrifugation at 10,000 rpm for 15 min., the supernatant was lyophilized. 40 µl of the extract was injected at 2-day intervals three times. Two crabs are shown for the growth of 1° LB. The injection of acid treated 2° LB extract did not inhibit the growth, while the positive control (2° LB extract from the same animal without acid incubation) did inhibit the growth (data not shown). Arrow indicates the day of the first injection.

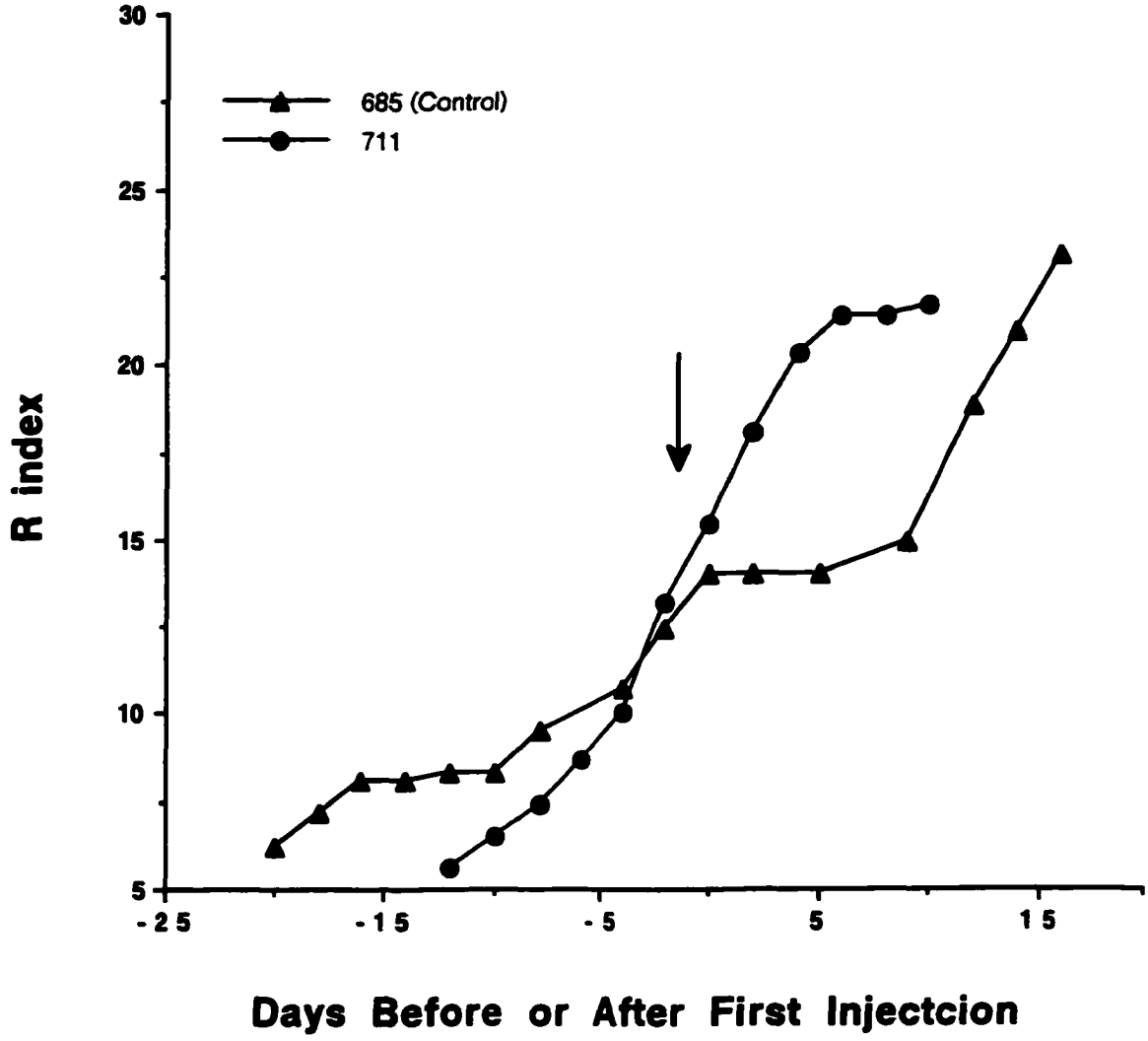


Fig.2-14. LAFpro is proteinase K sensitive. 2° LB extract (six limb bud equivalents) was split in half (three limb bud equivalents each) in 120 µl. One half was incubated with proteinase K at 0.5 µg/µl at 50 °C for 1.5 h. Proecdysial animals were injected with 40 µl of the treated and untreated (as positive control) extracts three times at 2-day intervals. Animals with the injection of proteinase K treated 2° LB extract showed no inhibition of growth (#711 as an example), while the controls with the untreated extract inhibited the growth (# 685).Arrow indicates the day of the first injection.

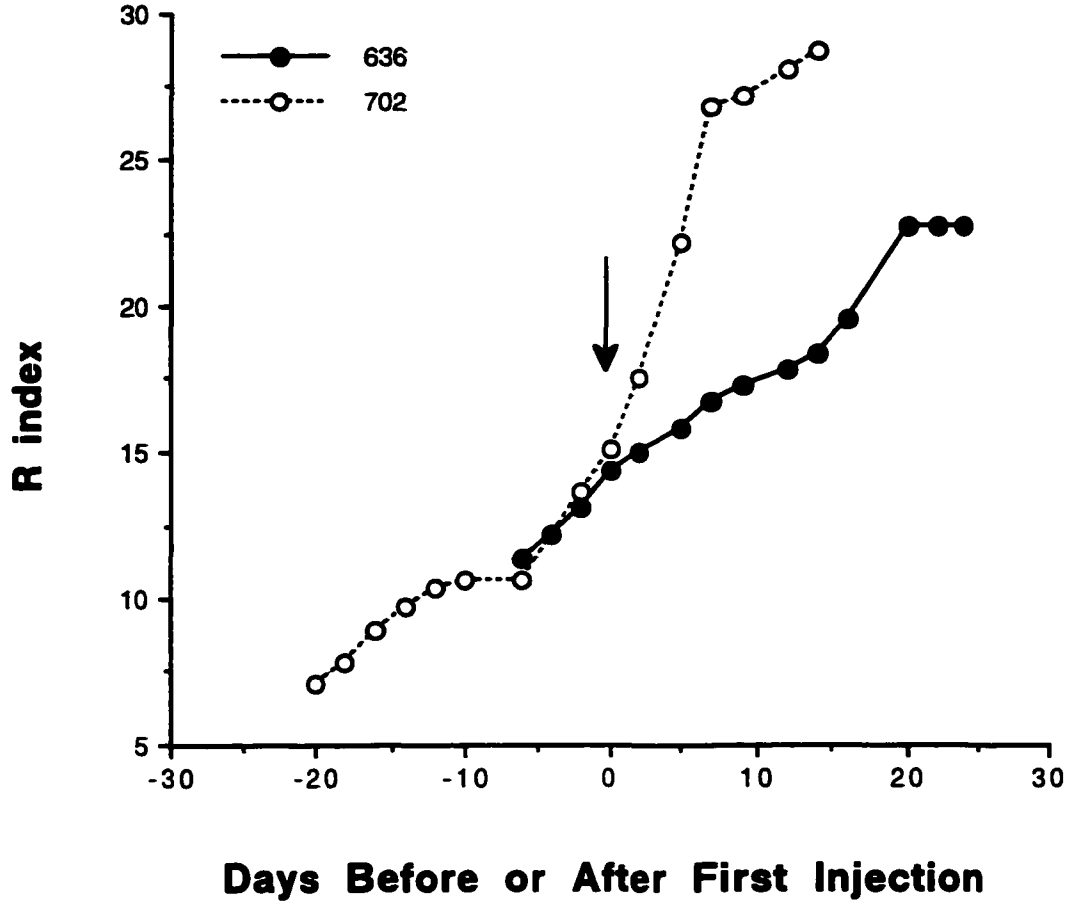
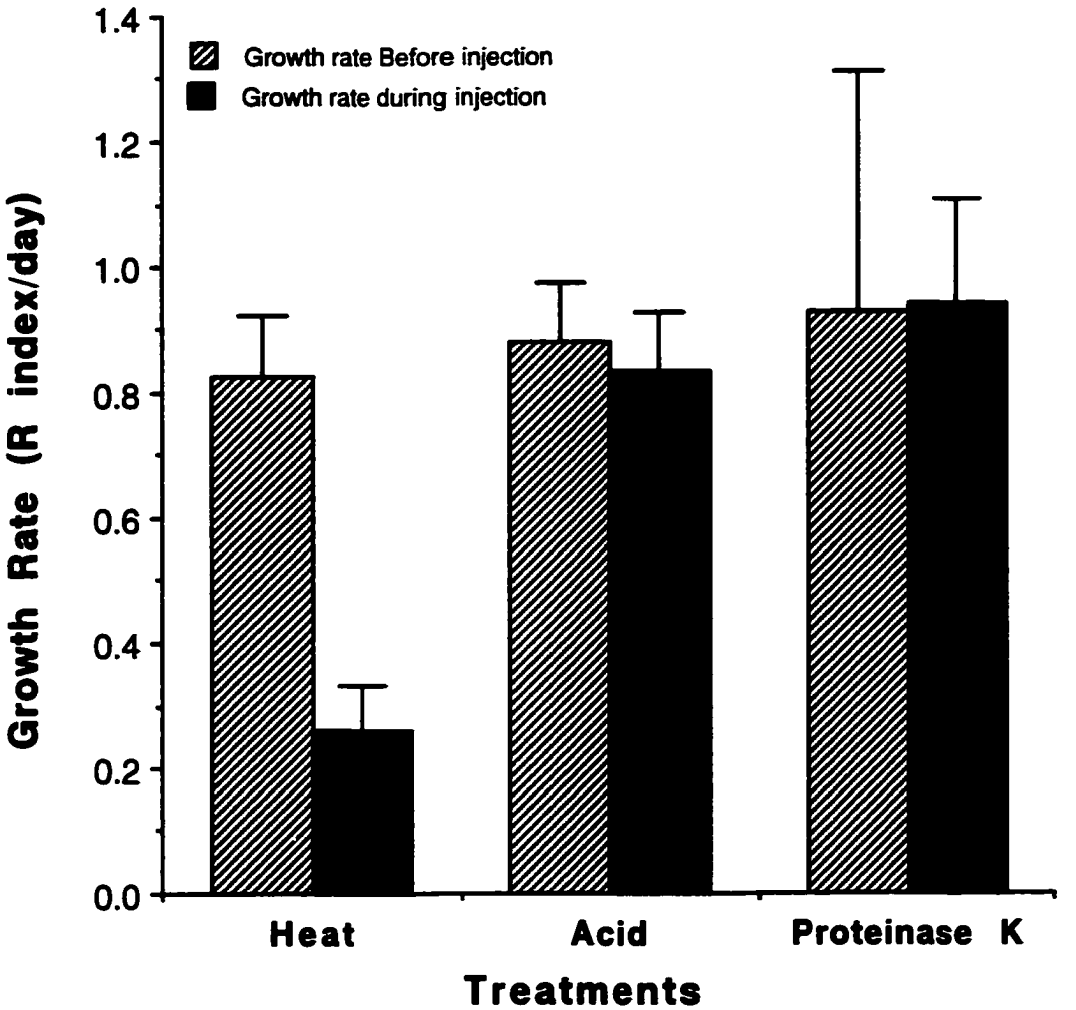


Fig.2-15. Effects of treatments on the activity of LAFpro in secondary limb buds. The mean growth rates of 1° LBs of the crabs with the injections of treated 2° LB extracts were compared one week before and after the injection. The X-axis shows the three different treatments of the extracts. The Y-axis indicates the average growth rate. There is a significant difference at the 5% level on the growth rate for the heated LAFpro injection animals (P-value <0.05, n=10). However, after incubation the 2° LB extract with 0.1 M acetic acid or proteinase K (0.5 μg/μl), the inhibiting effect of the extract (LAFpro) has lost, reflected by the non-significant change in growth rates. The P-values for the acid treated and the proteinase K treated injections were 0.37 (n= 8) and 1.78 (n=7), respectively.



Chapter Three

Cloning and Characterization of a Lobster Muscle Specific Calpain

ABSTRACT

Crustacean muscles contain four calcium-dependent cysteine proteinases (CDPs or calpains) which are involved in the degradation of myofibrillar proteins during a programmed atrophy associated with molting. Using nested PCR and inverse PCR, a full-length (1977 bp) lobster calpain gene (Ha-CalpM) was isolated and sequenced from a deep abdominal muscle cDNA library. The deduced amino acid sequence of the polypeptide (575 aa, 66.3 kDa) has high sequence identity to other calpains, particularly the *Drosophila* Calpain B (Dm-CalpB) gene. Domain I has a unique N-terminal sequence of 110 aa; domain II consists of a highly-conserved cysteine protease sequence with the conserved catalytic triad-Cys 141, His 299 and Asn 329; and domain III contains an extremely acidic region (an expanded stretch of 17 aspartates) which may bind Ca^{2+} and function as an "electrostatic switch" involved in Ca^{2+}– dependent activation. Ha-CalpM, like Dm-CalpA' lacks a calmodulin-like domain (Domain IV) found in the ubiquitous m-calpain and μ-calpain in mammals. Gene expression analysis revealed that Ha-CalpM is highly expressed in skeletal muscle, with little or no expression in other tissues. Real-time PCR showed that it is up regulated during the premolt stage in claw muscles undergoing atrophy. A polyclonal antibody raised against a unique amino acid sequence (residues #53-80) in Domain I was used to determine the location and relative amounts of Ha-CalpM protein expressed in lobster tissues. It detected 62-kDa and 68-kDa proteins in western blots. Immunocytochmistry indicated that Ha-CalpM is localized in both the cytoplasm and nuclei of muscle fibers.

INTRODUCTION

In animals such as vertebrate and nematodes, calpain forms a large gene family comprising more than 10 members (Jekely and Friedrich, 1999b; Dear *et al.*, 2000). Calpain isoforms have been found in all kinds of living organisms, including animals, plants, fungi, yeast, and bacteria. This strongly suggests that calpain-type protease must have ubiquitous, distinct, and essential functions required for the maintenance of life. In invertebrates, calpain genes have only been identified in *Drosophila*, *C. elegance* and *Schistosoma*. The limited knowledge of invertebrate calpains indicates that they share sequence homologies at all domains with vertebrate calpains, and they don't possess the small regulatory subunits (Jekely and Friedrich, 1999b).

Four calpains (Calcium-dependent proteases, CDPs) have been characterized biochemically in decapod crustaceans (review: Mykles, 1998). CDPs play a major role in the degradation of myofibrillar proteins in crustacean muscle. They degrade all the myofibrillar proteins to acid-soluble products (Mykles and Skinner, 1982, 1983, 1986b; Mattson and Mykles, 1993). Total CDP activity is increased about twofold in atrophic muscles (Mykles and Skinner, 1982). Muscle atrophy is tightly linked to molting and the study of atrophy mechanism and the enzymes involved will contribute to our understanding of the entire process. Here I describe the cloning of a crustacean calpain homologue with a highly specific pattern of expression in skeletal muscle tissues.

METHODS

Cloning of lobster calpain (Ha-CalpM)

Cloning of a full-length cDNA sequence of Ha-CalpM involved a three-step approach. First, a partial sequence within the protease domain of calpain was obtained with nested PCR. Second, inverse PCR (IPCR) was used to obtain the 5' and 3' ends of the sequence. Third, the full-length sequence was amplified by PCR using primers derived from the IPCR sequence

(1) Isolation of lobster calpain partial sequences by nested PCR

The deduced amino acid sequences of the calpain catalytic subunit from diverse species were aligned to identify conserved sequences using the Clustal W program (Fig. 3-1). The degenerate primers were selected from the regions of high amino acid sequence identity. Two pairs of primers were synthesized for nested PCR, covering a sequence segment of 553 amino acids (equivalent to #423-975 in *Drosophila* CalpA). The external primers were: 1F (sense): 5'- C(T)TN GGN GAC(T) TGC (T) TGG C(T)TN C(T)T-3' and 1B (antisense): 5'-C(G)T(A) CAT CCA G(A)AA T(C)TC NCC G(A)TC-3'. The internal primers were: 1F'(sense): 5'-TAC(T) GCN GGN ATI TTC (T)CAC(T) TT-3' and 1B' (antisense): 5'-NCC CCA NGG G(A)TT NCT (G) NAT (A)(G) NC-3'. The primer locations are indicated in Fig. 3-1.

Plasmid DNA (pcDNAII, Invitrogen, Fig. 3-23) of a lobster cDNA library made from deep abdominal muscle (Shean and Mykles, 1995) was used as template, and the external primers (1F and 1B) were used for the first PCR reaction. A Perkin-Elmer DNA thermal cycler model 9600 was used for all the PCR reactions. The final concentrations of the reaction components were: 1X reaction buffer (2.5 mM Mg^{2+}, 10 mM Tris-HCl, pH 9.0, 50 mM KCl and 0.1% Triton X-100), 200 µM each of dNTPs, 1 µM of each primer (1F and 1B), and 2.5 units of *Taq* polymerase (Promega). The reaction volume was 25 µl. Initial denaturation was at 94°C for 3 min, followed by 30 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 54.8°C and extension for 1 min at 72°C. The final extension was at 72°C for 6 min. PCR reaction (0.1 µl) was used as template for subsequent

amplification with the internal primers (1F' and 1B'). The reaction conditions were the same as those for the first-round PCR.

(2) Cloning of 5' and 3' regions of the calpain gene

For inverse PCR I used the Expand™ High Fidelity PCR System (Boehringer Mannheim). Inverse primers were synthesized based on the lobster partial calpain sequence: antisense, 5'-CAA GCC TTC CAT TGT ATG TG-3' (Ha-CalpM #602-619); sense: 5'-TCA TGC TTA CTC CAT CAC CC-3' (Ha-CalpM #909-928). The plasmid DNA from the lobster cDNA library (deep abdominal muscle) was used as template. The final concentrations of components in the reaction were: 1X reaction buffer (1.5 mM $MgCl_2$ 2mM Tris-HCl, pH 7.5, 10 mM KCl, 0.1 mM DTT, 0.01 mM EDTA, 0.05% Tween-20), 200 μM each of dNTPs, 300 nM of each primer, and 2.6 units of Expand™ High Fidelity PCR System enzyme mix. The final volume was 50 μl. Initial denaturation was at 94°C for 2 min, followed by 10 cycles of denaturation at 94°C for 15 sec, annealing at 59°C for 30 sec and extension at 68°C for 4 min. The reaction then underwent 20 cycles of denaturation at 94°C for 15 sec, annealing at 55°C for 30 sec, and extension at 68°C for 4 min. The final completion time was 6 min at 72°C. I then cloned the 5-kb IPCR product into pGEM-T Easy vector. Through a restriction digestion with *Eco*RI, the longest clone was selected for sequencing with vector primers T7 and Sp6.

(3) Isolation of full-length cDNA encoding lobster calpain

Based on the sequence from IPCR, specific primers to the 5' and 3' ends of lobster calpain were synthesized: sense, 5'-AGGAGCAACAACAGGATG-3' (Ha-CalpM #20-37); antisense, 5'-TGCACAGCCCATCATTAG -3' (Ha-CalpM #1978-1995). The PCR conditions were the same as those used for IPCR. The PCR product was cloned into pGEM-T Easy vector and sequenced.

(4) Cloning of PCR products

PCR products were electrophoresed on a 1.0 % (w/v) agarose gel, excised and purified using QIAGEN Gel-Purification Kit (QIAGEN Inc.). The DNA was concentrated in a Savant Speedvac concentrator and ligated into the pGEM-T Easy Vector (Promega, Fig. 3-24). The ligation was achieved by incubating 1 µl DNA, 1 µl vector, and 1 µl T4 DNA ligase (3 Weiss units) in 1X ligation buffer (2X rapid ligation buffer: 60 mM Tris-HCl, pH 7.8, 20 mM $MgCl_2$, 20 mM DTT, 1 mM ATP, and 10% PEG) at room temperature for one hour or overnight at 4 °C in a final volume of 10 µl. DH5α Electro-competent cells were transformed with plasmid by Electro-transformation and plated on LB agar plates containing ampicillin (100 µg/ml; Sigma) using standard procedures. Positive colonies were selected and grown overnight and plasmid DNA was isolated using a QIAGEN plasmid mini-prep kit. Plasmid DNA containing insert was sequenced from both directions using the promoter primers Sp6 and T7 and gene-specific primers.

(5) Sequence analysis

Calpain amino acid sequences were retrieved from GenBank at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). Sequence homology search was done by using the BLAST program at NCBI. Multiple alignment and phylogenetic analysis were done using Lasergene biocomputing software (Clustal V method, DNASTAR Inc.), and ClustalW (1.18) (http://www.ebi.ac.uk).

Lobster calpain gene expression

(1) Northern Blot

Total RNA extraction

Total RNA from various lobster tissues was extracted using **TRIZOL** Reagent (GIBCO BRL). The procedures were essentially the same as recommended by the manufacturer. Tissues were homogenized in 10 volumes **TRIZOL** reagent (500 mg tissue in 5 ml) using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) for about 1 min or until the tissues were completely homogenized. For muscle tissue, insoluble material was removed by centrifugation at 12,000 x g for 10 min at 4°C. One ml chloroform was added to each sample after incubating the homogenized samples at room temperature for 5 min. Samples were mixed well and centrifuged at 12,000 x g for 15 min at 4°C. RNA in the aqueous phase was precipitated with 2.5 ml of isopropyl alcohol (10 min at room temperature) and pelleted by centrifugation at 12,000 x g for 10 min at 4 °C. The precipitated RNA was washed with 75% ethanol and dissolved in 50 µl deionized formamide. All RNA samples were stored at -80°C.

Blotting and detection

Total RNA (20 µg) was separated on a 1.0% agarose gel containing 0.5 M formaldehyde buffered with 1X MOPS (5X MOPS: 200 mM MOPS, pH 7.0, 50 mM sodium acetate, 5 mM EDTA, pH 8.0) at 70 V for 3 h. The RNA was transferred onto Hybond-N nylon membrane (Amersham) overnight in 20 X SSC (3M NaCl, 0.3 M sodium citrate). The blot was cross-linked under UV light at 120 Joules for 30 sec and hybridized overnight at 68°C in 20 ml hybridization buffer (Boehringer Mannheim) containing 50% deionized formamide, 5X SSC, 0.1% N-lauroylsarcosine, 0.02% SDS, 1% Blocking Reagent, and 1 µg probe. The probe was a 600 bp DIG-labeled RNA (by *in vitro* transcription, Boehringer Mannheim) derived from the middle part of the lobster calpain gene (Ha-CalpM #646-1129). After washing and blocking, the membrane was incubated with anti-DIG-AP (anti-digoxygenin-alkaline phosphatase) antibody at 1:10,000 dilution for 30 min. CSPD™ (Roche Molecular Biochemicals), a chemiluminescent substrate for alkaline phosphatase activity, was used for the detection of the hybridized probe.

(2) RT-PCR

Total RNA in formamide was precipitated with 0.1 volume of 4 M LiCl and 3 volumes of 100% ethanol. The RNA was washed in 75% ethanol and dissolved in DEPC-treated H_2O. Reverse transcription and PCR were carried out using the Access RT-PCR Introductory System (Promega). RNA was first treated with DNase (Amplification grade, GIBCO BRL) to remove contaminating DNA, then 0.4 μg of RNA was reverse-transcribed and amplified using primer pairs directed to the 5' end of the Ha-CalpM gene: sense, 5'-AGGAGCAACAACAGGATG-3' (Ha-CalpM #20-37); antisense, 5'-CAA GCC TTC CAT TGT ATG TG-3' (Ha-CalpM #602-619). The reaction contained 1X AMV/Tfl buffer, 200 μM dNTP, 0.9 μM of each primer, 2.5 mM Mg^{2+}, 5 units of AMV-RT and 5 units of Tfl DNA polymerase. The final volume was 50 μl. The reaction was carried out at 48°C for 45 min for reverse transcription. After synthesis of first-strand cDNA, PCR amplification followed at 92°C for 2 min, and 40 cycles of 94°C for 30 sec, 60°C for 30 sec and 68°C for 1 min. The final extension time was 6 min at 72°C.

(3) Real-time PCR

First-strand cDNA was synthesized from RNAs of claw muscles at various molting stages using SuperScript™II RNase H^- Reverse Transcriptase (GIBCO BRL). The reaction consisted of 2.5 μg of Oligo $(dT)_{12-18}$, 2.5 mM dNTP, 1X First-Strand Buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 5 mM DTT, 2.5 units of RNase inhibitor (GIBCO BRL),10 μg total RNA, and 200 units SUPERSCRIPT™ II RNase H^- Reverse Transcriptase. The reaction was incubated at 42°C for 50 min and stored at -20°C.

The LightCycler-DNA Master SYBR Green I reaction mix for PCR (Roche Molecular Biochemicals) was used for the amplification of Ha-CalpM using a LightCycler

Instrument. Ha-CalpM primers were synthesized based on the recommendations by the manufacturer. The sense primer (RTCalp-F1), 5'-CAACAACAGGATGTCGCTTAGTGAG-3' (Ha-CalpM #25-49) and the antisense primer (RT-Calp-B1), 5'-TTCTGAAGGGAGACGGACTCTATG-3' (Ha-CalpM #145-168). The GC content of each primer was balanced (48 % of GC in RTCalp-F1 and 50 % of GC in RTCalp-B1). The PCR master mix was assembled on ice containing 1X LightCycler-DNA Master SYBR Green I [SYBR Green I 10X contains *Taq* DNA polymerse, reaction buffer, dNTP mix (with dUTP instead of dTTP), SYBR Green I dye, and 10 mM $MgCl_2$], 3 mM $MgCl_2$, 0.5 μM of each primer, and 1 unit Platinum *Taq* Antibody (GIBCO, BRL). The mixtures were placed in glass capillaries, followed by the addition of 2 μl (200 ng) of first-strand cDNA. The protocol used for the amplification consisted of four programs: denaturation of template DNA; amplification of the target DNA; melting curve analysis for product identification; and cooling the rotor and thermal chamber. Initial denaturation was at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 0 sec, annealing at 57°C for 5 sec and extension at 72°C for 10 sec. The parameters for melting curve analysis and cooling were set as the default values of the instrument.

Since the melting temperature of a DNA product is characteristic of its GC content, length, and sequence, melting temperature was used to identify PCR product. The peak area of the melting curve analysis was used to measure the relative amount of Ha-CalpM message. For comparison of the message levels during the molting cycle, the message levels of premolt and postmolt stages were normalized to that of the intermolt stage. The experiments were run three times, and the data were averaged. Anova single factor analysis was used to compare the means of data between molting stages.

(4) Western Blot

Antibody generation and purification

A 28-aa polypeptide at the N-terminal region of Ha-CalpM deduced amino acid sequence (#53-80: NH_2-SNDYTQKRIAKGGLKIPKKGFRTLRDEC-COOH) was synthesized by the Macromolecular Resource Facility at Colorado State University. The synthetic peptide was conjugated to keyhole limpet hemocyanin and used to raise polyclonal antibody in rabbits. Serum from the terminal bleed was used for immunochemical studies. Ha-CalpM antiserum was affinity-purified. The peptide was covalently bonded to Immobilon-AV membrane (Millipore Corp.). Briefly, 1 mg of peptide was dissolved in 2 ml 0.5 M potassium phosphate, pH 7.4 and a 2 x 2 cm square of Immobilon-AV membrane was incubated with this peptide solution at room temperature for 2 h. The membrane was washed with 1 M $NaHCO_3$ for 2 h, followed by two 30-min washes in PBS (phosphate buffered saline, 80 mM di-sodium hydrogen orthophosphate, 20 mM sodium dihydrogen orthophosphate, and 100 mM NaCl, pH 7.5) containing 0.1% Tween-20. The membrane was incubated with 2 ml of the Ha-CalpM antiserum for 3.5 h, rinsed three times with PBS, and bound antibody was eluted in 0.9 ml 100 mM glycine (pH 2.5) for 10 min. The solution was neutralized with 0.1 ml 1 M Tris (pH 8.0) and stored at 0°C with 0.02 % sodium azide. All incubations were at room temperature (Bowser, 1998).

Protein extraction

Tissues from lobster were homogenized in 6 volumes Buffer A [20 mM Tris-acetate, 20 mM KCl, 1 mM EDTA, 0.1 mM dithiothreitol (DTT), pH 7.5] containing 1X Protease Inhibitor Cocktail (SIGMA). The cocktail contained AEBSF (2 mM), EDTA (1 mM), bestatin (130 μM), E-64 (1.4 μM), leupeptin 1 (μM), and aprotinin (0.3 μM). The homogenates were centrifuged at 12,000 x g for 15 min at 4°C. The supernatant fractions

were removed and stored on ice. Protein concentration was determined by fluorescence emission with a Perkin-Elmer Filter Fluorimer (model LS-2) using BSA as standard.

Partial purification of lobster Ha-CalpM

Purification of Ha-CalpM followed the protocol by Mykles (1986). Claw and deep abdominal muscles were homogenized in Buffer A (20 mM Tris-acetate, 20 mM KCl, 1 mM EDTA, pH 7.5) and centrifuged at 12,000 x g for 15 min at 4°C. The supernatant was precipitated with 65% saturated ammonium sulfate, dialyzed overnight against two 2 liters Buffer A without DTT and KCl at 4°C, and chromatographed on an organomercurial-agarose (OMA) column. Active fractions from the OMA column were concentrated by a stirred ultrafiltration cell (Model 8200, AMICON) and dialyzed against 2 liters of Buffer B (20 mM Tris-acetate, 20 mM KCl, 1 mM EDTA, 100 mM NaCl, pH 7.5). A 5-ml sample (125 mg protein) was chromatographed on a Superdex 200 gel filtration column (Pharmacia) at 3 ml/min. Fractions (1.5 ml) were collected and stored at 4°C.

SDS-PAGE and Western blotting

After mixing (1:1) with sample buffer (2X: 150 mM Tris-HCl, pH 6.8, 2% SDS, 10 % glycerol, 20 mM DTT and 0.01 % bromophenol blue), samples (30 μg) were incubated for 5 min at 95°C and loaded onto discontinuous gels for SDS-PAGE (Laemmli, 1970). For the samples from gel filtration chromatography, 10 μl of fractions (#16-48) were analyzed. Gels were either stained with Coomassie blue or silver, or proteins were electrophoretically transferred to PVDF membrane (Hybond-P, Amersham).

The detection of Ha-CalpM on blots was performed using Biotin/Avidin System (Vector laboratories, Inc. Burlingame, CA). The membrane was blocked with 5% non-fat milk in TBS buffer (Tris-buffered saline, 20 mM Tris base, 137 mM NaCl, pH 7.5) at room temperature for 1 h. The primary antibody incubation was one hour with affinity-purified

Ha-CalpM polyclonal antiserum at 1:10,000 dilution in antibody solution [3% non-fat milk in TBS-T (0.01 % Tween-20 in TBS)]. The membrane was then washed in TBS-T three times at 5 min each. The secondary antibody was a biotinylated anti-rabbit IgG (1:10,000 dilution), the incubation time was 1 h. After washing three times at 5 min each, the membrane was incubated with ABC solution (10 µl avidin, 10 µl biotinylated hydrogen peroxidase in 10 ml TBS-T buffer) for 30 min. The final washes were 3 times of 5 min each. ECL Plus™ enzyme chemiluminescence (Amersham Pharmacia Biotech) was used for the detection of peroxidase activity.

(5) Immunocytochemistry

Tissues were fixed in 4% paraformaldehyde in PBS and embedded in paraffin. Sections (10 µm) were de-paraffinized in xylene and hydrated through an ethanol series. Immunodetection used Vectastain ABC *Elite* kit (Vector laboratories, Inc. Burlingame, CA) with minor modifications. After hydration, sections were washed twice in PBS buffer containing 15 mM glycine (PBS/Gly) for 5 min, and blocked with 1.5 % normal goat serum for 1 h. Sections were incubated with Ha-CalpM antiserum or pre-immune serum (1:5000 dilutions in 1.5% normal goal serum) overnight. After washing with PBS/Gly, sections were incubated with biotinylated goat anti-rabbit IgG (1:200 dilution in 1.5 % normal goat serum) for 2 h, followed by an incubation with ABC reagent (avidin/biotinylated horseradish peroxidase complex) for 30 min. Antibody was detected colormetrically by incubating the sections in staining solution [80% of DAB (diaminobenidine tetrahydrochloride), 40% of NiCl and 0.009% H_2O_2 in 20 ml 0.1 M Tris-HCl, pH 7.4). The sections were rinsed with deionized H_2O, dehydrated, cleared in xylene, and mounted in Permount® (Fisher Scientific).

RESULTS

Isolation of Ha-CalpM

Two pairs of degenerate primers were synthesized based on the conserved amino acid sequences from multiple alignments of calpain genes (Fig. 3-1). For the isolation of partial cDNA sequences of lobster calpains, nested PCR was used with the plasmid DNA of a lobster cDNA library from deep abdominal muscle as template. A 600-bp PCR product was amplified, purified and cloned into a pGEM vector (Fig. 3-2). Five partial cDNA clones ranging in size between 500 and 600 bp were isolated (Fig. 3-3) and sequenced. The sequences of the clones revealed a high homology with members of the calpain gene family, especially with *Drosophila* calpains (Fig. 3-4). These partial sequences also share significant sequence identity with each other (Fig. 3-5).

In order to obtain a full-length cDNA sequence of lobster calpain, inverse PCR (IPCR) was used to amplify the sequences flanking the initial PCR product. Inverse primers were synthesized based on the sequence of one of the five partial clones. Since the templates were plasmid DNA from lobster deep abdominal muscle cDNA library, the expected size of the IPCR products would be the length of the calpain gene plus the vector (3-kb). A 5-kb product (Fig. 3-6) was amplified. The IPCR product was cloned and the longest insert containing clone was used for sequencing the ends (Fig. 3-7). The end sequences of lobster calpain are located and primers synthesized. The final full-length calpain gene was amplified with the 3' and 5' end primers (Fig. 3-8, 3-9) and sequenced.

The full-length Ha-CalpM cDNA consists of 1977 nucleotides (Fig. 3-10). It contains a potential initiation codon that obeys Kozak's rule (Kozak, 1984). The position of the first methionine codon is similar to that of *Drosophila* CalpA and CalpB (Theopold *et al.* 1995;, Jekely *et al.* 1999). The Ha-CalpM cDNA has a 5' UTR of 15 bp and a 3' UTR of 234 bp. There is a high percentage of A's (40%) and T's (35%) within the 3' UTR. No poly-A tail was identified, although a sequence of "AATAA" (similar to "AATAAA, the polyadenylation signal) is located at the 3' end of the sequence. The high percentage of A's suggests that the Oligo (dT) primers used for cDNA synthesis annealed to this region, rather than at the poly (A) tail located further downstream. The ORF (open reading frame: #16-1743) of 1725 bp encodes a protein of 575 amino acids with a predicted molecular weight of 66.3 kDa. The domain structure of Ha-CalpM was assigned by sequence homology to *Drosophila* CalpA and CalpB. Domain I (#1-110) has a unique N-terminal sequence (#1-47) that has no known sequence homology with other calpains. Domain II (#111-396) consists of a highly-conserved cysteine protease sequence with the catalytic triad (Cys 141, His 299, Asn 329) and a putative Ca^{2+}-binding region (#366-376); Domain III (#397-575) contains an acidic loop with an expanded stretch of an additional 7 aspartates (#454-470) that forms an acidic loop may bind Ca^{2+} and function as an "electrostatic switch" for activating the protease (Strobl et al., 2000). Ha-CalpM is a truncated form with domain IV (a calmodulin-like domain) absent (Fig. 3-10, 3-13).

Alignment of the deduced amino acid sequence of Ha-CalpM with *Drosophila* and human calpains is shown in Fig. 3-11. Ha-CalpM has the highest sequence homology with *Drosophila* calpains (A and B, 50% of identity, 67% similarity). Besides *Drosophila* calpains, Ha-CalpM also has significant sequence similarities with calpain 9, digestive tract-specific calpain (64%, AF022799), and calpain 3, skeletal muscle specific calpain (62%, AF127765) (sequence not shown).

The similarity to human m-calpain is 60%. A gene tree was generated comparing the domain II sequence of Ha-CalpM with that of selected calpains representing diverse phyla (Fig. 3-12). Ha-CalpM clusters with *Drosophila* CalpA and CalpB; the human p94, m-calpain, and mouse nCL-4 form a distinct, but related group; mouse Calp5 and *tra-3* calpain are more divergent. *Shistosoma* calpain served as a outgroup. The domain organizations Ha-CalpM and other calpains are compared in Fig. 3-13.

Ha-CalpM Expression in tissues

To determine Ha-CalpM tissue specificity and its size of transcripts, Northern blotting was used with RNAs from various tissues. The membrane was hybridized with a DIG-labeled RNA probe synthesized from the conserved region of Ha-CalpM (domain II and III) under high stringency. The results show that Ha-CalpM is predominantly expressed in skeletal muscle (Fig. 3-14, lane a-c). Two major transcripts were detected ranging in size of 6.1 kb and 3.3 kb. A minor 4-kb band was also present in the fast muscles (cutter claw and deep abdominal). The probe hybridized in a broad smear to RNA from intestine, suggesting that the binding was nonspecific (Fig. 3-14, lane f).

RT-PCR was used to detect low levels of expression and to determine if the hybridization of the probe to intestine RNA was specific. Primers were synthesized to amplify the sequence encoding the unique N-terminal sequence of Ha-CalpM. As expected, a large amount of PCR product (about 700 bp) was synthesized from muscle RNAs (Fig. 3-15, lane a-c). Low amounts were generated from gill, intestine, and integument (Fig. 15, lanes, e, f, h), indicating that the hybridization in the Northern blot to intestine RNA was non-specific. No product was detected from

nerve cord, antennal gland, or ovary (Fig. 3-15, lanes g, l). Surprisingly, a large amount of PCR product was obtained from digestive gland RNA (Fig.15, lane d), even though the Northern blot showed no hybridization to the Ha-CalpM probe (Fig. 3-14, lane d). This discrepancy may be due to different primers/probe used for the two methods. The probe for the Northern blot was from part of the catalytic domain and the primers for the RT-PCR are located at the 5' end of the gene.

Since calpains are involved in molt-induced claw muscle atrophy, RT-PCR and real-time PCR were used to quantify Ha-CalpM mRNA level during the molting cycle. Total RNA was isolated and the cDNA were synthesized. RT-PCR showed very little changes of Ha-CalpM expression among the molting muscles (Fig. 3-16). For real-time PCR (Fig. 3-17), SYBR Green I fluorescent dye was included in the PCR reaction to quantity the synthesis of double-stranded DNA. The peak area of the melting curve analysis of PCR products was used to measure the relative amount of Ha-CalpM message. Ha-CalpM mRNA was lower in intermolt and postmolt stages, but higher in premolt stages. At premolt stage D_3, Ha-CalpM message reached the highest level, about 2.3-fold greater than that in intermolt. The difference of expression among molting claws is significant (P-value <0.001, Anova Single Factor analysis). The product specificity was determined by running the PCR samples on 1% agarose gel

For the analysis of Ha-CalpM protein expression, Western blotting and immunocytochemistry were used. A 28 aa polypeptide at the N-terminal of Ha-CalpM deduced amino acid sequence was synthesized and used to raise a polyclonal antibody in rabbit. Total protein from various tissues of lobster (at premolt stage) were extracted and separated. The gels were Coomassie blue stained, blotted onto PVDF membrane. The Ha-CalpM antibody detected the calpain protein only in skeletal muscle tissues-one 62 kDa band in cutter claw and crusher claw, and one 68 kDa protein in deep abdominal muscles (Fig. 3-18). The calpain proteins were

recognized both by crude antiserum and by antibodies that have been affinity purified. The detected 68-kDa protein is in good agreement with the size expected from the Ha-CalpM gene. The 62-kDa Ha-CalpM was expressed in claw muscles at all stages of the molting cycle (Fig. 3-19). As levels were quite variable in intermolt muscle, the higher levels of Ha-CalpM in premolt and postmolt claw muscles were not significantly different.

The mass of the deduced sequence (66.3 kDa) and masses of the Ha-CalpM proteins in Western blots (62 kDa and 68 kDa) suggested that Ha-CalpM cDNA encoded either CDP IIa (60 kDa subunit mass; 125 kDa native mass) or CDP III (59 kDa native mass). A soluble extract of intermolt lobster muscles (claws and abdominal) containing all four calpain activities was subjected to gel filtration chromatography. Samples of fractions #16-48 were separated on 10% SDS-PAGE and blotted onto PVDF membrane. The Ha-CalpM proteins eluted from the column at a position corresponding to CDP III. The 68-kDa protein was detected starting at fraction #32; the protein intensity increased to a peak at fraction #36 (Fig. 3-20, upper arrowhead). The 62-kDa protein was detected starting at fraction #42 (Fig. 3-20, lower arrowhead).

Immunocytochemistry on thoracic sections of juvenile lobster showed that the Ha-CalpM antibody stained ubiquitously among all tissues (Fig. 3-21). The cutter and crusher claw sections stained with anti Ha-CalpM antibody show that the staining was primarily cytoplasmic, although some nuclei were stained (Fig. 3-22).

DISCUSSION

I reported here the first identification of a crustacean calpain gene. This gene shares high sequence homology with other calpains. I showed that Ha-CalpM is predominantly expressed in skeletal muscle tissues and up-regulated in atrophied claw muscles associated with molting. The domain organization of Ha-CalpM is similar to that of other calpains. It

consists of three domains with domain IV absent. The first 75 amino acid residues of domain I is unique, showing no sequence identity with any calpain genes. The last 35 amino acid residues of domain I have about 40% sequence identity with the same region of *Drosophila* calpains. Domain II possesses the three amino acid residues (Cys 141, His 299, Asn 329) that are essential for the active site of cysteine proteases (Berti and Storer, 1995). The most striking feature of Ha-CalpM is that domain III contains an acidic stretch of 17 aspartates. An aspartate-rich domain has been reported in calsequestrin, a high-capacity, moderate-affinity, Ca^{2+}-binding protein (Treves *et al.*, 1992). Calsequestrin acts as an internal Ca^{2+} store in fast muscle fibers. Analysis of the crystal structure of m-calpain revealed that the acidic region may interact with Ca^{2+} and function as an "electrostatic switch" for protease activity (Strobl *et al.*, 2000). The X-ray structures of rat and human m-calpain also show that domain III is engaged in extensive ionic interactions with all the other domains within the large subunit and thus seems to function as an organizing center of calpain (Hosfield *et al.*, 1999b; Strobl *et al.*, 2000). These data suggest that domain III of Ha-CalpM may play a major role for activating the enzyme upon Ca^{2+} binding.

The overall similarities at the predicted amino acid sequence level between Ha-CalpM and others suggest its functional conservation. Ha-CalpM has the highest sequence homology with *Drosophila* calpains (50% of identity, 67% similarity). Ha-CalpM has high sequence similarities to mammalian calpains, such as calpain 9, digestive tract-specific calpain, calpain 3, skeletal muscle-specific calpain. Like *Drosophila* calpains, the Ha-CalpM gene product is not expected to form heterodimers with a small subunit (Theopold et al., 1995; Jekely et al., 1999). It lacks domain IV, which is required for interactions between the large and small subunits of mammalian μ- and m-calpains (Crawford *et al.*, 1993). Even though Ha-CalpM lacks a calmodulin-like domain, it is likely that the enzyme would have Ca^{2+}-dependent proteinase activity. Excision of domain III reduces enzyme activity, while having little effect on Ca^{2+} sensitivity (Vilei *et al.*, 1997). An expressed recombinant human m-calpain possessing only domain II retains Ca^{2+} dependency (Hata et al., 2001). Ca^{2+}-

binding regions in domains II and III are important in enzyme regulation. Based on the available evidence it is likely that Ha-CalpM function as a Ca^{2+}-dependent cysteine proteinase, although the demonstration of activity must be done on expressed recombinant Ha-CalpM with functional assays.

Ha-CalpM is classified as an "atypical" calpain, since it lacks a calmodulin-like region (domain IV). Other atypical calpains have been characterized in human (nCL-2', CAPN5), mouse (CAPN6), and *Drosophila* (Dm-CalpA') (Sorimachi et al., 1993, Dear et al. 1997, Theopold et al., 1995). These genes have various degrees of tissue specificity. The preferential expression of Ha-CalpM in skeletal muscle is not unusual. CAPN3 is skeletal muscle specific, Lp82, an alternative splice product of p94 was found only expressed in lenses; (Penny *et al.*, 1985; Ma *et al.*, 1998), and CAPN 9 is expressed in stomach and small intestine only.

Two major transcripts of Ha-CalpM (6.1 kb and 3.3 kb) were detected in Northern blots. An additional minor transcript (4 kb) is expressed in fast muscle. This suggests that the Ha-CalpM encodes the same full-length ORF but transcripts may differ in UTR's, perhaps due to alternative polyadenylation signals in the 3' UTR. RT-PCR confirmed the high levels of expression in skeletal muscle. RT-PCR also generated a large amount of product in digestive gland, even though little transcript was detected on Northern blots. A possible explanation of this discrepancy is that the probe for the Northern blot and the primers for the RT-PCR directed to different regions of Ha-CalpM cDNA. A segment of a 500-bp highly-conserved region in the protease domain was used for the synthesis of RNA probe for the Northern blots, whereas the RT-PCR primers were directed to the unique N-terminal domain at 5' end of the gene. A more likely explanation is that the RT-PCR was nonlinear, thereby producing comparable amounts of product from mRNA at levels in the digestive gland below the detection limits of the Northern blot analysis. Comparison of the domain II sequences of Ha-CalpM and mammalian calpains (Fig. 3-12) indicates that Ha-CalpM sequence is most similar to the mammalian skeletal muscle-specific calpain (CAPN3

or p94), which suggests that it is the lobster homolog of p94. Western blot analysis showed that Ha-CalpM proteins (62 kDa and 68 kDa) were only expressed in skeletal muscles. However, immunocytochemistry using the Ha-CalpM antiserum showed expression in all lobster tissues. This ubiquitous staining was observed with affinity-purified antibody, indicating that the binding of the antibody was specific. However, the anti-Ha-CalpM IgG titers in the antiserum are quite high, which necessitates high dilutions for immunocytochemistry (a 1:5,000 dilution was used in this study). Thus, the antiserum concentration may have to be diluted even further to show preferential staining of skeletal muscles. Taken together, these data indicate that Ha-CalpM is a muscle-specific calpain.

Western blots of gel filtration chromatography fractions show a major 68-kDa protein and a minor 62-kDa protein that elute from the column at the same position as CDP III (native mass 59 kDa). The other three calpains (CDP I, CDP IIa, and CDP IIb) elute earlier from the column. CDP IIb (native mass 195 kDa) is a homodimer of a 95-kDa subunit, while CDP IIa (native mass 125 kDa) is a homodimer of a 60-kDa subunit. The subunit composition of CDP I (native mass 310 kDa) is not known. The 68-kDa protein is similar to the predicted mass (66.3 kDa) of the deduced amino acid sequence of Ha-CalpM. These data indicate that the native CDP III is composed of a single polypeptide encoded by the Ha-CalpM gene. However, the identity of Ha-CalpM and CDP III remains to be established. The 62-kDa and 68-kDa protein are probably encoded by the same gene, since both proteins react specifically to antibody raised against the unique N-terminal sequence. The 62-kDa protein could either be the active form of calpain produced by autolysis of the 68 kDa protein or a second isoform generated by alternative splicing or post-translational processing.

Jekely et al. (1999) reported that autolysis is a prerequisite and not a consequence of the activation of *Drosophila* CalpB. The authors further addressed that the almost identical position of the autolytic cleavage site in vertebrate and *Drosophila* calpains reflects conserved structural features even in the sequentially divergent N-terminal region. This

conserved structural features even in the sequentially divergent N-terminal region. This conservation indicates certain functional constraints and therefore the characteristics of autolysis in *Drosophila* calpain point to similar regulation in the vertebrate enzymes (Jekely et al., 1999). I propose that a similar autolysis for activation can be applied to the Ha-CalpM. These results suggest that the regulation of lobster skeletal muscle calpain is similar to that observed in Japanese quail, in which calpain activity in skeletal muscle is regulated at three levels: regulation at the transcriptional level, regulation at the translational level, and regulation by autolytic activation.

Crustacean muscles contain four calcium-dependent cysteine proteinases (CDPs or calpains) that are involved in the degradation of myofibrillar proteins during programmed claw muscle atrophy associated with molting (Mykles, 1992). The mRNA level of Ha-CalpM increases during premolt, reaching its highest level about 2.3-fold greater at stage D_3 than that in intermolt claw muscle. Moreover, Western blot analysis indicates that the levels of the 62-kDa protein increase during late premolt and reach a maximum level in postmolt claw muscles. These data suggest that Ha-CalpM is involved in the termination of atrophy and/or regrowth of the muscle after ecdysis. This is analogous to the p94 calpain in mammalian skeletal muscle. The activity of p94 calpain is necessary to maintain muscle fiber viability and myofibrillar protein composition. Mutations in the p94 gene that result in reduced p94 protein are the underlying cause of limb girdle muscular dystrophy type 2A in humans. The p94 calpain appears to help maintain the structural integrity of the contractile apparatus through its interaction with titin.

In summary, the full-length cDNA encoding an atypical calpain expressed in lobster skeletal muscle has been characterized. This is the first calpain gene isolated from a crustacean species. It appears to encode the CDP III activity characterized in biochemical studies. Although the four crustacean calpains appear to be products of different genes, the

precise relationship between Ha-CalpM and CDPs I, IIa, and IIb will not be established until the full-length sequences of the other calpains are determined. The data suggest that Ha-CalpM plays a role in regulating skeletal muscle mass during the molting cycle.

Fig.3-1 Design of degenerate primers. The deduced amino acid sequences of calpain catalytic subunit from diverse species were aligned for the search of the conserved sequences. The "*" indicates the identical residues, the dot "." shows the residues are similar. Arrows indicate the sequences selected for the primers. The external primers are: 1F (forward): LGDCWL/FL [5' C(T)TN GGN GAC(T) TGC (T) TGG C(T)TN C(T)T 3'], 1B (backward): DGEFWMS [C(G)T(A) CAT CCA G(A)AA T(C)TC NCC G(A)TC 3']. The internal primers: 1F'(forward): YAGIFHF [5' TAC(T) GCN GGN ATI TTC (T) CAC(T) TT 3'], 1B' (backward): RM/I/LRNPWG [5' NCC CCA NGG G(A)TT NCT (G) NAT (A)(G) NC 3']. GeneBank accession numbers are in parenthesis. Mouse (AJ012475): mouse m-calpain. Schistosoma (P27730): *Schistosoma mansoni* calpain. Tra-3 (U14480): *Caenorhabditis elegance* calpain . nCL-4 human (AF022799): human digestive tract calpain Dm (Q11002): *Drosophila* melanogaster CalpA. *Coturnix* (AB011080): quail calpain.

Multiple Alignment of Calpains

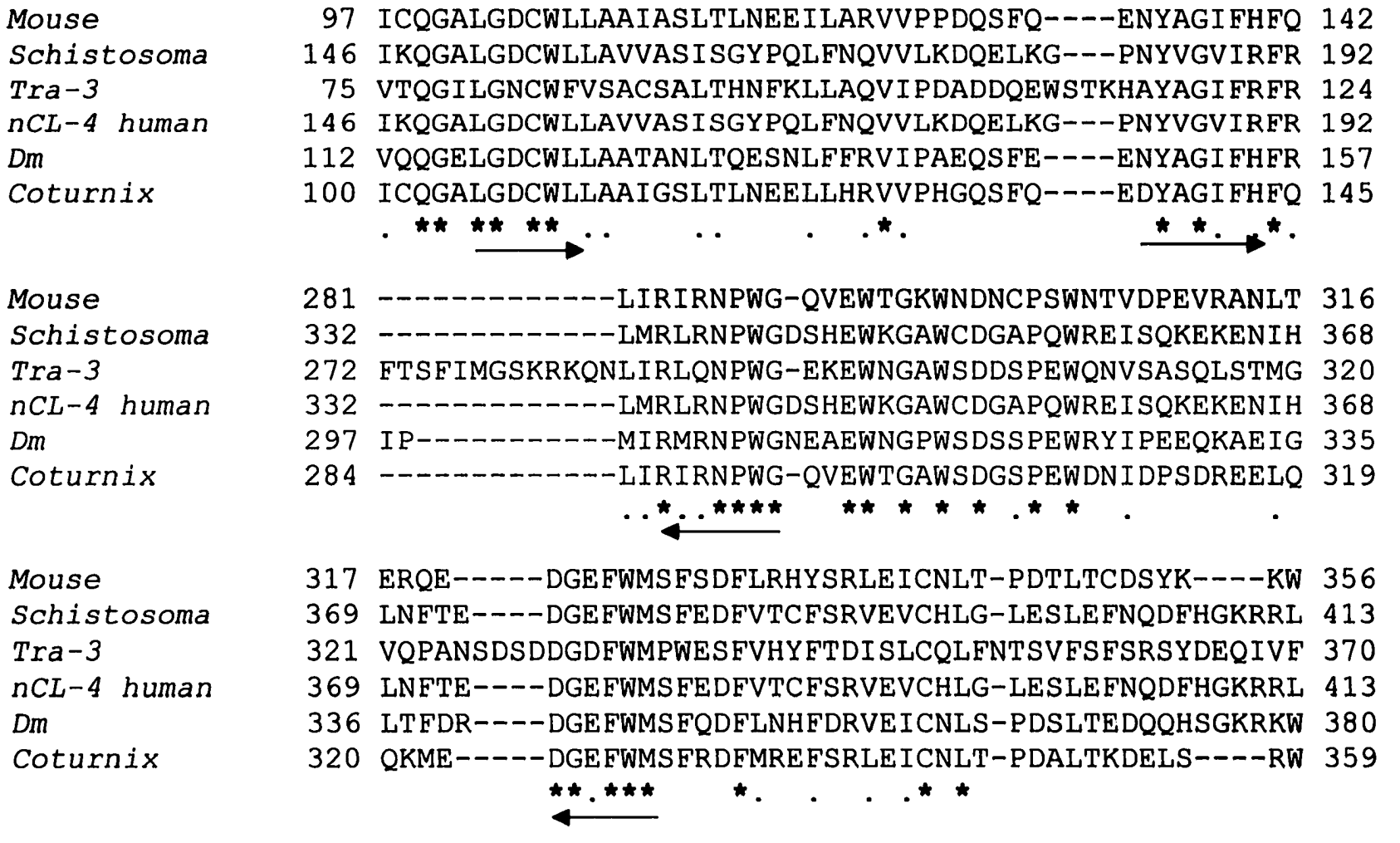


Fig. 3-2. Nested PCR using degenerate primers to highly conserved sequences in the protease domain of calpains. First round PCR was performed using the external primers (1F and 1B) on plasmid DNA from a cDNA library of lobster deep abdominal muscle. An aliquot (0.1 μl) of this PCR reaction was then re-amplified with the internal primers (1F' and 1B'). A single product of about 600 bp was generated (arrowhead). M, molecular markers.

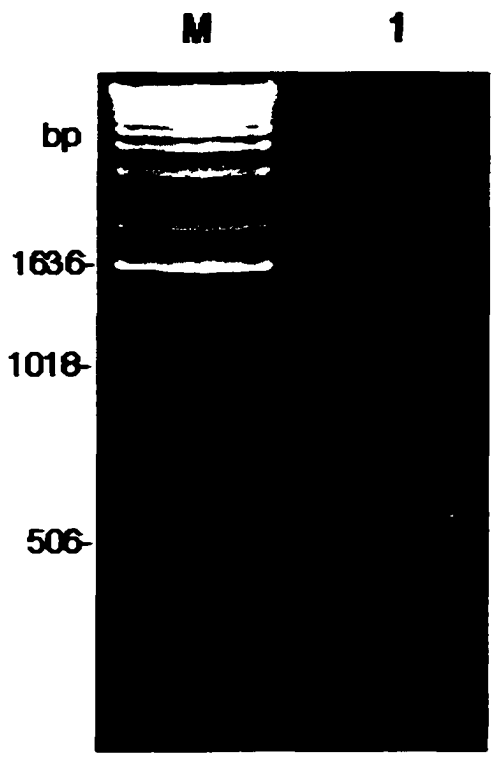


Fig. 3-3. Digestion of lobster calpain partial clones with *EcoRI*. Plasmid DNA was digested with *EcoR*I to release the insert. Lanes 1-6 show selected transformants with different sizes of inserts (about 700 bp, arrow). M, molecular weight markers.

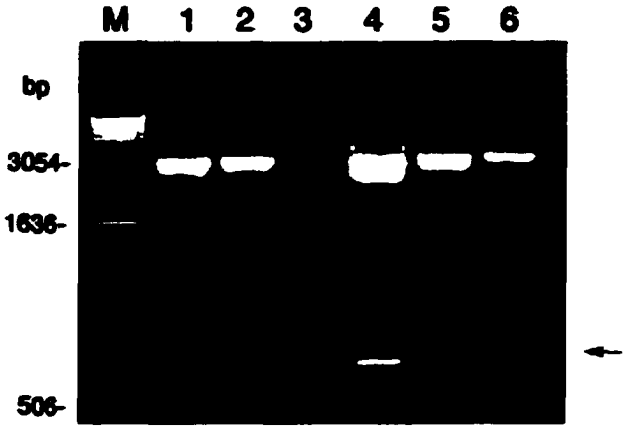


Fig. 3-4. BLAST results of the calpain partial clones. Four clones were sequenced on both strands. The deduced amimo acid sequences were compared in the database using BLAST. The sequences show high homology with Drosophila calpains. Query indicates the calpain partial sequences.

C500

```
gi|17137010|ref|NP_477047.1| (NM_057699) CalpA-P1 [Drosophila melanogaster]
 gi|7302479|gb|AAF57563.1| (AE003796) CalpA gene product [Drosophila melanogaster]
 gi|15291667|gb|AAK93102.1| (AY051678) LD22862p [Drosophila melanogaster]
          Length = 828

 Score =  335 bits (859), Expect = 2e-91
 Identities = 157/159 (98%), Positives = 158/159 (98%)
 Frame = +2
Query: 56  YAGIFHFRFWQYGKWVDVIIDDRLPTYNGELMYMHSTEKNEFWSALLEKAYARLHGSYEA 235
           YAGIFHFRFWQYGKWVDVIIDDRLPTYNGELMYMHSTEKNEFWSALLEKAYA+LHGSYEA
Sbjct: 173 YAGIFHFRFWQYGKWVDVIIDDRLPTYNGELMYMHSTEKNEFWSALLEKAYAKLHGSYEA 232
Query: 236 LKGGSTCEAMEDFTGGVSEWYDLKEAPGNLFTILQKAAERNSMMGCSIEPDPNVTEAGTP 415
           LKGGSTCEAMEDFTGGVSEWYDLKEAPGNLFTILQKAAERNSMMGCSIEPDPNVTEA TP
Sbjct: 233 LKGGSTCEAMEDFTGGVSEWYDLKEAPGNLFTILQKAAERNSMMGCSIEPDPNVTEAETP 292
Query: 416 QGLIRGHAYSITKVCLIDIVTPNRQGKIPMIRMRNPWGN 532
           QGLIRGHAYSITKVCLIDIVTPNRQGKIPMIRMRNPWGN
Sbjct: 293 QGLIRGHAYSITKVCLIDIVTPNRQGKIPMIRMRNPWGN 331
C4
gi|17137010|ref|NP_477047.1| (NM_057699) CalpA-P1 [Drosophila melanogaster]
gi|7302479|gb|AAF57563.1| (AE003796) CalpA gene product [Drosophila melanogaster]
gi|15291667|gb|AAK93102.1| (AY051678) LD22862p [Drosophila melanogaster]
          Length = 828
 Score =  263 bits (672), Expect = 2e-70
 Identities = 122/124 (98%), Positives = 123/124 (98%)
 Frame = +2
Query: 2   SYEALKGGSTCEAMEDFTGGVSEWYDLKEAPGNLFTILQKAAERNSMMGCSIEPDPNVTE 181
           SYEALKGGSTCEAMEDFTGGVSEWYDLKEAPGNLFTILQKAAERNSMMGCSIEPDPNVTE
Sbjct: 229 SYEALKGGSTCEAMEDFTGGVSEWYDLKEAPGNLFTILQKAAERNSMMGCSIEPDPNVTE 288
Query: 182 AGTPQGLIRGHAYSITRVCLIDIVTPNRQGKIPMIRMRNPWGNEAEWNGPWSDSSPEWRY 361
           A TPQGLIRGHAYSIT+VCLIDIVTPNRQGKIPMIRMRNPWGNEAEWNGPWSDSSPEWRY
Sbjct: 289 AETPQGLIRGHAYSITKVCLIDIVTPNRQGKIPMIRMRNPWGNEAEWNGPWSDSSPEWRY 348
Query: 362 IPEE 373
           IPEE
Sbjct: 349 IPEE 352
C2
gi|7294858|gb|AAF50189.1| (AE003550) CalpB gene product [Drosophila melanogaster]
          Length = 791
 Score =  103 bits (258), Expect = 3e-22
 Identities = 51/97 (52%), Positives = 67/97 (68%)
 Frame = +1
Query: 16  NEFWCALFEKAYAKLYGGYESLRGGNINESMVDLTGGVVEMIDLRNPPPKLFSTLWKAYR 195
           NEFW AL EKAYAKL+G YE+L+GG   E+M D TGGV E  D++  PP LFS + KA
Sbjct: 249 NEFWSALLEKAYAKLHGSYEALKGGTTCEAMEDFTGGVTEWYDIKEAPPNLFSIMMKAAE 308
Query: 196 RGALIXTAIEPGQSNIQAESIHSNGLIMRHAYSITRV 306
           RG+++  ++EP    ++AE+    GLI  HAYSIT+V
Sbjct: 309 RGSMMGCSLEPDPHVLEAET--PQGLIRGHAYSITKV 343
C5
gi|17647229|ref|NP_524016.1| (NM_079292) Calpain-B [Drosophila melanogaster]
gi|3820523|gb|AAD04331.1| (AF062404) calpain [Drosophila melanogaster]
          Length = 791
 Score =  140 bits (352), Expect = 3e-33
 Identities = 67/120 (55%), Positives = 87/120 (71%)
 Frame = -2
Query: 379 DDRLPTYNGRLVFMHSKTENEFWCALFEKAYAKLYGGYESLRGGNINESMVDLTGGVVEM 200
           DDRLPTYNG L++MHS  +NEFW AL EKAYAKL+G YE+L+GG   E+M D TGGV E
Sbjct: 230 DDRLPTYNGELIYMHSTEKNEFWSALLEKAYAKLHGSYEALKGGTTCEAMEDFTGGVTEW 289

Query: 199 IDLRNPPPKLFSTLRKAYRRGALIGTAIEPDQSNIQAESILSNGLIMRHAYSITRVTTVD 20
            D++  PP LFS + KA  RG+++G ++EPD    ++AE+    GLI  HAYSIT+V  +D
Sbjct: 290 YDIKEAPPILFSIMMKAAERGSMMGCSLEPDPHVLEAET--PQGLIRGHAYSITKVCLMD 347
```

Fig. 3-5. Alignment of amino acid sequences of the calpain partial clones. Clustal W (1.18) was used for the alignment. "*" indicates identical or conserved residues in all sequences in the alignment; ":" shows conserved substitutions, and "." indicates semi-conserved substitutions. The sequence names are on the left, and the numbers on the right indicate sequence positions.

```
CLUSTAL W (1.81) multiple sequence alignment


C500            YAGIFHFRFWQYGKWVDVIIDDRLPTYNGELMYMHSTEKNEFWSALLEKAYARLHGSYEA 60
C4              ------------------------------------------------------SYEA 4
C2              -----------------------------------NEFWSALLEKAYAKLHGSYEA 21
C5              -------------------DDRLPTYNGRLVFMHSKTENEFWCALFEKAYAKLYGGYES 40

.**:


C500            LKGGSTCEAMEDFTGGVSEWYDLKEAPGNLFTILQKAAERNSMMGCSIEPDPNVTEAGT-
119
C4              LKGGSTCEAMEDFTGGVSEWYDLKEAPGNLFTILQKAAERNSMMGCSIEPDPNVTEAGT- 63
C2              LKGGTTCEAMEDFTGGVTEWYDIKEAPPNLFSIMMKAAERGSMMGCSLEPDPHVLEAET- 80
C5              LRGGNINESMVDLTGGVVEMIDLRNPPPKLFSTLRKAYRRGALIGTAIEPDQSNIQAESI
100
                *:**.  *:* *:**** *  *::::.* :**: : ** .*.:::* ::***    :* :


C500            -PQGLIRGHAYSITKVCLIDIVTPNRQGKIPMIRMRNPWGN-------------------
159
C4              -PQGLIRGHAYSITRVCLIDIVTPNRQGKIPMIRMRNPWGNEAEWNGPWSDSSPEWRYIP
122
C2              -PQGLIRGHAYSITKV------------------------------------------- 95
C5              LSNGLIMRHAYSITRVTTVD--------------------------------------
120
                 .:***  ******:*


C500            --
C4              EE 124
C2              --
C5              --
```

Fig. 3-6. Inverse PCR (IPCR). A pair of inverse primers were synthesized based on the sequence of the nested PCR product. With the plasmid DNA of deep abdominal muscle cDNA library as template, IPCR resulted in a 5-kb product (arrowhead). This PCR product was cloned and sequenced with T7 and Sp6 vector primers to locate the 5' and 3' ends of the calpain sequence.

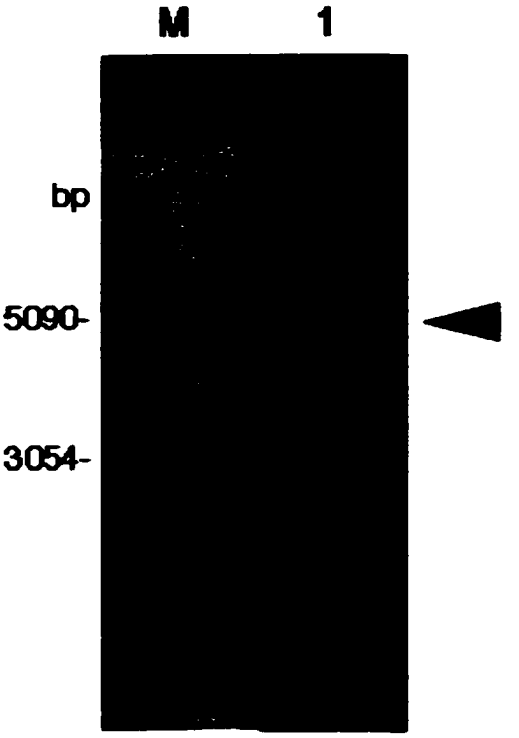


Fig. 3-7. Digestion of IPCR clones. Six individual clones of IPCR product were randomly selected for the digestion of EcoRI to release the inserts. The digestion released two fragments of the inserts (arrows). The longest insert containing clone was used for sequencing (lane 5). M, molecular weight markers.

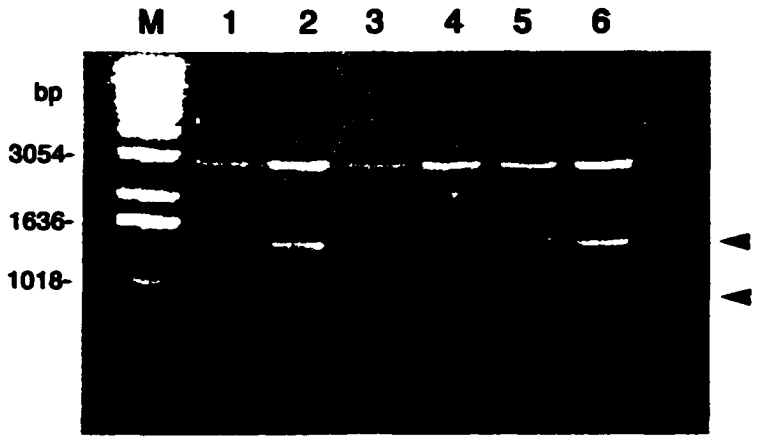


Fig. 3-8. PCR amplification of Ha-CalpM. A pair of 5' and 3' end lobster calpain sequence primers were synthesized, based on the sequences at the 3' and 5' ends of the calpain sequence obtained from IPCR. This primer pair was used for PCR amplification of a full-length sequence from a lobster deep abdominal muscle cDNA library. The product was about 2 kb (arrow).

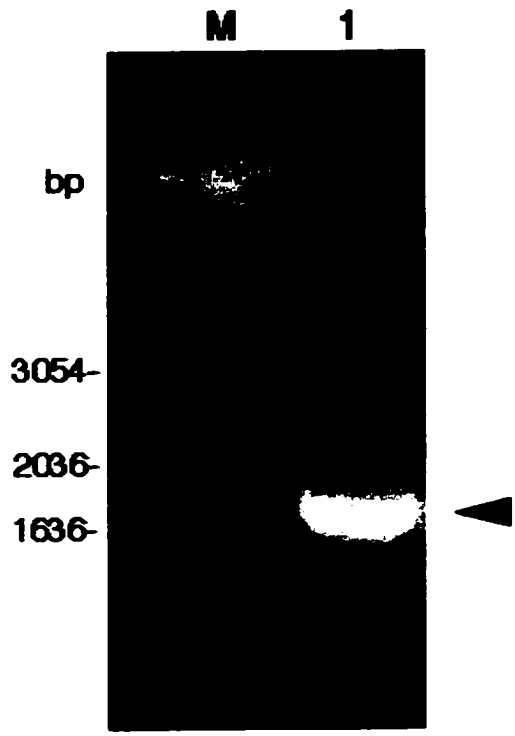


Fig. 3-9. Lobster calpain full-length clones cut with *EcoRI*. Six individual clones were used for the digestion; five of them contain the insert (arrow). Clone 6 was used for sequencing. M, molecular weight markers.

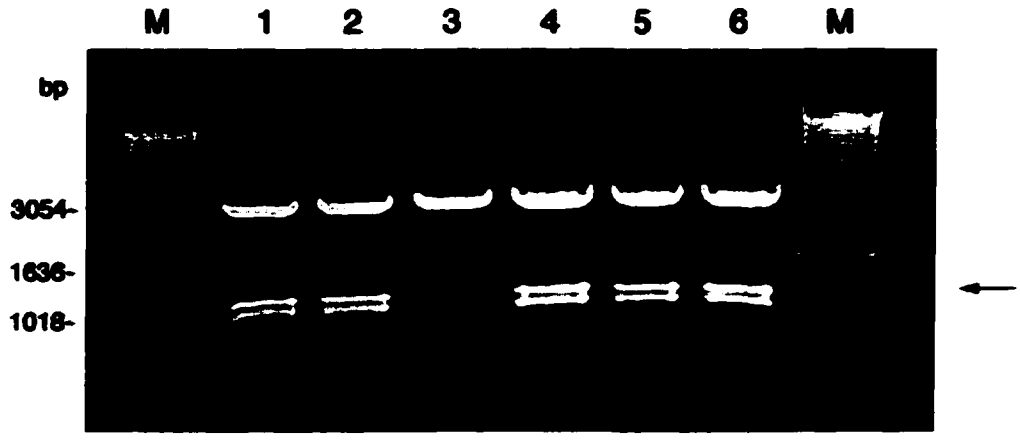


Fig. 3-10. The complete sequence of Ha-CalpM. The sequence (1977 bp) contains an open reading frame (#16-1743) encoding a protein of 575 amino acids with a predicted mass of 66.3 kDa. Ha-CalpM has three domains: domain I has a unique N-terminal sequence of 110 aa (#1-110); domain II (#111-386) consists of a highly conserved cysteine protease sequence; and domain III (#387-575) contains a highly acidic region, which includes an expanded stretch of 17 aspartates (bold). The three conserved amino acids (Cys 141, His 299, Asn 329) essential for hydrolase activity are indicated in bold. The numbers on the left indicate amino acid positions; those on the right indicate nucleotides. The amino acid sequences on which the two pairs of degenerate primers were based on are underlined. Ha-CalpM lacks a calmodulin-like domain (Domain IV) found in some calpain genes.

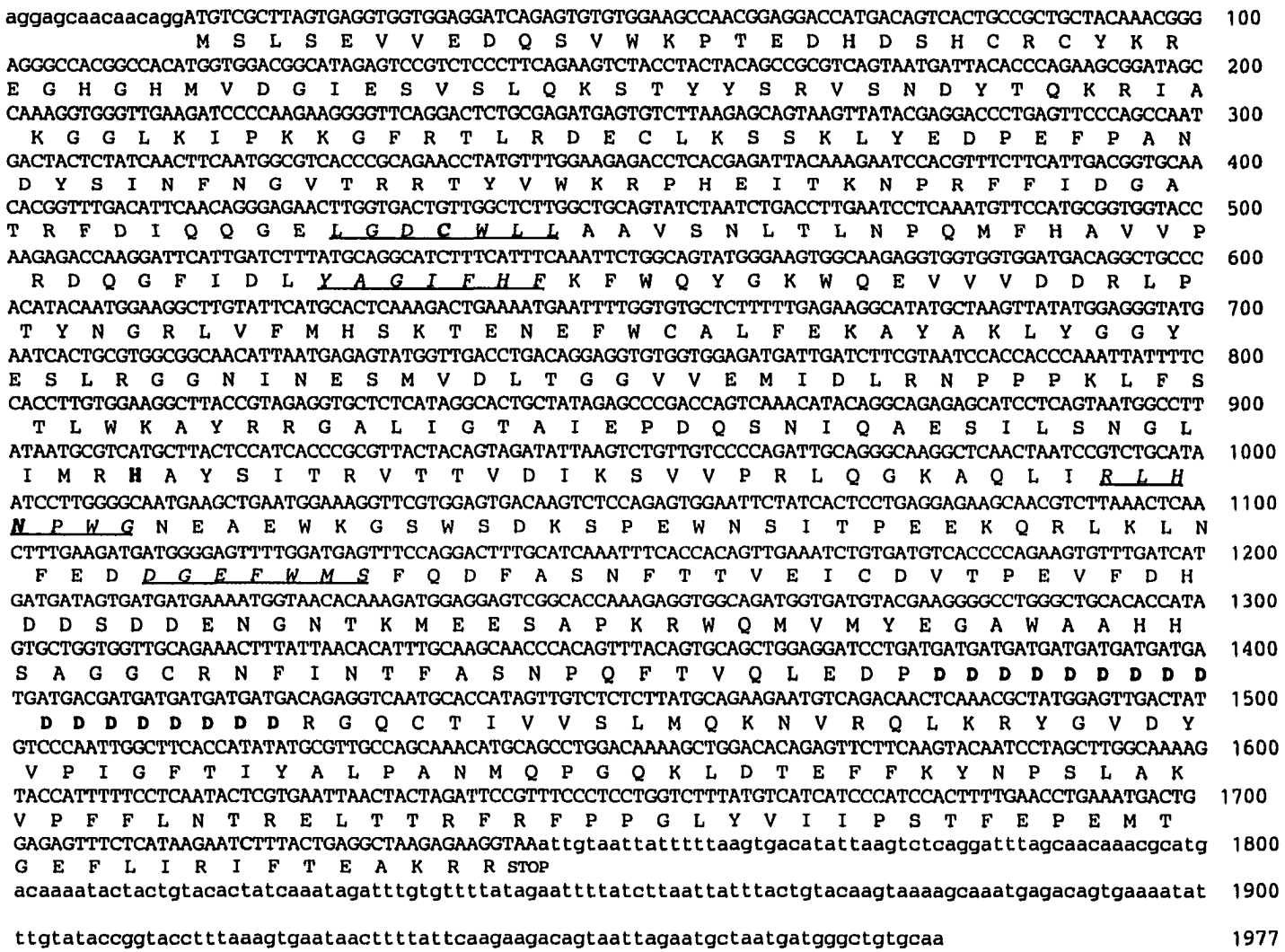
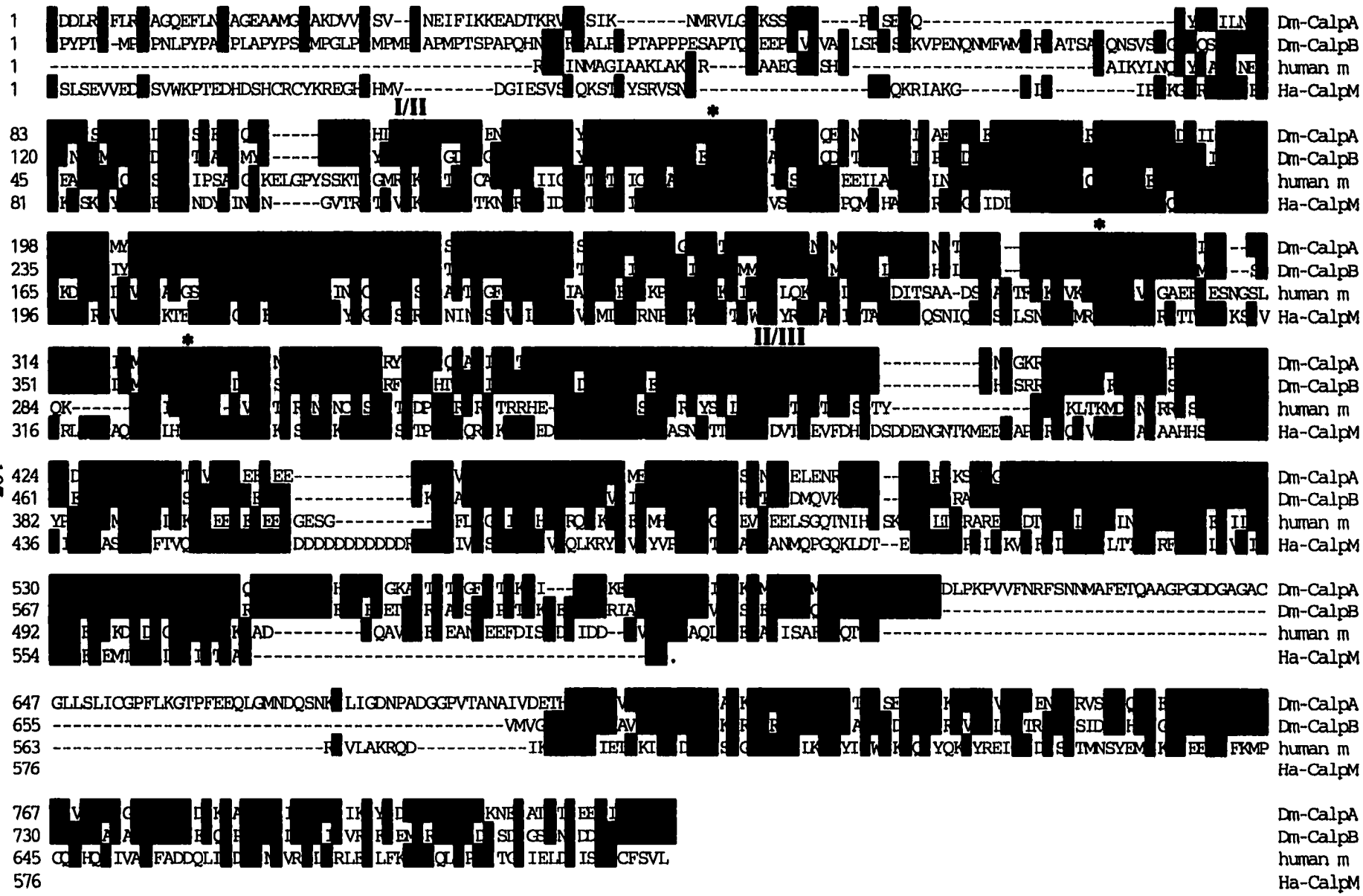


Fig. 3-11. Homology of Ha-CalpM with calpains from human and *Drosophila*. The deduced amino acid sequence of Ha-CalpM was aligned with *Drosophila* and human calpains by Clustal V method (DNASTAR). Amino acid residues that are identical to the consensus sequence are highlighted. Ha-CalpM is highly homologous to the *Drosophila* calpains (50% of identity, 67% similarity). The single-letter amino acid code is used; gaps (---) were introduced to optimize the alignment. The boundaries between domains I and II and II and III are indicated by I/II and II/III, respectively. The asterisk signs indicate the three amino acid residues of the catalytic triad of domain II. The GenBank accession numbers are Z46891 for *Drosophila* calpain A (Dm-CalpA), AF062404 for *Drosophila* calpain B (Dm-CalpB), and M23254 for human m-calpain (human m).



Decoration 'Decoration #1': Shade (with solid black) residues that match the Consensus exactly.

Fig. 3-12. The relationship of Ha-CalpM with other calpains as shown in gene tree. Amino acid sequences of domain II of calpains from selected species were compared using Clustal V method (DNASTAR). Branch lengths are proportional to the inferred phylogenetic distances. *Shistosoma* calpain domain II served as outgroup. Ha-CalpM was grouped together with *Drosophila* calpains (Dm-CalpA and CalpB), reflecting the high sequence similarity between the three sequences. GenBank accession numbers for *Drosophila* calpains and human m-calpain are as listed in the legend of Fig. 3-11; the accession numbers for the remaining calpains are X92523 for human p94, AF022799 for mouse nCL-4, M74233 for *Shistosoma mansoni* calpain, Y10656 for mouse calpain 5 (CAPN5), and U14480 for *C. elegance* tra-3.

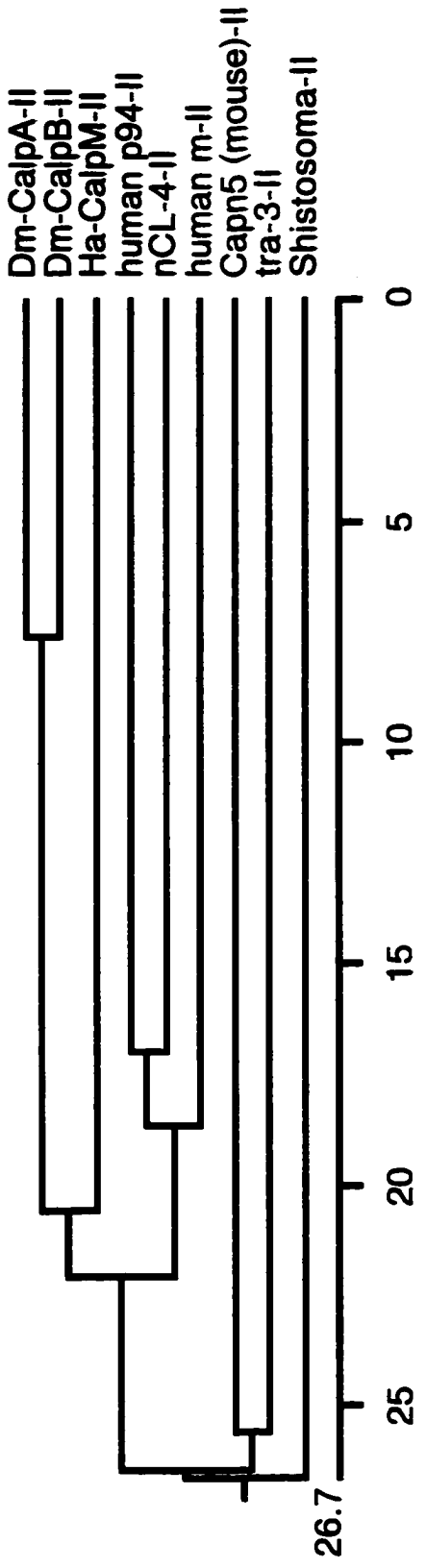


Fig. 3-13. Comparison of the gene organization of Ha-CalpM with other calpains. The organization of typical calpains consists of four domains (I, II, III and IV). The protease domain (II) is the most conserved with a catalytic triad consisting of cysteine (C), histidine (H), and asparagine (N). Domain III contains a C2-like motif incorporating an acidic loop, which is thought to mediate Ca^{2+}/phospholipid binding. Domain IV, when present, is a calmodulin-like Ca^{2+}-binding region containing five EF hand motifs. Ha-CalpM shares sequence homology in domains II and III with other calpains. It lacks domain IV.

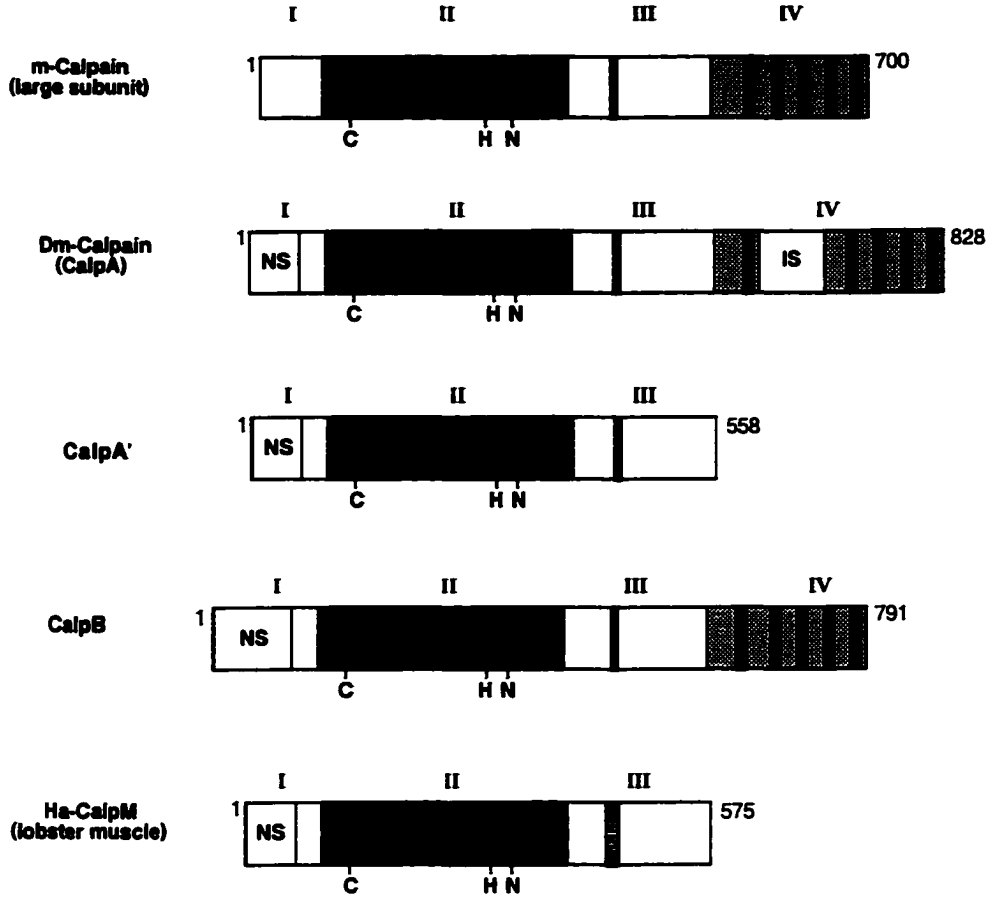


Fig. 3-14. Northern blot analysis. Total RNA (20 μg) was separated on a 1.0% agarose gel and blotted overnight. The membrane (A) was hybridized under high stringency with a DIG-labeled RNA probe synthesized from a region encompassing domains II and III of Ha-CalpM (see Materials and Methods). Ha-CalpM was highly expressed in cutter (Cu) and crusher (Cr) claw muscles and deep abdominal (DA) muscle (A, lane a-c). Three transcripts were detected (3.3, 4.0, 6.1 kb). The intestine (In; lane f) showed hybridization bands at high molecular weight range. Ha-CalpM mRNA was not detected in digestive gland (DG; lane d), gill (Gi; lane e), nerve cord (NC; lane g), integument (Ig; lane h), heart (ht; lane i), antennal gland (AG; lane j), or ovary (Ov; lane k). RNA loading was determined by staining the gel with ethidium bromide (B). M, molecular weight markers (sizes indicated at left of panel B).

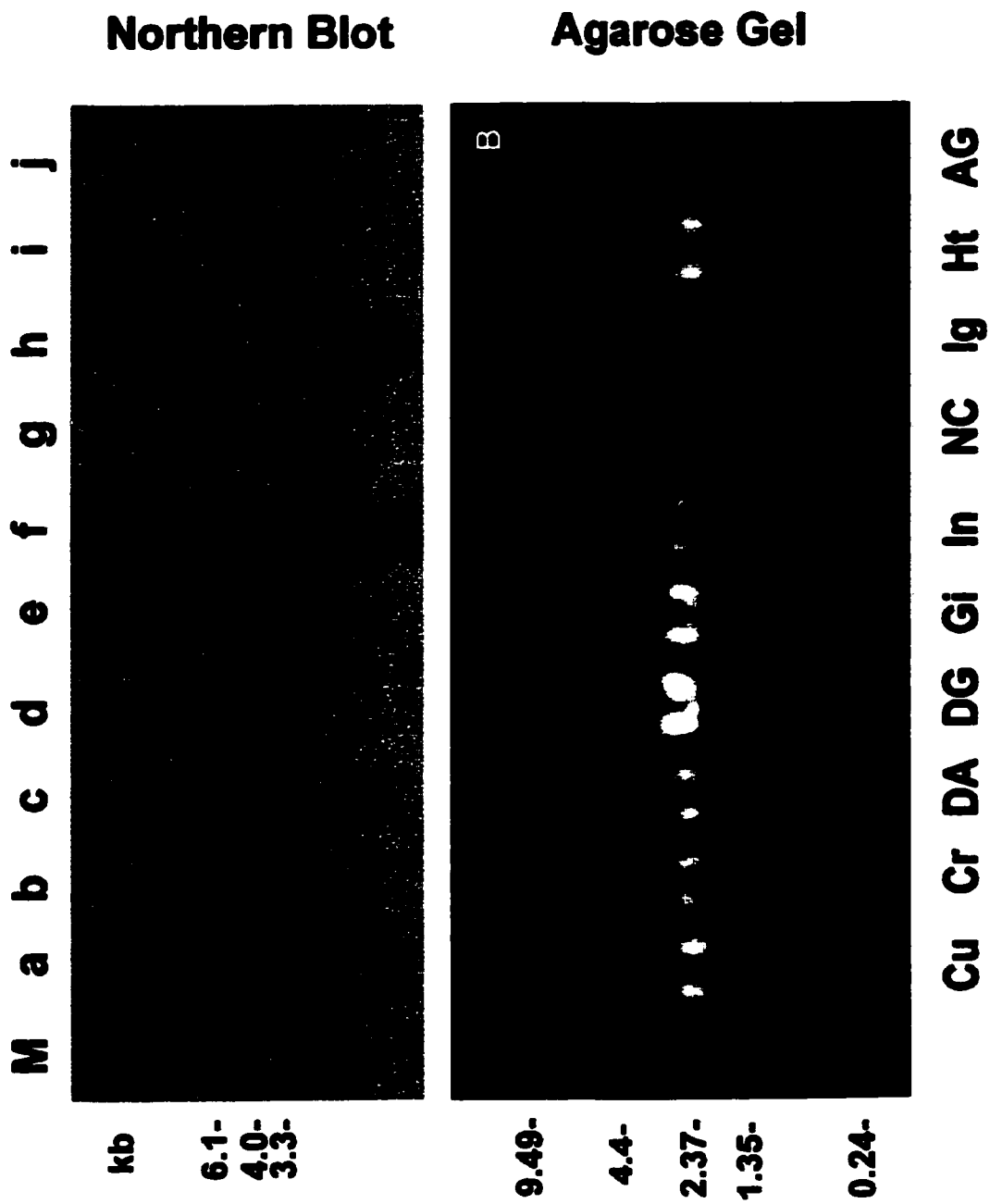


Fig. 3-15. RT-PCR of Ha-CalpM. Total RNA from various tissues were DNase treated, reverse transcribed, and PCR-amplified (see Materials and Methods). The primers were directed to the 5' end of Ha-CalpM sequence (about 700 bp). Shown is an image-inverted ethidium bromide-stained agarose gel of the PCR products. There were high amounts of Ha-CalpM message from cutter claw, crusher claw, deep abdominal muscle and digestive gland (lane a-d). Little or no product from other tissues was amplified (lane e-i). Same abbreviations as in the legend to Fig. 3-14.

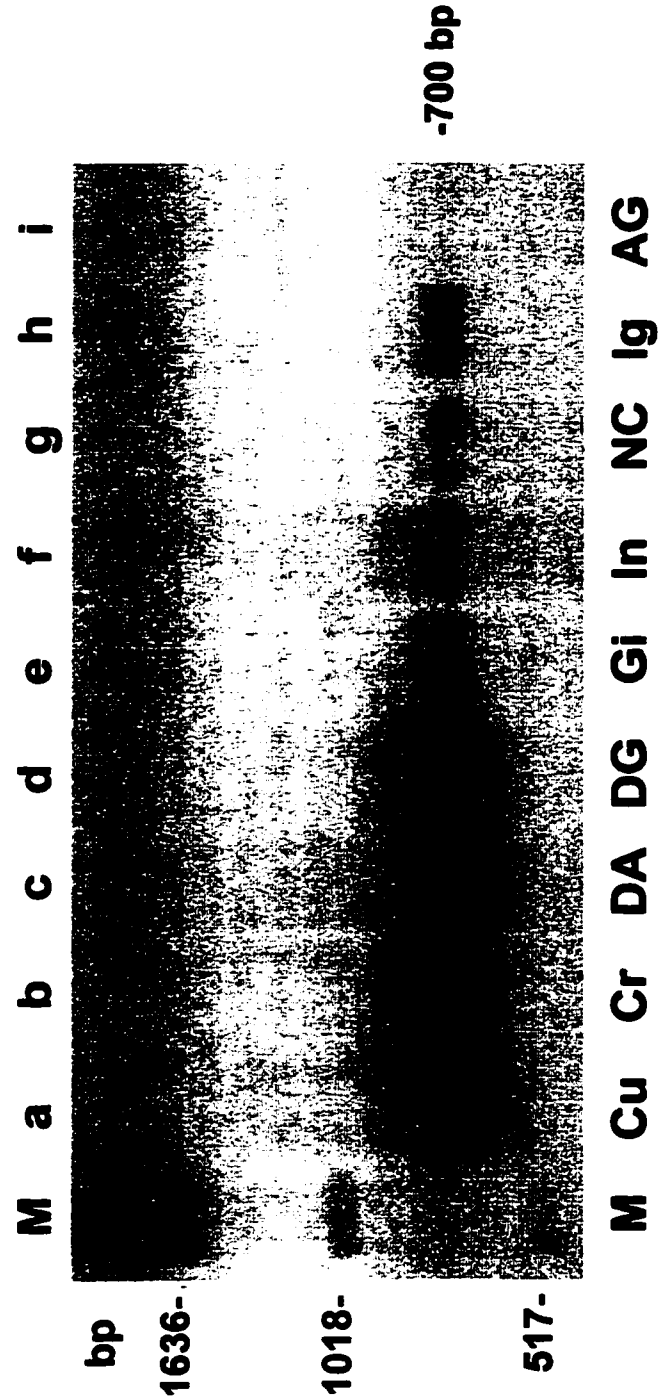


Fig. 3-16. RT-PCR of Ha-CalpM in Molting Claws. Total RNA samples from different molting claws were DNase treated, reverse transcribed and PCR-amplified. Shown is Ethidium-bromide-stained gel (image-inverted). All samples show the expected 700-bp product. The overall expression level shows very little change among the different stages of molting. There are also faint 500-bp bands on all the cutter claws. M, molecular weight marker; IM, Intermolt cutter claw; D_0, Premolt-D_0 cutter claw; D_3, Premolt-D_3 cutter claw; PM, Postmolt cutter claw. The first four lanes are cutter tissues. The last two lanes are: IM, Intermolt crusher claw; PM, Premolt-D_3 crusher claw.

RT-PCR of HaCalpM in Molting Claws

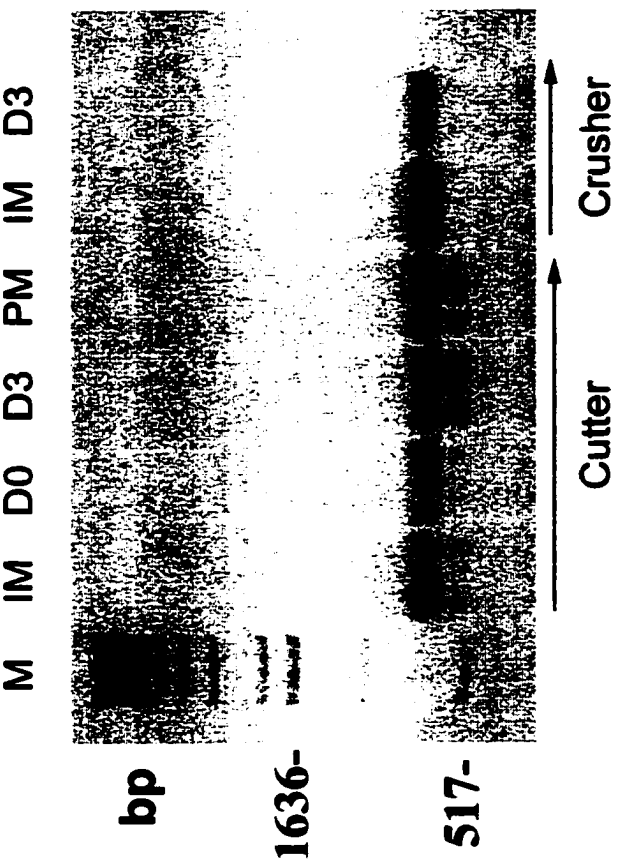
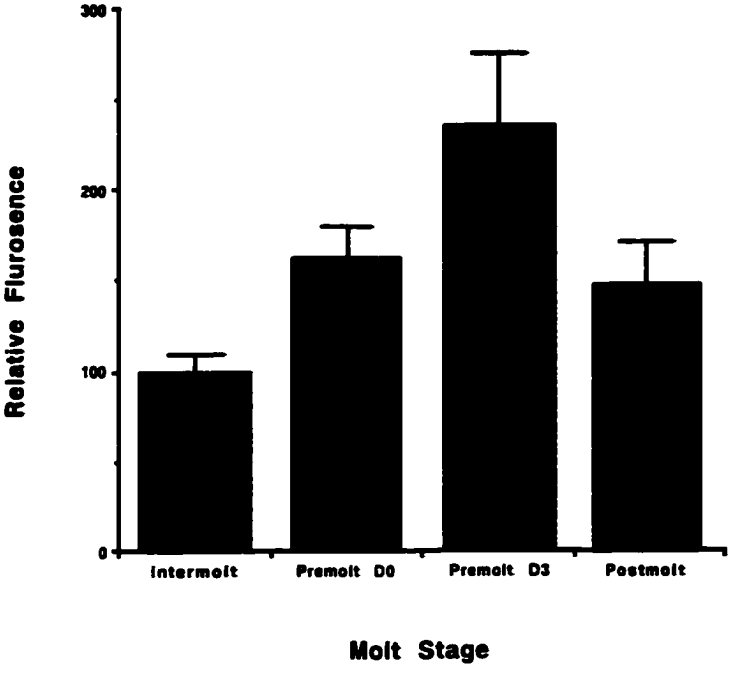


Fig. 3-17. Real-time PCR analysis of Ha-CalpM mRNA in lobster claw muscles during the molting cycle. Total RNA was DNase treated, reverse transcribed, and PCR-amplified (see Materials and Methods). SYBR Green I fluorescence dye was included in the amplification. The peak area of the melting curve analysis of the PCR products was used to measure the relative amount of Ha-CalpM message. The data was normalized to that of intermolt muscle. Three independent experiments were carried out, and the data are presented as mean ±1 standard deviation. Ha-CalpM mRNA increased during premolt, reaching its highest level at stage D3. The differences in the means were significant (P-value <0.001, Anova Single Factor analysis). The gel picture from one of the experiment was shown here for demonstrating the specificity of the products.



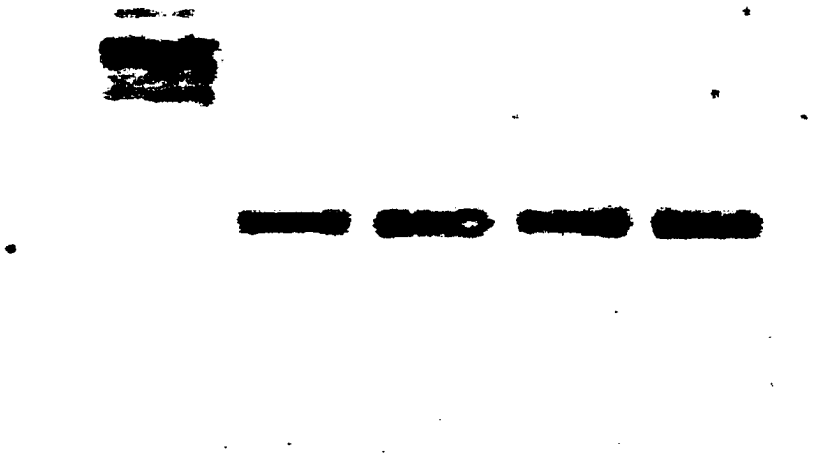


Fig. 3-18. SDS-PAGE and Western Blot on lobster tissues. Solution fractions (32 μg protein) from various tissues were separated on 6% SDS-polyacrylamide gels. The gels were either stained with Coomassie blue (C) or blotted onto PVDF membrane and probed with Ha-CalpM antiserum (A) or pre-immune serum (B). The Ha-CalpM antibody detected the calpain protein only in skeletal muscle: a 62-kDa protein in cutter claw (lane a) and crusher claw (lane b) and a 68 kDa protein in deep abdominal muscle (lane c). The two proteins co-migrated with 62- and 68-kDa proteins in fraction #48 from the gel filtration column (lane i). The non-specific staining of the antibody in lanes f-h are indicated by the asterisk signs. Molecular weight markers standards are indicated at left. Te, testis; all other abbreviations are the same as those in the legend to Fig. 3-14.

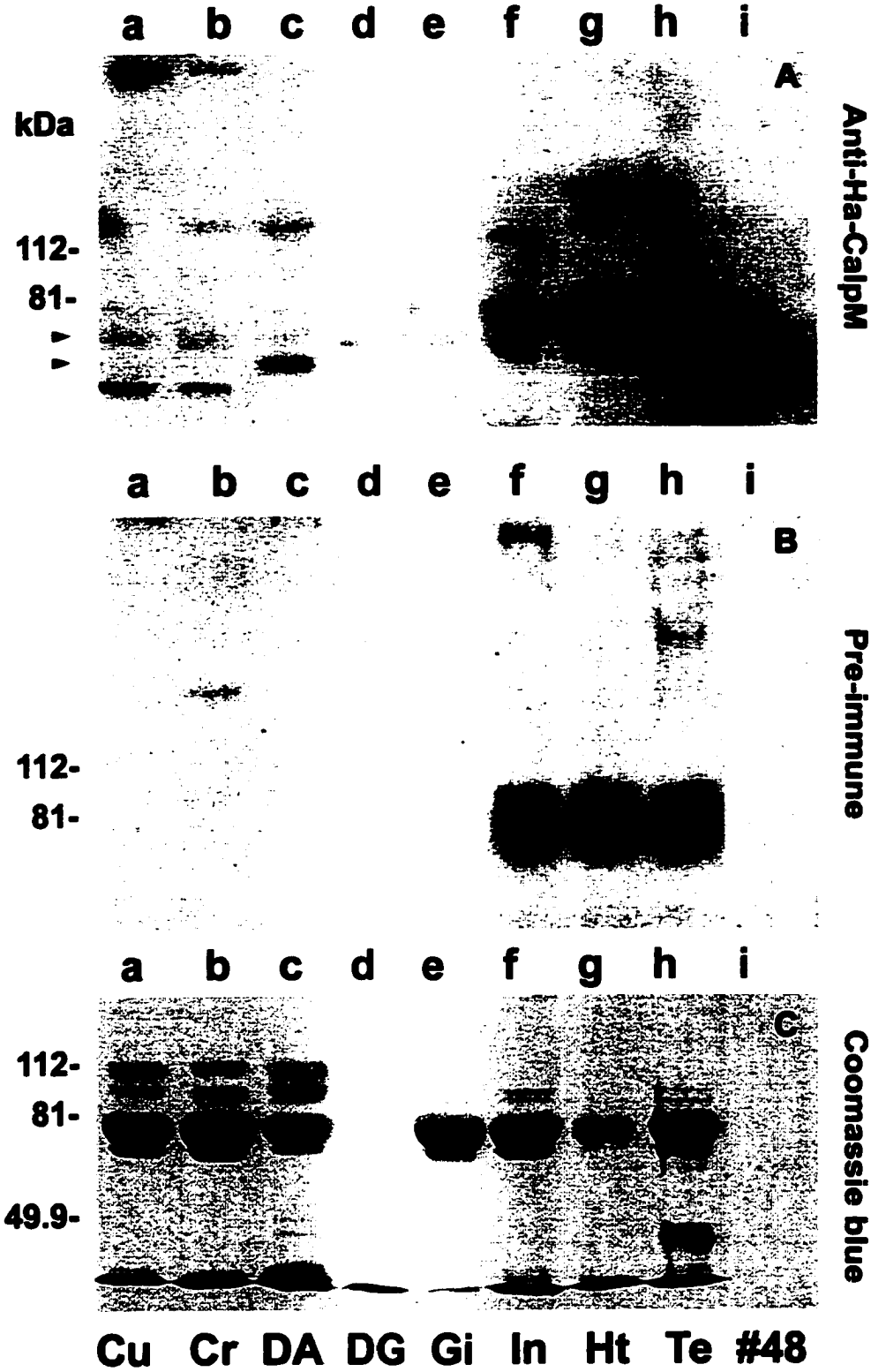


Fig. 3-19. Western Blot on molting claw muscles. The soluble proteins (32 μg) of claw muscles at different molting stages were separated on 6% SDS-polyacrylamid gels and either stained with Coomassie blue (C) or transferred onto PVDF membranes, and probed with Ha-CalpM antiserum (A) or pre-immune serum (B) at 1:10,000 dilution. A 62-kDa protein (arrowhead) was present at all molting stages: intermolt (lane a, b), premolt (lane c, d), and postmolt (lane e, f). Lane g, fraction #44 (10 μl) from gel filtration column. Position of molecular weight standards are indicated at left. I-1 and I-2, intermolt at 20% and 30% of the intermolt interval, respectively; D1, premolt stage D1; D3, premolt stage D3; P-1 and P-2, postmolt at 7% and 13% of the intermolt interval, respectively.

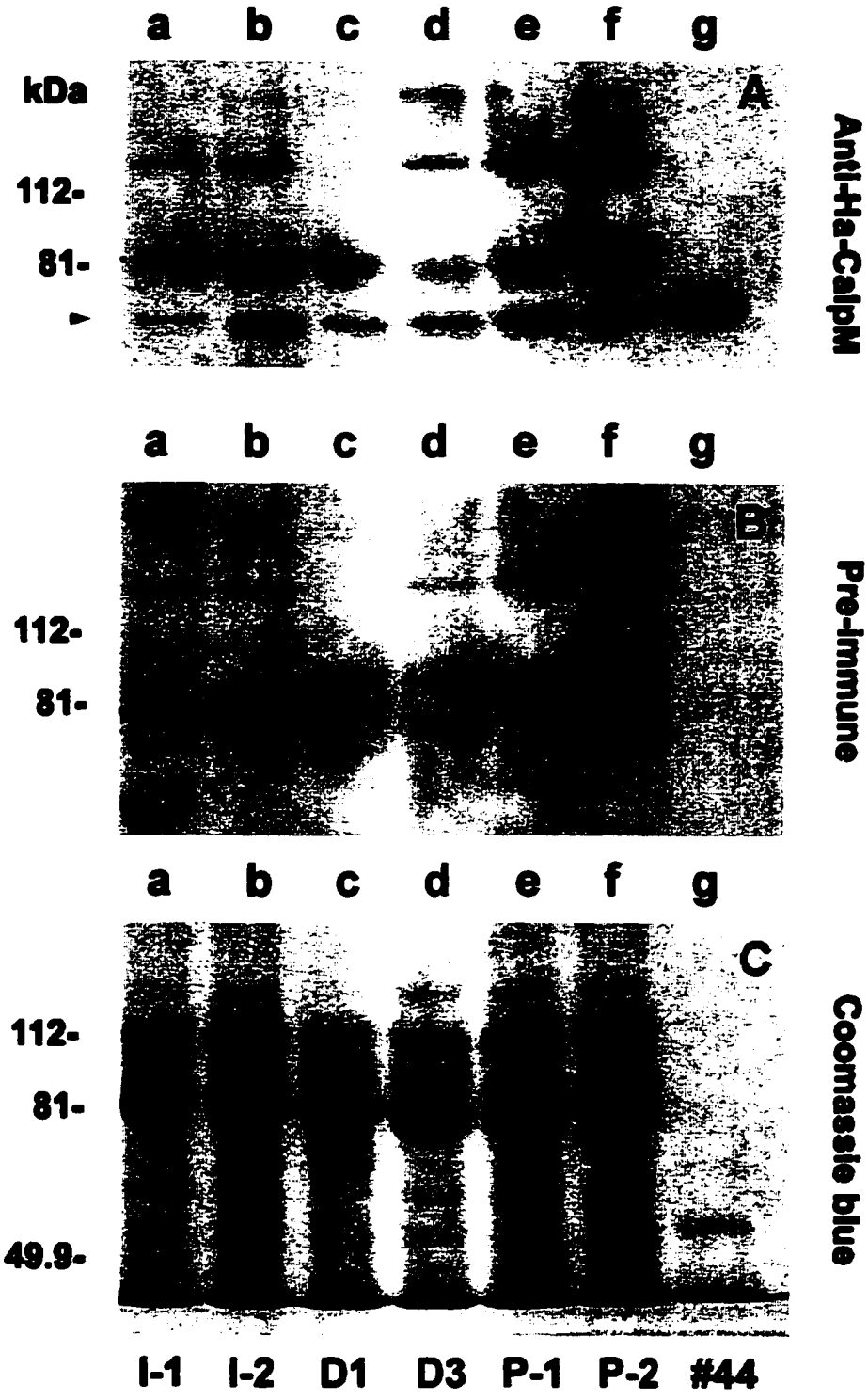


Fig. 3-20. SDS-PAGE and Western blot analysis of gel filtration chromatography fractions. A calpain fraction from organomercurial-agarose chromatography was separated on a Superdex 200 gel filtration column. Ten μl samples of fractions #16-48 were separated on 10% SDS-polyacrylamide gels, blotted onto PVDF membrane, and probed with an Ha-CalpM antiserum (1:10,000 dilution). A, Western blot showing the presence of two calpain proteins (arrowheads). A 68-kDa protein was detected starting at fraction #32 and reached a peak at fraction #36. A 62-kDa protein appeared at fraction of #42 and ended at fraction 48 (the last fraction collected). B, A silver-stained gel of the same fractions. Positions of molecular weight are indicated at left.

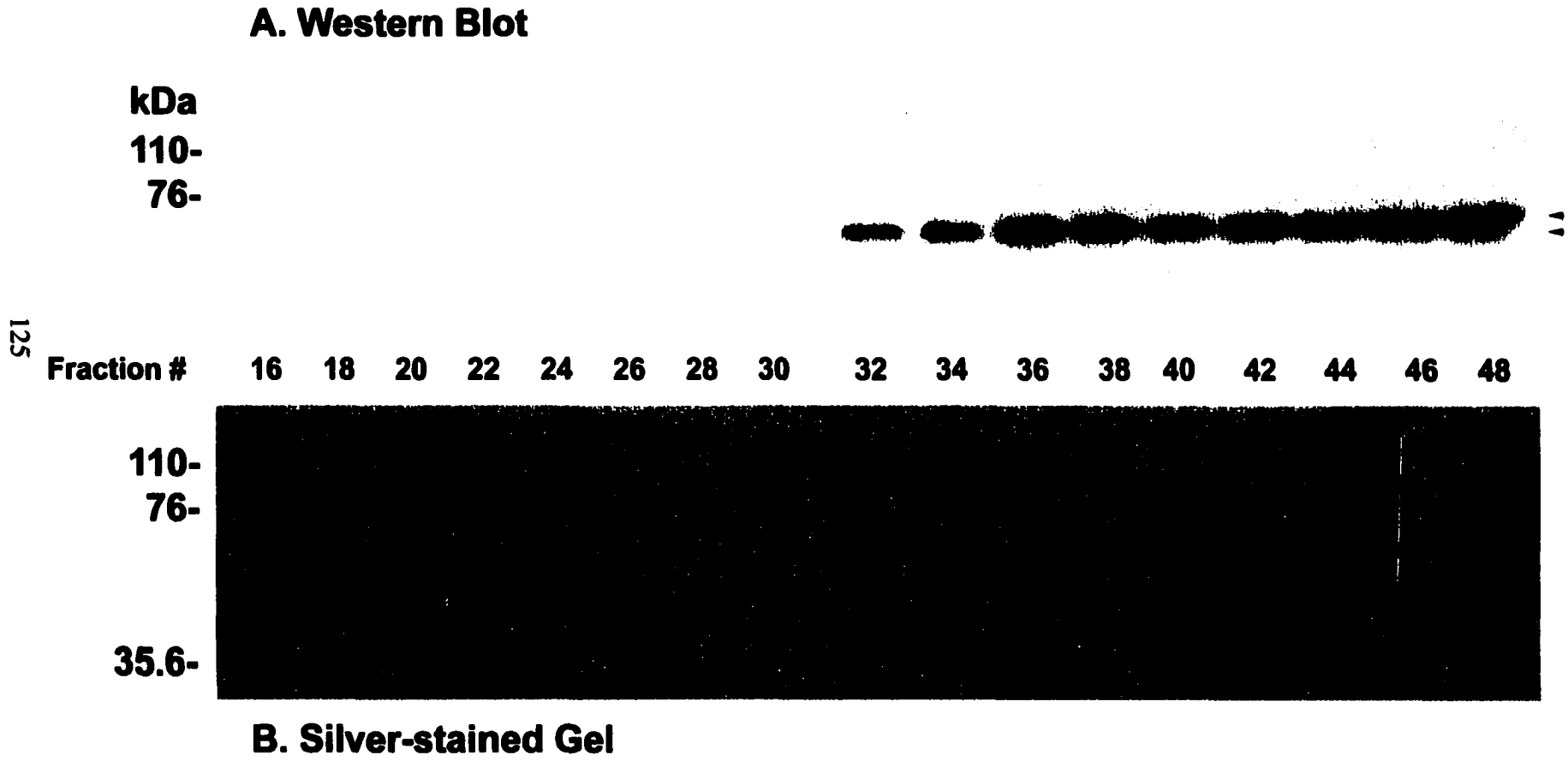


Fig. 3-21. Immunocytochemistry on the thoracic region of juvenile lobster. Sections were probed with pre-immune serum (A, 1:5,000 dilution), anti-myosin heavy chain antibody (B, 1:5,000 dilution), or Ha-CalpM antiserum (C, 1:5,000 dilution). There was weak non-specific binding associated with the digestive gland, midgut (intestine), and heart (A). The anti-myosin HC antibody showed strong staining of muscle and weak staining in other tissues (B). The Ha-CalpM antiserum stained all tissues at moderate levels (C). M, muscle; H, heart; DG, digestive gland; Mg, midgut (intestine); NC, nerve cord.

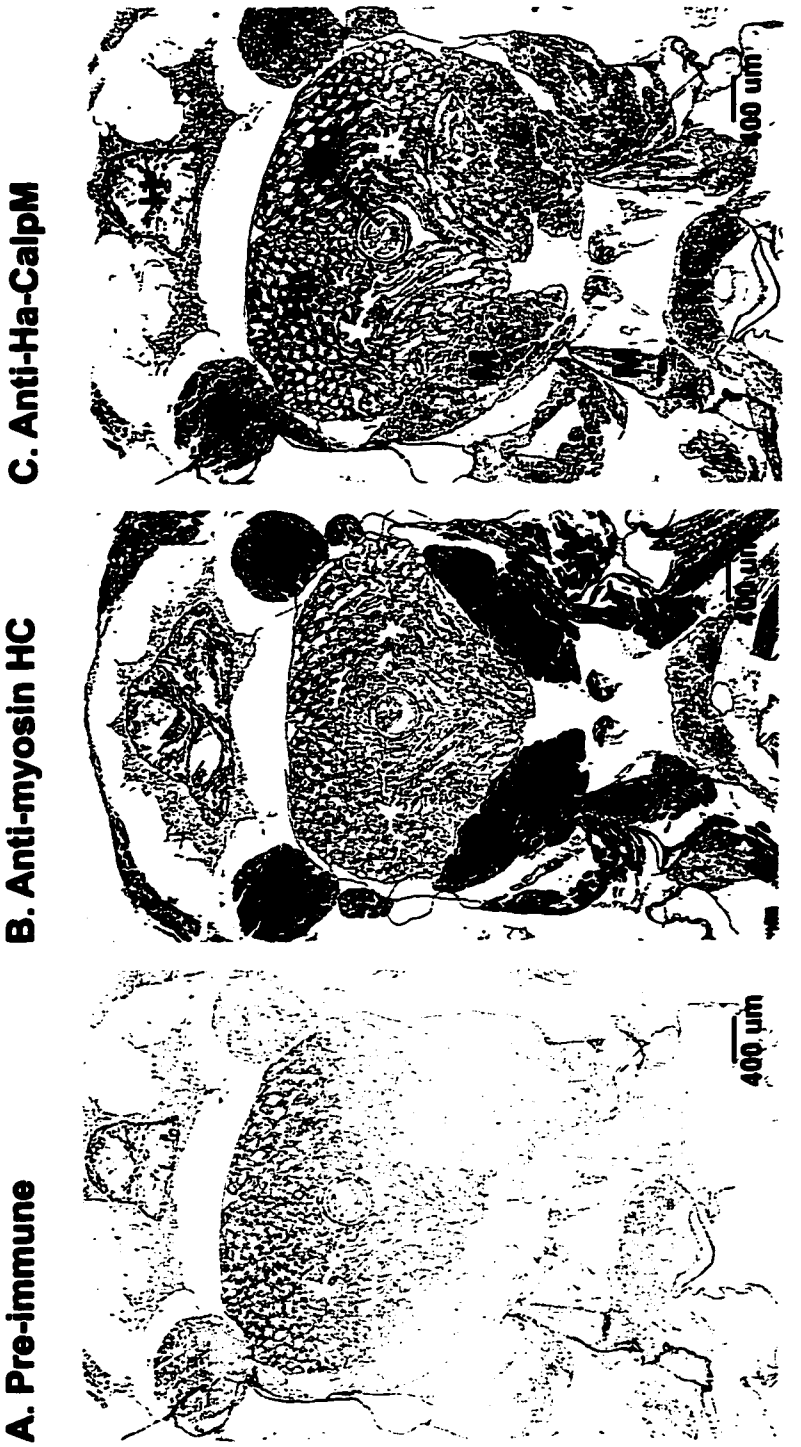


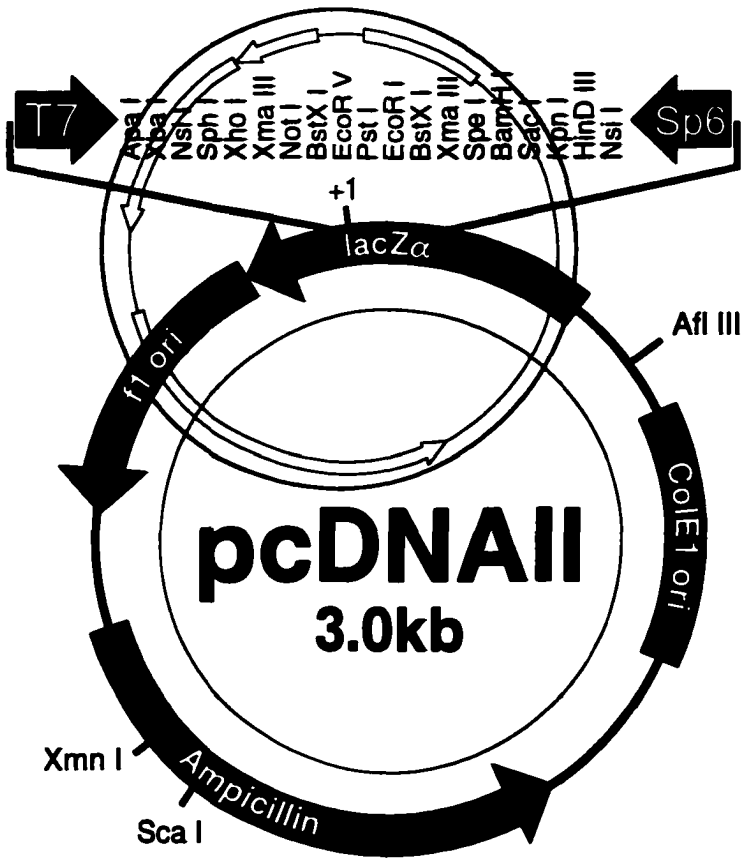
Fig. 3-22. Immunocytochemistry of lobster claw muscles. Sections of claw muscles were stained with hemtoxylin/eosin (B) or probed with pre-immune serum (A, 1:5000 dilution) or Ha-CalpM antiserum (C, D; 1:5,000 dilution). Nuclei (arrowheads) were associated with the sub-sarcolemmal cytoplasm of sarcolemal clefts (arrows). The Ha-CalpM antiserum stained the cytoplasm uniformly and some nuclei.

Fig. 3-23. The map of vector pcDNAII. pcDNAII contains lobster deep abdominal muscle cDNA library.

Comments for pcDNA II:
3013 nucleotides

Polylinker: bases 10-122
Sp6 promoter: bases 136-152
Ampicilin resistance: bases 1331-2191
F1 origin: bases 2377-2832
Lac Z gene: bases 2832-390
T7 promoter: bases 2993-3012

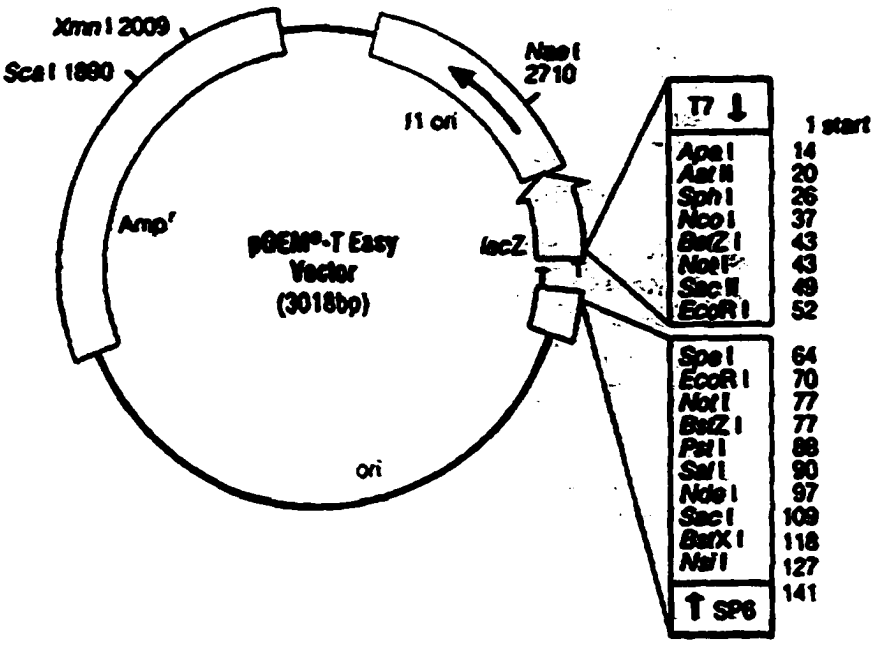
Invitrogen™
life technologies



vA-951003

The sequence of pcDNA II has been compiled from information in sequence databases, published sequences, and other sources. Portions of this vector have not yet been completely sequenced. If you suspect an error in the sequence, please contact Invitrogen's Technical Services Department at 800-955-6288.

Fig. 24. The map of vector pGEM-T easy. This vector was used for the PCR clonings.



SUMMARY

The control on molting is through the interaction between molt inhibitory hormone (MIH) from the X-organ sinus gland complex which predominates during the intermolt period, and the molting hormone ecdysone synthesized and secreted from the Y-organs, which stimulates growth when present in a favorable concentration with respect to the antagonistic MIH. Although there must be many fine tuning on the growth and differentiation of various tissues in the whole animal, the interaction of these two hormones have been thought to be primary in regulating "growth" *vs.* "non-growth".

The studies done by Holland and Skinner (1970, 1972) showed that the loss of more than four appendages is an adequate stimulus to molting preparations in intermolt land crab. Removal of a critical number of limbs is more potent for inducing precocious molting than eyestalk ablation (Skinner, 1985). In many crustaceans, limb regeneration occurs *de novo*. The process of autotomy, the self-amputation of the limb at a preformed breakage plane at the base of the ischial joint, leaves an essentially clean area, free of cut muscles or other tissues (Skinner, 1985). After leg autotomy, tissue differentiates so that the completed papilla at the end of basal growth is a miniature limb with epidermis, muscle, and nerves clearly distinguishable. Regenerates have complete motor innervation even when they are very small papillae (R Index = 2). Autotomy of only one (or more) of the ensuing primary regenerates is effective in inhibiting further preparations for molt (Holland and Skinner, 1976). During the period of inhibition a second blastema forms at the autotomy plane of each missing primary regenerate. The magnitude of the inhibitory effect depends on the stage of the animal in its molting preparation. Prior to a certain critical time (late D_0), the growth of the remaining primary regenerates "catch up". After the critical time (late D_1 to

ecdysis) autotomy of partial regenerates does not influence the remaining regenerates or the duration of the molting period (Holland and Skinner, 1976). I obtained similar results in my study in that autotomy of 1° LBs could delay molting as long as 30 days as compared with control animals. I also found that premolt period is lengthened further by autotomy of 2° LBs. The 3° LBs were even smaller than the secondary; the inhibition effect to the remaining 1° LB was the same as the 2° LBs.

In decapod crustaceans the changes in molting hormone ecdysone follows a general pattern in that at the beginning of proecdysis, the ecdysone level is low, then gradually increases to a peak value in stage D_3, drops precipitously at stage D_4, and remains at low levels though postmolt and intermolt periods. I showed that in land crab the hemolymph ecdysone level drops in response to limb bud autotomy and remains low during the basal growth stage. The increasing level of ecdysone during proecdysial period is interrupted by the autotomy of 1° LBs at R index of 13-15. The ecdysone level remains low when the regenerated 2° LBs were in the inhibiting stage. This suggests that LAFpro inhibits ecdysone synthesis or secretion.

The existence of LAFpro in secondary regenerates was demonstrated by a series of injection experiments. Extracts of 2° LBs were injected into proecdysial animals before (R index 13-15) or near (R index 16-17) the critical period. Growth of 1° LB was inhibited either partially or completely. However, the injection of 1° LB extracts did not have any inhibiting effects. LAFpro is heat stable, acid labile, and proteinase K sensitive. It appears to be a small peptide related to MIH, however, its identity is unknown at this point. LAFpro may be synthesized in neurons in the thoracic ganglion and/or nerve roots and transported via axonal processes to the 2° LBs.

The existence of LAFpro challenges the central paradigm in crustacean endocrinology that all proecdysial processes are coordinated by the eyestalk neurosecretory centers. Proecdysis is also suspended by LBA in eyestalk-ligated crabs (Holland and Skinner, 1976), suggesting that there are extra-eyestalk sources of MIH (Snyder and

Chang, 1986). Extra eyestalk sites of neuropeptide synthesis and secretion are not uncommon (Chung *et al.*, 1999) (Webster *et al.*, 2000) (Dircksen *et al.*, 2001) (Chang *et al.*, 1998). LAFpro could directly act on the Y-organs by inhibiting the ecdysone synthesis and secretion, or it may act on peripheral tissues (epidermis, 1° LBs, claw muscles) by inhibiting the action of 20-HE on peripheral targets.

Besides multiple limb regeneration for the preparation of molting, another important physiological aspect related to molting is claw muscle atrophy. Atrophy facilitates the withdrawl at ecdysis of the very large muscle mass through the narrow aperture of the old exoskeleton (Mykles and Skinner, 1990). Studies of atrophy related enzymes may help us understand the mechanism of muscle atrophy. For this purpose, I cloned and characterized the lobster muscle specific calpain Ha-CalpM. Sequence homology and phylogenetic analysis of Ha-CalpM with selected calpains have shown high similarity among them. Ha-CalpM has the highest sequence identity with *Drosophila* CalpA and CalpB. The overall similarities between the whole amino acid sequence and individual domain suggest the functional conservation of calpains. Like other calpains, Ha-CalpM must play an important role in the cells, although so far it has not been elucidated. Although lacking of domain IV, the calmodulin-like calcium binding domain, Ha-CalpM could still be a functional calpain for its possession of the long acidic region in domain III. The X-ray structure has revealed the significance and central role of domain III (Ho *et al.*, 1999; Stroble *et al.*, 2000). It has been shown that domain III is engaged in extensive ionic interactions with all the other domains within the large subunit and thus seems to function as an organizing center of calpain.

Ha-CalpM is highly expressed in skeletal muscle tissues. In mammals, muscle specific calpain p94 (CAPN3) was cloned (Sorimachi *et al.*, 1989). The p94 gene encodes a protein of 821 amino acids and it has significant sequence homology with both μ- and m-calpains. Mutation in CAPN 3 causes limb girdle muscle dystrophy type 2A. The high sequence similarity between Ha-CalpM and CAPN3 indicates that Ha-CalpM may be

lobster homolog of p94. Ha-CalpM is up-regulated (increase 2.3-fold) during premolt stage D_3 suggesting it may play a role in claw muscle atrophy. Previous work has shown that crustacean CDPs play a major role in the breakdown of myofibrillar proteins in muscle. Total CDP activity is increased about twofold in atrophic muscles (Mykles and Skinner, 1982). The results from gel filtration chromatography western blot suggests Ha-Calp M is CDP III previously purified biochemically.

An interesting aspect of calpain function is the modulation of the enzymatic activity of this protein. Calpain may directly affect cell behavior thorough proteolytic cleavage of cytoskeletal components. As a calcium-regulated protease that acts on proteins of various key cellular components (transcriptional factors, protein kinase C, etc), calpain should be able to integrate the signals of different transudation pathways by receiving the activating Ca^{2+} signal from one pathway and permanently modifying components of another. This activity may also be modulated though control of the autolytic activation and degradation of the enzyme and through differential splicing. Cloning of the lobster calpain homolog not only contributes to the already complex calpain family, especially the atypical calpain, but it may also help in elucidating the function of calpain, which remains the central question of calpain study. As for the muscle atrophy study, Ha-CalpM will lead the study in deciphering the enzymatic mechanisms of atrophy and eventually help to understand molting and its related physiological changes.

APPENDIX

The results of this part of work has been accepted for publication in:
Ubiquitin and actin expression in claw muscles of land crab, *Gecarcinus, lateralis*, and American lobster, *Homarus americanus*: differential expression of ubiquitin in two slow muscle fiber types during molt-induced atrophy. Annette Koenders, Xiaoli Yu, Ernest S. Chang, and Donald L. Mykles. Journal of Experimental Zoology, 2002.

The claw muscles of decapod crustaceans undergo atrophy to enable the animal to withdrawal the appendage at ecdysis (Mykles and Skinner, 1990). Both polyUb mRNA and Ub-protein conjugates increase during claw muscle atrophy. Ubiquitin (Ub), a highly-conserved 8.5-kDa protein, is convalently bonded to ε-amino groups on lysine side chains of a protein by an ATP-dependent ubiquitin-conjugating system; subsequent addition of Ub monomers via isopeptide linkages from a multiubiquitin chain that targets the protein for degradation by proteasome (Mykles, 1998). In lobster, α-actin is constitutively expressed at relatively constant levels during premolt stage (Whiteley and El Haj, 1997; Spees *et al.*, 2001).

Using RT-PCR and real-time PCR, the expression level ubiquitin and α-actin at different molt stage of claw muscle were analyzed. The partial polyUb cDNA clone (1.7 kb) containing about 7 Ub monomers was isolated from a lobster fast muscle library ((Shean and Mykles, 1995). A cDNA encoding the complete sequence of a lobster muscle α-actin was obtained (named Ha-actin1, GeneBank Accession number: AF399872) while screening a fast muscle library for calpain genes (Chapter 3 of this dissertation). The complete sequence and its deduced amino acid sequence are shown in Fig. A-1. The cDNA was 1386 bp in length with an open reading frame containing the complete actin polypeptide sequence

(377 amino acid residues) and an estimated mass of about 42 kDa. The deduced amino acid sequence had highest sequence identity (97%) with tiger shrimp (*Penaeus monodon*) actin1 (Fig. A-2). There was high sequence identity with a partial sequence of an actin cDNA from Homarus *gammarus* (92% identity over a 229-amino acid sequence equivalent to residues #30 through #258 in the H. americanus α-actin1; Harrison and El Haj, 1993). It also showed high sequence identity with α-actins from puffer fish (90%, Fig. A-2) and other vertebrate species (data not shown). Both strands of the Ha-actin1 cDNA were sequenced by Davis Sequencing (Davis, CA) using the vector primers SP6 and T7, and the sequence-specific primers at positions #380-398 and #1098-1079 in the sequence.

The methods for both RT-PCR and real-time PCR are essentially the same as described in Chapter 3. The polyUb primers were synthesized based on the sequence of the 3' region of the lobster polyUb cDNA (clone #116; accession number L22645; see Fig. 1B in Shean and Mykles, 1995). Sense (bp #31-53): CCA AGA CAA GGA AGG TAT TCC CC; antisense (bp #400-379): TAC ATC CAG ACC AGT AGC AAG CAC. For real-time PCR the primers are sense: (bp #54-75) CAG ACC AGT AGC AAG CAC CAC TC; antisense (bp #193-174): CCG ACG ATG GAG GGG AAT AC. The melting temperature, which is a measure of GC content, length, and sequence, was used to identify the PCR product. The peak area of the PCR product accumulating during the exponential phase of PCR was sued to quantify the relative amount of Ha-act1 mRNA level. The message level was expressed relative to the level in muscles from anecdysial (intermolt) animals. The data were averaged between two experiments.

The PCR amplification of the polyUb mRNA generated a family of discrete products due to the presence of repeated Ub-coding sequences in the gene (Fig. 3A in Shean and Mykles, 1995). The smallest product was the size predicted (225 bp) based on the primer locations. Larger products were multiples of 228 bp, which is the distance between potential binding sites of the primer in each Ub-coding sequence. The amount of amplified product varied according to the molting stage. The greatest amount occurred at

stage D_0 (early proecdysis), with a higher proportion of larger products that were amplified. There were lesser amounts at stage D_3 (late proecdysis); (Fig. A-3A, lanes d and g). The lowest amounts occurred at anecdysis (intermolt; Fig. A-3A, lanes b and f) and metecdysis (postmolt; Fig. A-3A, lane e). Due to the heterogeneity of the products, real-time PCR could not be used to quantify the polyUb mRNA. For lobster α-actin1 RT-PCR, a single product (401 bp) was amplified. The low amount of message at stage D0 (Fig. A-3B, lane c) apparently resulted from a lower amount of RNA in the reaction, since real-time PCR showed only relatively small changes over the intermolt cycle (Fig. A-4).

In summary, a cDNA encoding a full-length lobster muscle α-actin was identified. It expression remains relatively stable during molting stage. The up-regulated polyUb gene in premolt stage suggesting its role in atrophying claw muscle. The α-actin could be used as a useful molecular marker in studying muscle atrophy in crustacean.

Fig. A-1. Nucleotide and deduced amino acid sequences of cDNA encoding lobster (*Homarus americanus*) muscle α-actin (Ha-actin1). The cDNA (1386 bp), which was isolated from a fast muscle cDNA library derived from deep abdominal muscle mRNA, encodes a protein of 377 amino acids (ORF: #33-1163). The deduced amino acid sequence has a predicted mass of about 42 kDa. The locations and directions of primers used for RT-PCR are indicated by unidirectional arrows above the DNA sequence.

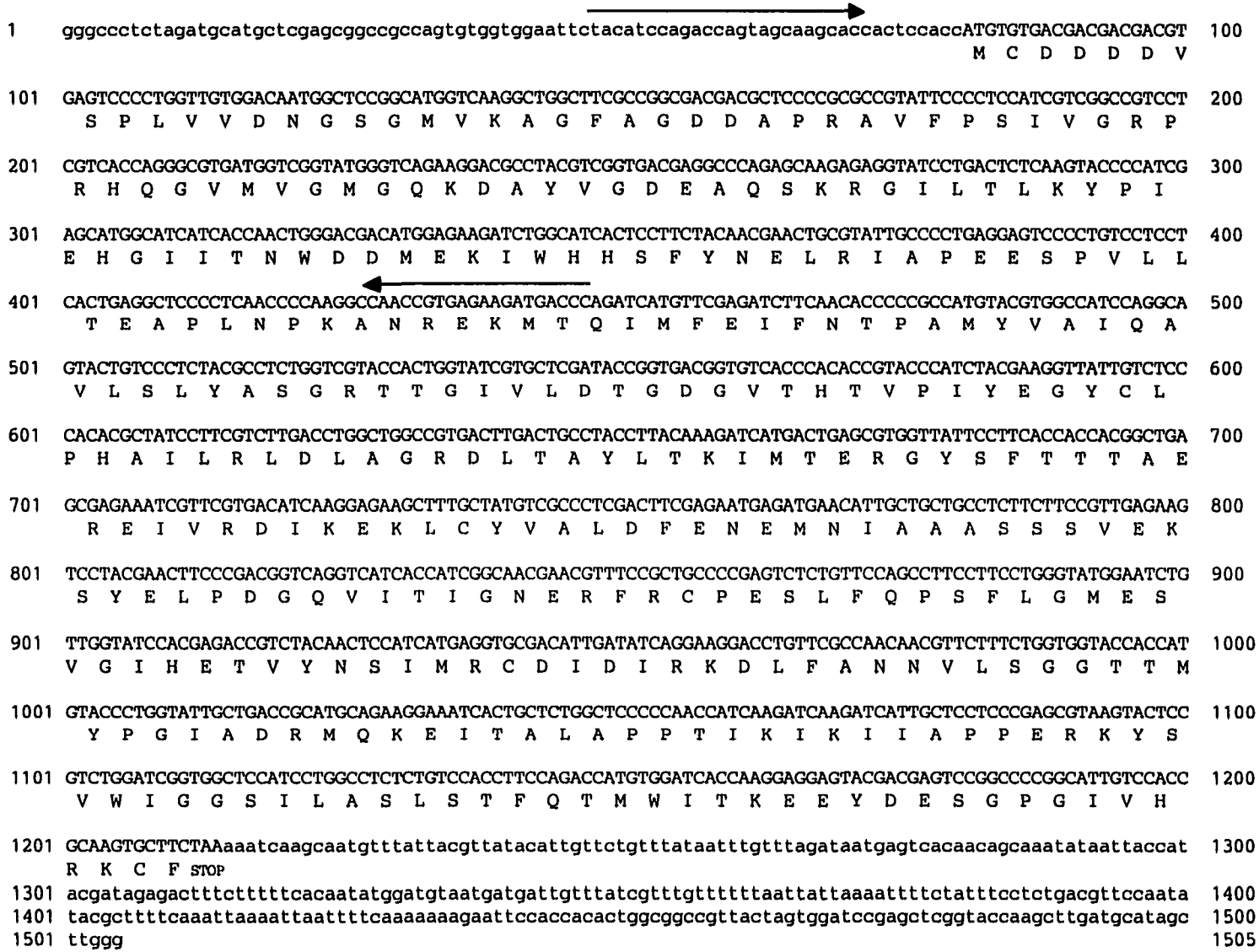
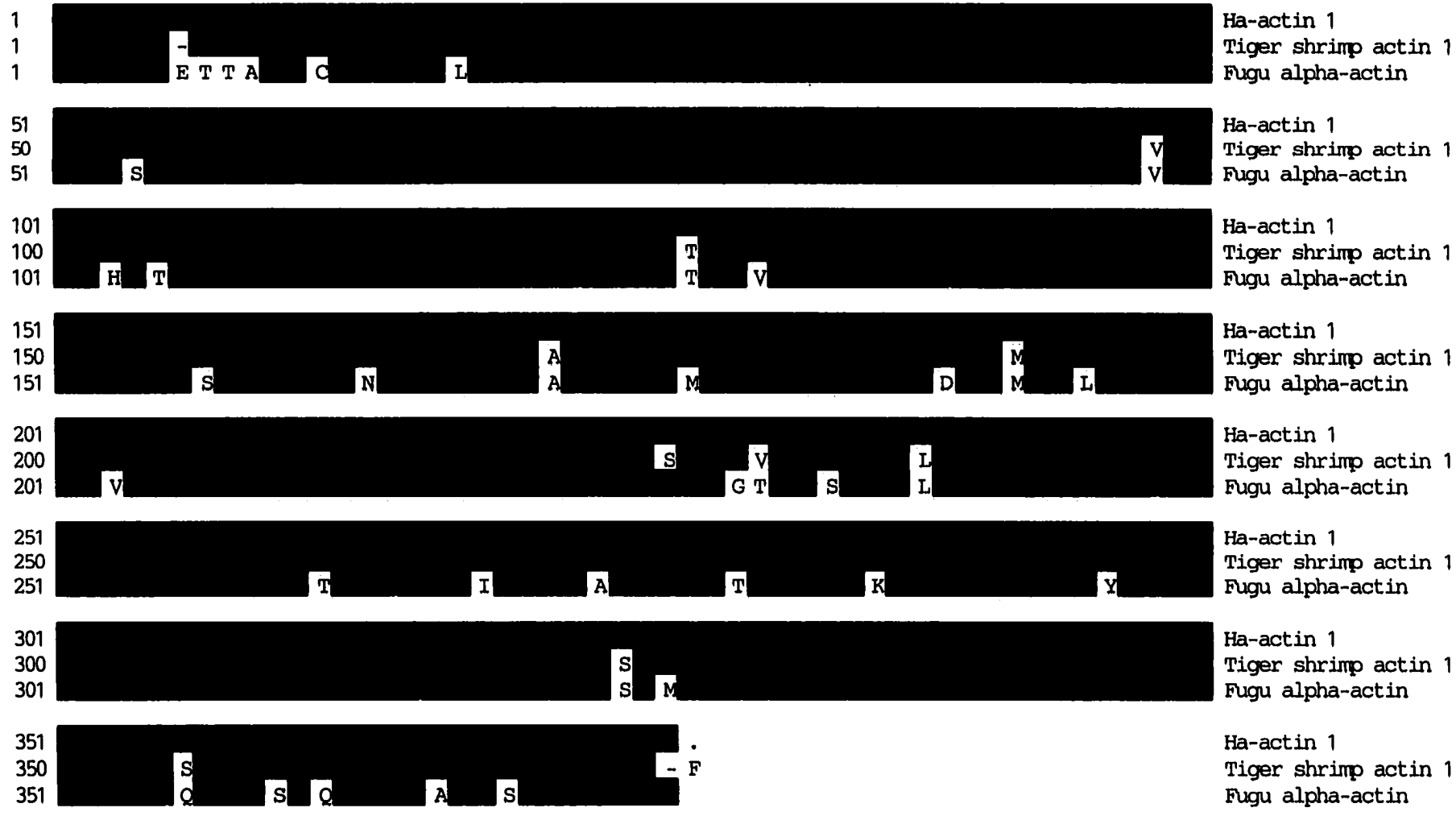


Fig. A-2. Comparison of deduced amino acid sequences of α-actins from lobster, tiger shrimp, and puffer fish. Ha-actin1 (accession number AF399872) is 97% identical to actin1 of tiger shrimp (*Penaeus monodon*; accession number AF100986) and 90% identical to α-actin from puffer fish (Fugu; accession number P53482) muscles at the amino acid level. Residues in the tiger shrimp and puffer fish sequences that are identical to those in the Ha-actin1 sequence are indicated with black shading.



Decoration 'Decoration #1': Shade (with solid black) residues that match Ha-actin 1 exactly.

Fig. A-3. RT-PCR of polyubiquitin and Ha-actin1 mRNAs in lobster claw muscle during the intermolt cycle. Total RNA from cutter and crusher claw closer muscles was reverse-transcribed into cDNA using oligonucleotide primers specific for ubiquitin (A) or actin (B), followed by PCR amplification and agarose gel electrophoresis (see Materials and Methods). The figure is a negative image of ethidium bromide-stained gels. RT-PCR of polyubiquitin produced multiple products due to the presence of repeated copies of Ub in the mRNA (Shean and Mykles, 1995). The smallest product (225 bp) was the size expected from amplification from antisense and sense primers hybridizing to template at the 3' UTR and the adjacent Ub-coding region, respectively. Larger products at multiples of 228 bp (453 bp, 681 bp, 909 bp, 1137 bp, etc.) were the sizes expected from the sense primer hybridizing to Ub coding sequences located further toward the 5' end. The greatest amount of products, particularly those with higher molecular weights, was obtained from stage D_0 (lane c) indicating higher levels of polyUb expression during claw muscle atrophy. RT-PCR of Ha-actin1 showed no consistent differences in expression of actin at various molt stages. M, molecular weight marker; I, intermolt (anecdysis); D0, stage D_0 or early proecdysis; D3, stage D_3 or late proecdysis; and P, postmolt (metecdysis). Labels for lanes, molt stages, and claw type (cutter or crusher) apply to both panels.

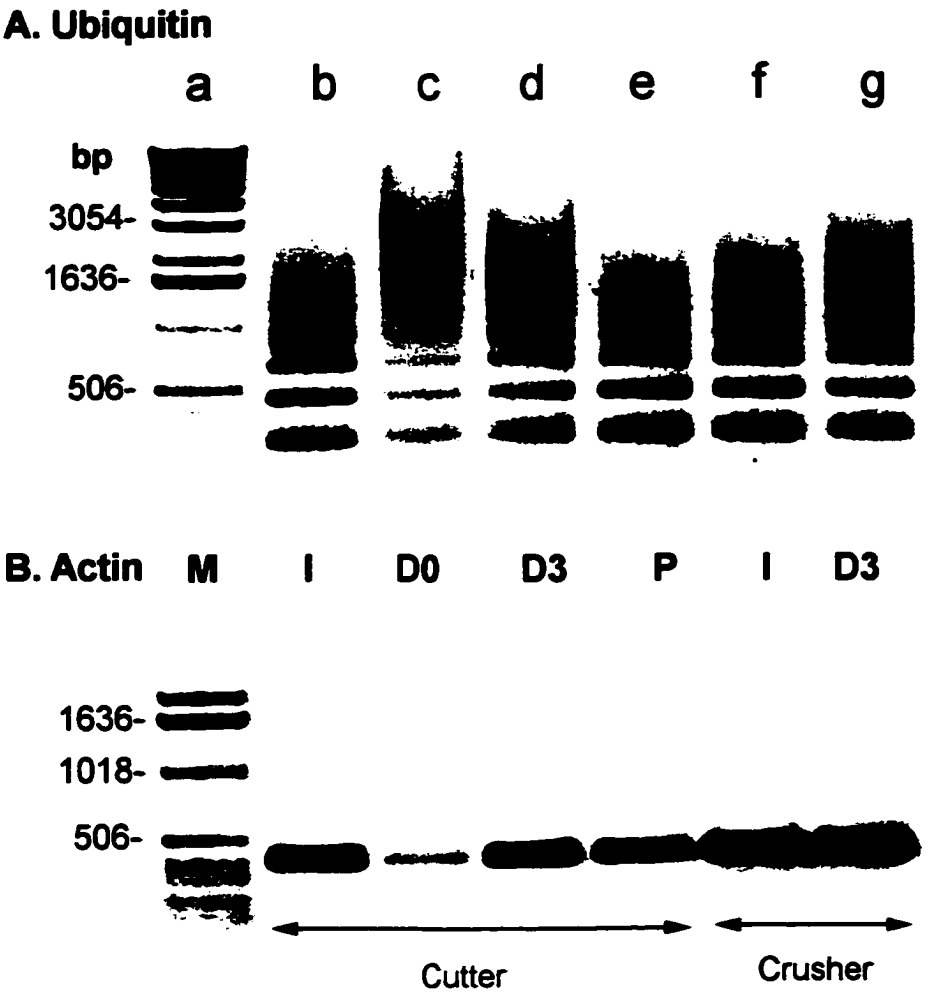
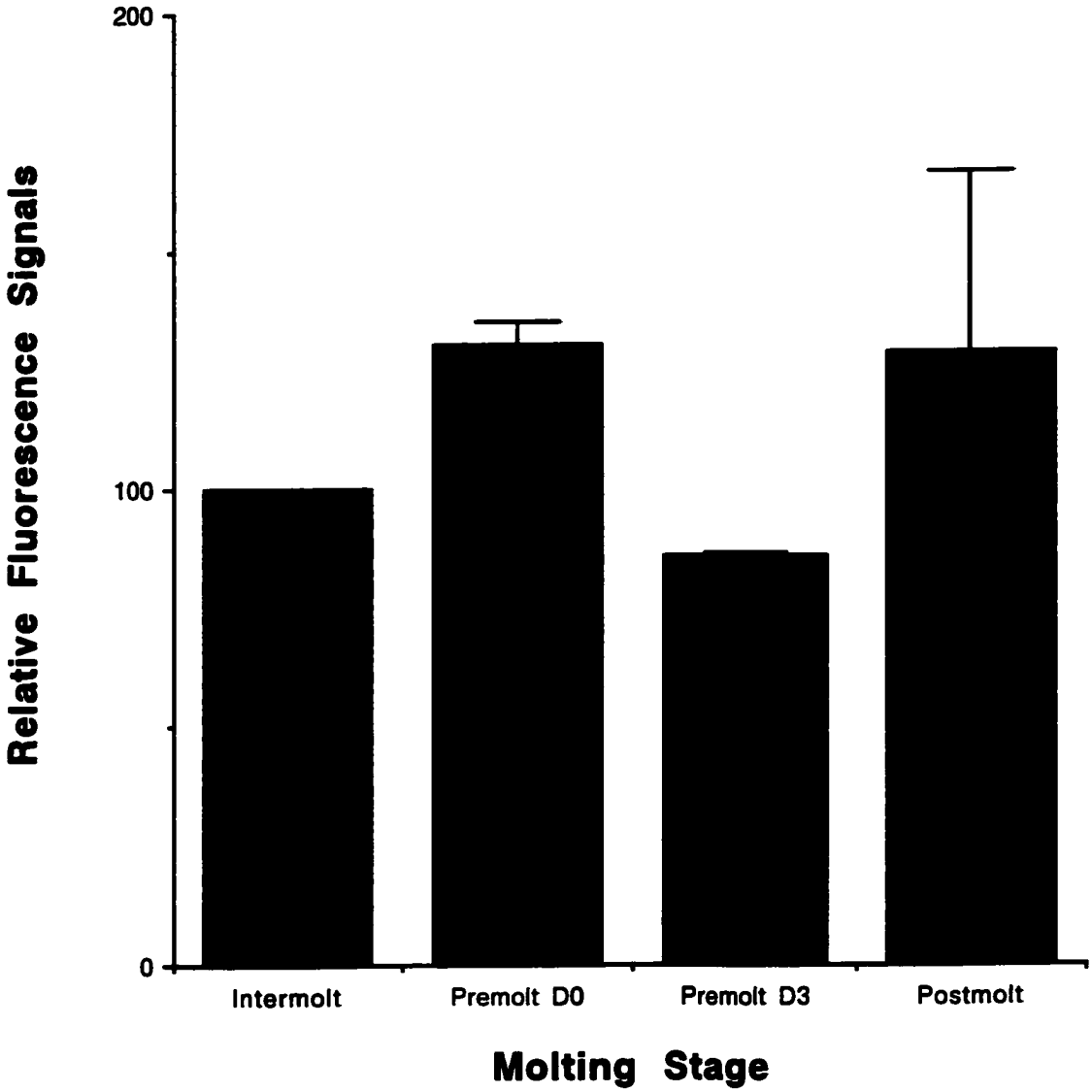


Fig. A-4. Real-time PCR quantification of Ha-act1 mRNA levels in lobster claw muscles during the intermolt cycle. Total RNA was isolated and cDNA synthesized for PCR using Ha-actin1-specific primers. The peak area from the melting curve analysis was used to quantify the amount of Ha-act1 mRNA. The abundance of Ha-actin1 mRNA is expressed relative to the peak area from anecdysial (intermolt) animals. The results were averaged from analyses of two sets of RNA preparations from the four molt stages. The range between the values is indicated by the vertical bar. Actin1 mRNA showed small fluctuations (less than 30% of anecdysial levels) during the intermolt cycle.



REFERENCES

Arthur, J. S. C., J. S. Elce, C. Hegadorn, K. Williams and P. A. Greer (2000). Disruption of the murine calpain small subunit gene, *Capn4*: Calpain is essential for embryonic development but not for cell growth and division. Molecular and Cellular Biology **20**: 4474-4481.

Azuma, M., E. Inoue, T. Oka and T. R. Shearer (1995). Proteolysis by calpain is an underlying mechanism for formation of sugar cataract in rat lens. Curr.Eye Res. **14**: 27-34.

Baghdassarian, D., N. De Bessé, B. Saïdi, G. Sommé and F. Lachaise (1996). Neuropeptide-induced inhibition of steroidogenesis in crab molting glands: Involvement of cGMP-dependent protein kinase. General and Comparative Endocrinology **104**: 41-51.

Baki, A., P. Tompa, A. Alexa, O. Molnár and P. Friedrich (1996). Autolysis parallels activation of μ-calpain. Biochemical Journal **318**: 897-901.

Barnes, T. M. and J. Hodgkin (1996). The *tra-3* sex determination gene of *Caenorhabditis elegans* encodes a member of the calpain regulatory protease family. EMBO J. **15**: 4477-4484.

Bartus, R. T., R. L. Dean, K. Cavanaugh, D. Eveleth, D. L. Carriero and G. Lynch (1995a). Time-related neuronal changes following middle cerebral artery occlusion: Implications for therapeutic intervention and the role of calpain. J.Cereb.Blood Flow Metab. **15**: 969-979.

Bartus, R. T., P. J. Elliott, N. J. Hayward, R. L. Dean, S. Harbeson, J. A. Straub, Z. Li and J. C. Powers (1995b). Calpain as a novel target for treating acute neurodegenerative disorders. Neurol.Res. **17**: 249-258.

Berti, P. J. and A. C. Storer (1995). Alignment/phylogeny of the papain superfamily of cysteine proteases. Journal of Molecular Biology **246**: 273-283.

Beyette, J. R., Y. Emori and D. L. Mykles (1997). Immunological analysis of two calpain-like Ca^{2+}-dependent proteinases from lobster striated muscles: Relationship to mammalian and *Drosophila* calpains. Archives of Biochemistry and Biophysics **337**: 232-238.

Beyette, J. R., J.-S. Ma and D. L. Mykles (1993). Purification and autolytic degradation of a calpain-like calcium-dependent proteinase from lobster (*Homarus americanus*) striated muscle. Comparative Biochemistry and Physiology [B] **104B**: 95-99.

Beyette, J. R. and D. L. Mykles (1997). Autolysis and biochemical properties of a lobster muscle calpain-like proteinase. Journal of Experimental Zoology **277**: 106-119.

Blais, C., M. Sefiani, J.-Y. Toullec and D. Soyez (1994). In vitro production of ecdysteroids by Y-organs of Penaeus vannamei (Crustacea, Decapoda). Correlation with

hemolymph titers. SO - Invertebrate Reproduction & Development. 26(1). 1994. 3-11.

Bliss, D. E. (1956). Neurosecretion and the control of growth in a decapod crustacean. Bertil Hanstron, Zoological Papers in Honor of His Sixty-fifth Birthday. K. G. Eingstrand. Lund, Sweden, Zool. Inst.: 56-57.

Bliss, D. E., S. M. E. Wang and E. A. Martinez (1966). Water balance in the land crab, *Gecarcinus lateralis*, during the intermolt cycle. American Zoologist **6**: 197-212.

Bowser, J. M. (1998). Immunochemical characterization of a kinesin-like calmodulin-binding protein in plants. Graduate Degree Program in Cell and Molecular Biology. Fort Collins, Colorado State University.

Carlisle, D. B. and P. F. R. Dohrn (1953). Studies on Lysmata seticaudata Risso (Crustacea Decapoda). II. Experimental evidence for a growth- and moult-accelerating factor obtainable from eyestalks. Pubbl. Staz. Zool. Nopoli **24**: 69-83.

Chan, S. M., X. G. Chen and P. L. Gu (1998). PCR cloning and expression of the molt-inhibiting hormone gene for the crab (*Charybdis feriatus*). Gene **224**: 23-33.

Chang, E. S. (1993). Comparative endocrinology of molting and reproduction: Insects and crustaceans. Annu.Rev.Entomol. **38**: 161-180.

Chang, E. S. (2001). Crustacean hperglycemic hormone family: old paradigms and new perspectives. Am. Zool. **41**: 380-388.

Chang, E. S., M. J. Bruce and R. W. Newcomb (1987). Purification and amino acid composition of a peptide with molt-inhibiting activity from the lobster, *Homarus americanus*. General and Comparative Endocrinology **65**: 56-64.

Chang, E. S., S. A. Chang, R. Keller, P. S. Reddy, M. J. Snyder and J. L. Spees (1999). Quantification of stress in lobsters: Crustacean hyperglycemic hormone, stress proteins, and gene expression. American Zoologist **39**: 487-495.

Chang, E. S., R. Keller and S. A. Chang (1998). Quantification of crustacean hyperglycemic hormone by ELISA in hemolymph of the lobster, *Homarus americanus*, following various stresses. General and Comparative Endocrinology **111**: 359-366.

Chang, E. S. and J. D. O'Connor (1977). Secretion of α-ecdysone by crab Y-organs *in vitro*. Proceedings of the National Academy of Sciences USA **74**: 615-618.

Chang, E. S. and J. D. O'Connor (1979). Arthropod molting hormones. Methods of Hormone Radioimmunoassay. B. M. B. M. J. a. H. R. B. M. J. Jaffe and H. R. Behrman. New York, Academic Press: 797-814.

Chang, E. S., G. D. Prestwich and M. J. Bruce (1990). Amino acid sequence of a peptide with both molt-inhibiting and hyperglycemic activities in the lobster, *Homarus americanus*. Biochem.Biophys.Res.Commun. **171**: 818-826.

Chittleborough, R. G. (1975). Environmental factors affecting growth and survival of juvenile western rock lobsters *Panulirus longipes* (Milne-Edwards). Australian Journal of Marine and Freshwater Research **26**: 117-196.

Chung, A. C. K., D. S. Durica, S. W. Clifton, B. A. Roe and P. M. Hopkins (1998a). Cloning of crustacean ecdysteroid receptor and retinoid-X receptor gene homologs and elevation of retinoid-X receptor mRNA by retinoic acid. Mol.Cell.Endocrinol. **139**: 209-227.

Chung, A. C. K., D. S. Durica and P. M. Hopkins (1998b). Tissue-specific patterns and steady-state concentrations of ecdysteroid receptor and retinoid-X-receptor mRNA during the molt cycle of the fiddler crab, *Uca pugilator*. General and Comparative Endocrinology **109**: 375-389.

Chung, J. S., H. Dircksen and S. G. Webster (1999). A remarkable, precisely timed release of hyperglycemic hormone from endocrine cells in the gut is associated with ecdysis in the crab *Carcinus maenas*. Proceedings of the National Academy of Sciences USA **96**: 13103-13107.

Connat, J. L., R. Lafont and P. A. Diehl (1986). Metabolism of [3H]ecdysone by isolated tissues of the female ixodid tick Amblyomma hebraeum (Ixodoidea; Ixodidae). Mol Cell Endocrinol **47**(3): 257-67.

Crawford, C., N. R. Brown and A. C. Willis (1993). Studies of the active site of m-calpain and the interaction with calpastatin. Biochemical Journal **296**: 135-142.

Croall, D. E. and G. N. DeMartino (1991). Calcium-activated neutral protease (calpain) system: Structure, function, and regulation. Physiol.Rev. **71**: 813-847.

Croall, D. E. and K. S. McGrody (1994). Domain structure of calpain: Mapping the binding site for calpastatin. Biochemistry **33**: 13223-13230.

Dauphin-Villemant, C., D. Böcking and D. Sedlmeier (1995). Regulation of steroidogenesis in crayfish molting glands: Involvement of protein synthesis. Mol.Cell.Endocrinol. **109**: 97-103.

De Kleijn, D. P. V. and F. Van Herp (1995). Molecular biology of neurohormone precursors in the eyestalk of Crustacea. Comparative Biochemistry and Physiology [B] **112B**: 573-579.

Dear, N., K. Matena, M. Vingron and T. Boehm (1997). A new subfamily of vertebrate calpains lacking a calmodulin-like domain: Implications for calpain regulation and evolution. Genomics **45**: 175-184.

Dear, T. N. and T. Boehm (1999). Diverse mRNA expression patterns of the mouse calpain genes *Capn5*, *Capn6* and *Capn11* during development. Mech.Dev. **89**: 201-209.

Dear, T. N., N. T. Meier, M. Hunn and T. Boehm (2000). Gene structure, chromosomal localization, and expression pattern of *Capn12*, a new member of the calpain large subunit gene family. Genomics **68**: 152-160.

Dear, T. N., A. Möller and T. Boehm (1999). CAPN11: A calpain with high mRNA levels in testis and located on chromosome 6. Genomics **59**: 243-247.

Debiasi, R. L., M. K. T. Squier, B. Pike, M. Wynes, T. S. Dermody, J. J. Cohen and K. L. Tyler (1999). Reovirus-induced apoptosis is preceded by increased cellular calpain activity and is blocked by calpain inhibitors. J.Virol. **73**: 695-701.

Dircksen, H., D. Böcking, U. Heyn, C. Mandel, J. S. Chung, G. Baggerman, P. Verhaert, S. Daufeldt, T. Plösch, P. P. Jaros, E. Waelkens, R. Keller and S. G. Webster (2001). Crustacean hyperglycaemic hormone (CHH)-like peptides and CHH-precursor-related peptides from pericardial organ neurosecretory cells in the shore crab, *Carcinus maenas*, are putatively spliced and modified products of multiple genes. Biochemical Journal **356**: 159-170.

Drach, P. (1939). Meu et cycle d'intermue chez les Crustaces Decapodes. Ann. Inst. Oceanogr. **19**: 103-391.

Durica, D. S. and P. M. Hopkins (1996). Expression of the genes encoding the ecdysteroid and retinoid receptors in regenerating limb tissues from the fiddler crab, *Uca pugilator*. Gene **171**: 237-241.

El Haj, A. J. and D. F. Houlihan (1987). *In vitro* and *in vivo* protein synthesis rates in a crustacean muscle during the moult cycle. J. Exp. Biol. **127**: 413-426.

Elce, J. S., C. Hegadorn, J. Simon and C. Arthur (1997). Autolysis, Ca^{2+} requirement, and heterodimer stability in m-calpain. The Journal of Biological Chemistry **272**: 11268-11275.

Emori, Y., H. Kawasaki, S. Imajoh, Y. Minami and K. Suzuki (1988). All four repeating domains of the endogenous inhibitor for calcium-dependent protease independently retain inhibitory activity. Expression of the cDNA fragments in Escherichia coli. J Biol Chem **263**(5): 2364-70.

Emori, Y. and K. Saigo (1994). Calpain localization changes in coordination with actin-related cytoskeletal changes during early embryonic development of *Drosophila*. The Journal of Biological Chemistry **269**: 25137-25142.

Eto, A., Y. Akita, T. C. Saido, K. Suzuki and S. Kawashima (1995). The role of the calpain-calpastatin system in thyrotropin-releasing hormone-induced selective down-regulation of a protein kinase C isozyme, nPKCε, in rat pituitary GH_4C_1 cells. The Journal of Biological Chemistry **270**: 25115-25120.

Fingerman, M. (1987). The endocrine mechanisms of crustaceans. Journal of Crustacean Biology **7**: 1-24.

Fingerman, M. and S. W. Fingerman (1974). The effects of limb removal on the rates of ecdysis of eyed and eyestalkless fiddler crabs, *Uca pugilator*. Zool.Jb.Physiol. **78**: 301-309.

Freeman, J. A. and J. D. Costlow (1979). Hormonal control of apolysis in barnacle mantle tissue epidermis, in vitro Journal of Experimental Zoology **210**: 333-346.

Goll, D. E., V. F. Thompson, R. G. Taylor and J. A. Christiansen (1992). Role of the calpain system in muscle growth. Biochimie **74**: 225-237.

Gonen, H., D. Shkedy, S. Barnoy, N. S. Kosower and A. Ciechanover (1997). On the involvement of calpains in the degradation of the tumor suppressor protein p53. FEBS Lett. **406**: 17-22.

Grynspan, F., W. R. Griffin, A. Cataldo, S. Katayama and R. A. Nixon (1997). Active site-directed antibodies identify calpain II as an early-appearing and pervasive component of neurofibrillary pathology in Alzheimer's disease. Brain Research **763**: 145-158.

Hata, S., H. Sorimachi, K. Nakagawa, T. Maeda, K. Abe and K. Suzuki (2001). Domain II of m-calpain is a Ca2+-dependent cysteine protease. Febs Letters **501**(2-3): 111-114.

Hatzizisis, D., C. Gaitanaki and I. Beis (2000). Degradation of myofibrillar proteins by a calpain-like proteinase in the arm muscle of *Octopus vulgaris*. J.Comp.Physiol.[B] **170**: 447-456.

Herasse, M., Y. Ono, F. Fougerousse, E. Kimura, D. Stockholm, C. Beley, D. Montarras, C. Pinset, H. Sorimachi, K. Suzuki, J. S. Beckmann and I. Richard (1999). Expression and functional characteristics of calpain 3 isoforms generated through tissue-specific transcriptional and posttranscriptional events. Molecular and Cellular Biology **19**: 4047-4055.

Hirai, S., H. Kawasaki, M. Yaniv and K. Suzuki (1991). Degradation of transcription factors, c-Jun and c-Fos, by calpain. FEBS Lett. **287**: 57-61.

Hoffmann, J. and M. Porchet (1984). Biosynthesis, metabolism, and mode of action of invertebrate hormones. Berlin ; New York, Springer-Verlag.

Holland, C. A. and D. M. Skinner (1976). Interactions between molting and regeneration in the land crab. Biological Bulletin **150**: 222-240.

Hopkins, P. M. (1989). Ecdysteroids and regeneration in the fiddler crab *Uca pugilator*. Journal of Experimental Zoology **252**: 293-299.

Hopkins, P. M. (1993). Regeneration of walking legs in the fiddler crab *Uca pugilator*. American Zoologist **33**: 348-356.

Hopkins, P. M. (2001). Limb regeneration in the fiddler crab, *Uca pugilator*: hormonal and growth factor control. Am. Zool. **41**: 389-398.

Hopkins, P. M., D. E. Bliss, S. W. Sheehan and J. R. Boyer (1979). Limb growth-controlling factors in the crab *Gecarcinus lateralis*, with special reference to the limb growth-inhibiting factor. General and Comparative Endocrinology **39**: 192-207.

Horikawa, Y., N. Oda, N. J. Cox, X. Q. Li, M. Orho-Melander, M. Hara, Y. Hinokio, T. H. Lindner, H. Mashima, P. E. H. Schwarz, L. Del Bosque-Plata, Y. Oda, I. Yoshiuchi, S. Colilla, K. S. Polonsky, S. Wei, P. Concannon, N. Iwasaki, T. Schulze, L. J. Baier, C. Bogardus, L. Groop and E. Boerwinkle (2000). Genetic variation in the gene encoding calpain-10 is associated with type 2 diabetes mellitus. Nature Genet. **26**: 163-175.

Horn, D. H. S., J. S. Wilkie, B. A. Sage and J. D. O'Connor (1976). A high affinity antiserum specific for the ecdysone nucleus. Journal of Insect Physiology **22**: 901-905.

Hosfield, C. M., J. S. Elce, P. L. Davies and Z. Jia (1999a). Crystal structure of calpain reveals the structural basis for Ca(2+)-dependent protease activity and a novel mode of enzyme activation. Embo J **18**(24): 6880-9.

Hosfield, C. M., J. S. Elce, P. L. Davies and Z. C. Jia (1999b). Crystal structure of calpain reveals the structural basis for Ca^{2+}-dependent protease activity and a novel mode of enzyme activation. EMBO J. **18**: 6880-6889.

Huang, J. and N. E. Forsberg (1998). Role of calpain in skeletal-muscle protein degradation. Proceedings of the National Academy of Sciences USA **95**: 12100-12105.

Ismail, S. Z. M. and D. L. Mykles (1992). Differential molt-induced atrophy in the dimorphic claws of male fiddler crabs, *Uca pugnax*. Journal of Experimental Zoology **263**: 18-31.

Jekely, G. and P. Friedrich (1999a). Characterization of two recombinant Drosophila calpains. CALPA and a novel homolog, CALPB. J Biol Chem **274**(34): 23893-900.

Jekely, G. and P. Friedrich (1999b). The evolution of the calpain family as reflected in paralogous chromosome regions. J Mol Evol **49**(2): 272-81.

Jia, J., Q. Han, N. Borregaard, K. Lollike and M. Cygler (2000). Crystal structure of human grancalcin, a member of the penta-EF-hand protein family. J Mol Biol **300**(5): 1271-81.

Johnson, P. and J. L. Hammer (1990). Inhibitory effects of spermine and spermidine on muscle calpain II. Experientia **46**: 276-278.

Karcz, S. R., R. B. Podesta, A. A. Siddiqui, G. A. Dekaban, G. H. Strejan and M. W. Clarke (1991). Molecular cloning and sequence analysis of a calcium-activated neutral protease (calpain) from *Schistosoma mansoni*. Mol.Biochem.Parasitol. **49**: 333-336.

Karlson, P. and D. M. Skinner (1960). Attempted extraction of crustacean moulting hormone from isolated Y-organs. Nature **185**: 543-544.

Kinbara, K., S. Ishiura, S. Tomioka, H. Sorimachi, S. Y. Jeong, S. Amano, H. Kawasaki, B. Kolmerer, S. Kimura, S. Labeit and K. Suzuki (1998a). Purification of native p94, a muscle-specific calpain, and characterization of its autolysis. Biochemical Journal **335**: 589-596.

Kinbara, K., H. Sorimachi, S. Ishiura and K. Suzuki (1998b). Skeletal muscle-specific calpain, p94 - Structure and physiological function. Biochem.Pharmacol. **56**: 415-420.

Klein, J. M., S. Mangerich, D. P. V. De Kleijn, R. Keller and W. M. Weidemann (1993). Molecular cloning of crustacean putative molt-inhibiting hormone (MIH) precursor. FEBS Lett. **334**: 139-142.

Kleinholz, L. H. (1975). Purified hormones from the crustacean eyestalk and their physiological specificity. Nature **258**: 256-257.

Koenders, A., X. L. Yu, E. S. Chang and D. L. Mykles (2002). Ubiquitin and actin expression in claw muscles of land crab, *Gecarcinus lateralis*, and American lobster, *Homarus americanus*: differential expression of ubiquitin in two slow muscle fibre types during moult-induced atrophy. Journal of Experimental Zoology **in press**.

Kuo, W. N., J. B. Jn-Baptiste, R. N. Kanadia, M. L. D. McNabb, L. Zhai, K. Weeks, N. Dopson and M. C. Chambers (1996). Immunoreactivities of m-calpain, calpastatin, nitric oxide synthase, myelin basic protein and dynamin II in baker's yeast, wheat germ and lobster tail muscle. Cytobios **87**: 251-263.

Kuo, W. N., T. W. Ku, D. L. Jones and J. Jn-Baptiste (1995). Calpain immunoreactivity in baker's yeast, lobster and wheat germ. Cytobios **81**: 175-180.

Kuris, A. M. and M. Mager (1975). Effect of limb regeneration on size increase at molt of the shore crabs *Hemigrapsus oregonensis* and *Pachygrapsus crassipes*. Journal of Experimental Zoology **193**: 353-360.

Lachaise, A., A. Le Roux, M. Hubert and R. Lafont (1993). The molting gland of crustaceans: localization, activity, and endocrine control (a review). Journal of Crustacean Biology **13**: 198-234.

Lachaise, F., G. Sommé, G. Carpentier, E. Granjeon, S. Webster and D. Baghdassarian (1996). A transaldolase - An enzyme implicated in crab steroidogenesis. Endocrine **5**: 23-32.

Lacombe, C., P. Grève and G. Martin (1999). Overview on the sub-grouping of the crustacean hyperglycemic hormone family. Neuropeptides **33**: 71-80.

Lee, K. J., T. S. Elton, A. K. Bej, S. A. Watts and R. D. Watson (1995). Molecular cloning of a cDNA encoding putative molt-inhibiting hormone from the blue crab, *Callinectes sapidus*. Biochem.Biophys.Res.Commun. **209**: 1126-1131.

Lee, W. J., Y. Adachi, M. Maki, M. Hatanaka and T. Murachi (1990). Factors influencing the binding of calpain I to human erythrocyte inside-out vesicles. Biochem.Int. **22**: 163-172.

Leid, M., P. Kastner and P. Chambon (1992). Multiplicity generates diversity in the retinoic acid signalling pathways. Trends Biochem Sci **17**(10): 427-33.

Lu, W. Q., G. Wainwright, S. G. Webster, H. H. Rees and P. C. Turner (2000). Clustering of mandibular organ-inhibiting hormone and moult-inhibiting hormone genes in the crab, *Cancer pagurus*, and implications for regulation of expression. Gene **253**: 197-207.

Ma, H., C. Fukiage, M. Azuma and T. R. Shearer (1998). Cloning and expression of mRNA for calpain Lp82 from rat lens: Splice variant of p94. Invest.Ophthalmol.Vis.Sci. **39**: 454-461.

Ma, H., H. Q. Yang, E. Takano, M. Hatanaka and M. Maki (1994). Amino-terminal conserved region in proteinase inhibitor domain of calpastatin potentiates its calpain

inhibitory activity by interacting with calmodulin-like domain of the proteinase. The Journal of Biological Chemistry **269**: 24430-24436.

Maki, M., H. Bagci, K. Hamaguchi, M. Ueda, T. Murachi and M. Hatanaka (1989). Inhibition of calpain by a synthetic oligopeptide corresponding to an exon of the human calpastatin gene. The Journal of Biological Chemistry **264**: 18866-18869.

Maki, M., S. V. L. Narayana and K. Hitomi (1997). A growing family of the Ca^{2+}-binding proteins with five EF-hand motifs. Biochemical Journal **328**: 718-720.

Mattson, J. M. and D. L. Mykles (1993). Differential degradation of myofibrillar proteins by four calcium-dependent proteinase activities from lobster muscle. Journal of Experimental Zoology **265**: 97-106.

Mattson, M. P. (1986). New insights into neuroendocrine regulation of the crustacean molt cycle. Zoological Science **3**: 733-744.

Mattson, M. P. and E. Spaziani (1985). Characterization of molt-inhibiting hormone (MIH) action on crustacean Y-organ segments and dispersed cells in culture and a bioassay for MIH activity. Journal of Experimental Zoology **236**: 93-102.

Mattson, M. P. and E. Spaziani (1986). Regulation of Y-organ ecdysteroidogenesis by molt-inhibiting hormone in crabs: involvement of cyclic AMP-mediated protein synthesis. General and Comparative Endocrinology **63**: 414-423.

McCarthy, J. F. and D. M. Skinner (1977). Interruption of proecdysis by autotomy of partially regenerated limbs in the land crab, *Gecarcinus lateralis*. Developmental Biology **61**: 299-310.

McConaugha, J. R. (1991). Limb regeneration in juveniles of the mud crab *Rhithropanopeus harrisii* following removal of developing limb buds. Journal of Experimental Zoology **257**: 64-69.

Mehdi, S. (1991). Cell-penetrating inhibitors of calpain. Trends in Biochemical Science **16**: 150-153.

Mellgren, R. L. and T. Murachi (1990). Intracellular Calcium-Dependent Proteolysis. Boca Raton, CRC Press.

Molinari, M., J. Anagli and E. Carafoli (1994). Ca^{2+}-activated neutral protease is active in the erythrocyte membrane in its nonautolyzed 80-kDa form. The Journal of Biological Chemistry **269**: 27992-27995.

Mykles, D. L. (1990). Calcium-dependent proteolysis in crustacean claw closer muscle maintained in vitro. Journal of Experimental Zoology **256**: 16-30.

Mykles, D. L. (1998). Intracellular proteinases of invertebrates: calcium-dependent and proteasome/ubiquitin-dependent systems. International Review of Cytology **184**: 157-289.

Mykles, D. L. (1999). Proteolytic processes underlying molt-induced claw muscle atrophy in decapod crustaceans. American Zoologist **39**: 541-551.

Mykles, D. L. (2001). Interactions between limb regeneration and molting in decapod crustaceans. Am. Zool. **41**: 399-406.

Mykles, D. L. and D. M. Skinner (1981). Preferential loss of thin filaments during molt-induced atrophy in crab claw muscle. Journal of Ultrastructure Research **75**: 314-325.

Mykles, D. L. and D. M. Skinner (1982). Molt cycle-associated changes in calcium-dependent proteinase activity that degrades actin and myosin in crustacean muscle. Developmental Biology **92**: 386-397.

Mykles, D. L. and D. M. Skinner (1983). Ca^{2+}-dependent proteolytic activity in crab claw muscle. Effects of inhibitors and specificity for myofibrillar proteins. The Journal of Biological Chemistry **258**: 10474-10480.

Mykles, D. L. and D. M. Skinner (1986a). Four Ca^{2+}-dependent proteinase activities isolated from crustacean muscle differ in size, net charge, and sensitivity to Ca^{2+} and inhibitors. The Journal of Biological Chemistry **261**: 9865-9871.

Mykles, D. L. and D. M. Skinner (1986b). Four Ca^{2+}-dependent proteinases in crustacean muscles: possible regulation by activators and inhibitors. Journal of Cell Biology **103**: 401a.

Mykles, D. L. and D. M. Skinner (1990). Atrophy of crustacean somatic muscle and the proteinases that do the job. A review. Journal of Crustacean Biology **10**: 577-594.

Nakayama, S. (1995). [Evolution of EF-hand proteins]. Seikagaku **67**(2): 131-7.

Naya, Y., M. Ohnishi, M. Ikeda, W. Miki and K. Nakanishi (1989). What is molt-inhibiting hormone? The role of an ecdysteroidogenesis inhibitor in the crustacean molting cycle. Proceedings of the National Academy of Sciences USA **86**: 6826-6829.

Needham, A. E. (1965). Regeneration in the arthropoda and its endocrine control. Regener. Anim. Relat. Probl., Int. Symp.,.

Ohno, S., S. Minoshima, J. Kudoh, R. Fukuyama, Y. Shimizu, S. Ohmi-Imajoh, N. Shimizu and K. Suzuki (1990). Four genes for the calpain family locate on four distinct human chromosomes. Cytogenet.Cell Genet. **53**: 225-229.

Patel, Y. M. and M. D. Lane (1999). Role of calpain in adipocyte differentiation. Proceedings of the National Academy of Sciences USA **96**: 1279-1284.

Penny, I. F., M. A. Taylor, A. G. Harris and D. J. Etherington (1985). Purification and immunological characterisation of two calcium-activated neutral proteinases from rabbit skeletal muscle. Biochim Biophys Acta **829**(2): 244-52.

Ranga Rao, K. (1965). Isolation and partial characterization of the molt-inhibiting hormone of the crustacean eyestalk Experientia **21**: 593-594.

Richard, I., O. Broux, V. Allamand, F. Fougerousse, N. Chiannilkulchai, N. Bourg, L. Brenguier, C. Devaud, P. Pasturaud, C. Roudaut, D. Hillaire, M.-R. Passos-Bueno, M. Zatz, J. A. Tischfield, M. Fardeau, C. E. Jackson, D. Cohen and J. S. Beckmann (1995). Mutations in the proteolytic enzyme calpain 3 cause limb-girdle muscular dystrophy type 2A. Cell **81**: 27-40.

Richard, I., C. Roudaut, A. Saenz, R. Pogue, J. E. M. A. Grimbergen, L. V. B. Anderson, C. Beley, A. M. Cobo, C. De Diego, B. Eymard, P. Gallano, H. B. Ginjaar, A. Lasa, C. Pollitt, H. Topaloglu, J. A. Urtizberea, M. De Visser, A. Van der Kooi, K. Bushby, E. Bakker, A. L. De Munain, M. Fardeau and J. S. Beckmann (1999). Calpainopathy - A survey of mutations and polymorphisms. Am.J.Hum.Genet. **64**: 1524-1540.

Rizo, J. and T. C. Sudhof (1998). C2-domains, structure and function of a universal Ca2+-binding domain. J Biol Chem **273**(26): 15879-82.

Saido, T. C., H. Sorimachi and K. Suzuki (1994). Calpain: New perspectives in molecular diversity and physiological-pathological involvement. FASEB Journal **8**: 814-822.

Saito, K.-I., J. S. Elce, J. E. Hamos and R. A. Nixon (1993). Widespread activation of calcium-activated neutral proteinase (calpain) in the brain in Alzheimer disease: A potential molecular basis for neuronal degeneration. Proceedings of the National Academy of Sciences USA **90**: 2628-2632.

Salamino, F., M. Averna, I. Tedesco, R. De Tullio, E. Melloni and S. Pontremoli (1997). Modulation of rat brain calpastatin efficiency by post-translational modifications. FEBS Lett. **412**: 433-438.

Sato, K. and S. Kawashima (2001). Calpain function in the modulation of signal transduction molecules. Biological Chemistry **382**(5): 743-751.

Schmitt, J. and H. G. Stunnenberg (1993). The glucocorticoid receptor hormone binding domain mediates transcriptional activation in vitro in the absence of ligand. Nucleic Acids Res **21**(11): 2673-81.

Scott, J. C. and D. P. McManus (2000). Characterization and expression of a cDNA encoding the 80-kDa large subunit of *Schistosoma japonicum* calpain. Parasitology International **48**: 205-214.

Sedlmeier, D. and R. Fenrich (1993). Regulation of ecdysteroid biosynthesis in crayfish Y-organs: I. Role of cyclic nucleotides. Journal of Experimental Zoology **265**: 448-453.

Shean, B. S. and D. L. Mykles (1995). Polyubiquitin in crustacean striated muscle: Increased expression and conjugation during molt-induced claw muscle atrophy. Biochim.Biophys.Acta Gene Struct.Expression **1264**: 312-322.

Shields, D. C. and N. L. Banik (1999). Pathophysiological role of calpain in experimental demyelination. Journal of Neuroscience Research **55**: 533-541.

Skinner, D. M. (1962). The structure and metabolism of a crustacean integumentary tissue during a molt cycle. Biological Bulletin **123**: 635-647.

Skinner, D. M. (1965). Amino acid incorporation into protein during the molt cycle of the land crab, *Gecarcinus lateralis*. Journal of Experimental Zoology **160**: 225-234.

Skinner, D. M. (1966). Breakdown and reformation of somatic muscle during the molt cycle of the land crab, *Gecarcinus lateralis*. Journal of Experimental Zoology **163**: 115-124.

Skinner, D. M. (1985). Molting and regeneration. The Biology of Crustacea. D. E. Bliss and L. H. Mantel. New York, Academic Press: 43-146.

Skinner, D. M. and D. E. Graham (1970). Molting in land crabs: stimulation by leg removal. Science **169**: 383-385.

Skinner, D. M. and D. E. Graham (1972). Loss of limbs as a stimulus to ecdysis in Brachyura (true crabs). Biological Bulletin **143**: 222-233.

Snyder, M. J. and E. S. Chang (1986). Effects of sinus gland extracts on larval molting and ecdysteroid titers of the American lobster, *Homarus americanus*. Biological Bulletin **170**: 244-254.

Sorimachi, H., S. Imajoh-Ohmi, Y. Emori, H. Kawasaki, S. Ohno, Y. Minami and K. Suzuki (1989). Molecular cloning of a novel mammalian calcium-dependent protease distinct from both m- and μ-types. Specific expression of the mRNA in skeletal muscle. The Journal of Biological Chemistry **264**: 20106-20111.

Sorimachi, H., S. Ishiura and K. Suzuki (1997). Structure and physiological function of calpains. Biochemical Journal **328**: 721-732.

Sorimachi, H., T. C. Saido and K. Suzuki (1994). New era of calpain research: Discovery of tissue-specific calpains. FEBS Lett. **343**: 1-5.

Sorimachi, H. and K. Suzuki (2001). The structure of calpain. Journal of Biochemistry (Tokyo) **129**: 653-664.

Sorimachi, H., N. Toyama-Sorimachi, T. C. Saido, H. Kawasaki, H. Sugita, M. Miyasaka, K. Arahata, S. Ishiura and K. Suzuki (1993). Muscle-specific calpain, p94, is degraded by autolysis immediately after translation, resulting in disappearance from muscle. The Journal of Biological Chemistry **268**: 10593-10605.

Spaziani, E., T. C. Jegla, W. L. Wang, J. A. Booth, S. M. Connolly, C. C. Conrad, M. J. Dewall, C. M. Sarno, D. Stone and R. Montgomery (2001). Further studies on signaling pathways for ecdysteroidogenesis in crustacean Y-organs. American Zoologist **41**(3): 418-429.

Spaziani, E., M. P. Mattson, W. N. L. Wang and H. E. McDougall (1999). Signaling pathways for ecdysteroid hormone synthesis in crustacean Y-organs. American Zoologist **39**: 496-512.

Spees, J. L., S. A. Chang, D. L. Mykles, M. J. Snyder and E. S. Chang (2001). Molt cycle-dependent molecular chaperone and polyubiquitin gene expression in lobster. in preparation.

Stoffel, L. A. and J. H. Hubschman (1974). Limb loss and the molt cycle in the freshwater shrimp, *Palaemonetes kadiakensis*. Biological Bulletin **147**: 203-212.

Strobl, S., C. Fernandez-Catalan, M. Braun, R. Huber, H. Masumoto, K. Nakagawa, A. Irie, H. Sorimachi, G. Bourenkow, H. Bartunik, K. Suzuki and W. Bode (2000). The crystal structure of calcium-free human m-calpain suggests an electrostatic switch mechanism for activation by calcium. Proceedings of the National Academy of Sciences USA **97**: 588-592.

Suzuki, K., T. C. Saido and S. Hirai (1992a). Modulation of cellular signals by calpain. Ann.NY Acad.Sci. **674**: 218-227.

Suzuki, K., K. Shimizu, T. Hamamoto, Y. Nakagawa, T. Murachi and T. Yamamuro (1992b). Characterization of proteoglycan degradation by calpain. Biochemical Journal **285**: 857-862.

Suzuki, K., S. Tsuji, S. Kubota, Y. Kimura and K. Imahori (1981). Limited autolysis of Ca^{2+}-activated neutral protease (CANP) changes its sensitivity to Ca^{2+} ions. Journal of Biochemistry (Tokyo) **90**: 275-278.

Takano, E., H. Ma, H. Q. Yang, M. Maki and M. Hatanaka (1995). Preference of calcium-dependent interactions between calmodulin-like domains of calpain and calpastatin subdomains. FEBS Lett. **362**: 93-97.

Theopold, U., M. Pintér, S. Daffre, Y. Tryselius, P. Friedrich, D. R. Nässel and D. Hultmark (1995). *CalpA*, a *Drosophila* calpain homolog specifically expressed in a small set of nerve, midgut, and blood cells. Molecular and Cellular Biology **15**: 824-834.

Tompa, P., Y. Emori, H. Sorimachi, K. Suzuki and P. Friedrich (2001). Domain III of calpain is a Ca^{2+}-regulated phospholipid-binding domain. Biochem.Biophys.Res.Commun. **280**: 1333-1339.

Traub, M., G. Gellissen and K. D. Spindler (1987). 20 (OH) ecdysone-induced transition from intermolt to premolt protein biosynthesis patterns in the hypodermis of the crayfish, Astacus leptodactylus, in vitro. Gen Comp Endocrinol **65**(3): 469-77.

Treves, S., B. Vilsen, P. Chiozzi, J. P. Andersen and F. Zorzato (1992). Molecular cloning, functional expression and tissue distribution of the cDNA encoding frog skeletal muscle calsequestrin. Biochem J **283 (Pt 3)**: 767-72.

Tsai, Y. F., H. J. Wang, W. P. Pi and M. Y. Tai (1994). Age-related changes in the pattern of luteinizing hormone release induced in female rats by male rat urine. Neurosci Lett **180**(2): 179-82.

Tsuji, T., S. Shimohama, J. Kimura and K. Shimizu (1998). M-Calpain (calcium-activated neutral proteinase) in Alzheimer's disease brains. Neuroscience Letters **248**: 109-112.

Umphrey, H. R., K. J. Lee, R. D. Watson and E. Spaziani (1998). Molecular cloning of a cDNA encoding molt-inhibiting hormone (MIH) of the crab, *Cancer magister*. Molecular and Cellular Endocrinology **136**: 145-149.

Vilei, E. M., S. Calderara, J. Anagli, S. Berardi, K. Hitomi, M. Maki and E. Carafoli (1997). Functional properties of recombinant calpain I and of mutants lacking domains III and IV of the catalytic subunit. The Journal of Biological Chemistry **272**: 25802-25808.

Watson, R. D., K. J. Lee, S. Qiu, M. Lou, H. R. Umphrey, R. D. Roer and E. Spaziani (2001). Molecular cloning, expression, and tissue distribution of crustacean molt-inhibiting hormone. American Zoologist **41**: 407-417.

Watson, R. D., E. Spaziani and W. E. Bollenbacher (1989). Regulation of ecdysone biosynthesis in insects and crustaceans. Ecdysone, from chemistry to mode of action. J. Koolman. Stutgart, Georg Thieme Verlag: 188-203.

Webster, S. G. (1986). Neurohormonal control of ecdysteroid biosynthesis by *Carcinus maenas* Y-organs *in vitro*, and preliminary characterization of the putative molt-inhibiting hormone (MIH). General and Comparative Endocrinology **61**: 237-247.

Webster, S. G. (1993). High-affinity binding of putative moult-inhibiting hormone (MIH) and crustacean hyperglycaemic hormone (CHH) to membrane-bound receptors on the Y-organ of the shore crab *Carcinus maenus*. Proc.R.Soc.Lond.[Biol.] **251**: 53-59.

Webster, S. G., H. Dircksen and J. S. Chung (2000). Endocrine cells in the gut of the shore crab *Carcinus maenas* immunoreactive to crustacean hyperglycaemic hormone and its precursor-related peptide. Cell Tissue Res. **300**: 193-205.

Webster, S. G. and R. Keller (1986). Purification, characterisation and amino acid composition of the putative moult-inhibiting hormone (MIH) of *Carcinus maenas* (Crustacea, Decapoda). Journal of Comparative Physiology **156**: 617-624.

Weis, J. S. (1976). Regeneration in the fiddler crab, Uca pugilator, after injury or removal of limb buds. Journal of Experimental Zoology **197**: 21-30.

Whiteley, N. M. and A. J. El Haj (1997). Regulation of muscle gene expression over the moult in crustacea. Comparative Biochemistry and Physiology [B] **117B**: 323-331.

Willig, A. and R. Keller (1973). Molting hormone content, cuticle growth and gastrolith growth in the molt cycle of the crayfish *Orconectes limosus*. Journal of Comparative Physiology **86**: 377-388.

Yamaguchi, R., M. Maki, M. Hatanaka and H. Sabe (1994). Unphosphorylated and tyrosine-phosphorylated forms of a focal adhesion protein, paxillin, are substrates for calpain II in vitro: Implications for the possible involvement of calpain II in mitosis-specific degradation of paxillin. FEBS Lett. **356**: 114-116.