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# Cloning of a muscle-specific calpain from the American lobster *Homarus americanus*: expression associated with muscle atrophy and restoration during moulting

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**Summary**

**A cDNA (1977 bp) encoding a crustacean calpain (Ha-CalpM; GenBank accession no. AY124009) was isolated from a lobster fast muscle cDNA library. The open reading frame specified a 575-amino acid (aa) polypeptide with an estimated mass of 66.3 kDa. Ha-CalpM shared high identity with other calpains in the cysteine proteinase domain (domain II; aa 111–396) and domain III (aa 397–575), but most of the N-terminal domain (domain I; aa 1–110) was highly divergent. Domain II contained the cysteine, histidine and asparagine triad essential for catalysis, as well as two conserved aspartate residues that bind $Ca^{2+}$. In domain III an acidic loop in the C2-like region, which mediates $Ca^{2+}$-dependent phospholipid binding, had an expanded stretch of 17 aspartate residues. Ha-CalpM was classified as a non-EF-hand calpain, as it lacked domain IV, a calmodulin-like region containing five EF-hand motifs. Northern blot analysis, relative reverse transcription-polymerase chain reaction (RT-PCR) and real-time PCR showed that Ha-CalpM was highly expressed in skeletal muscles, but at much lower levels in heart, digestive gland, intestine, integument, gill, nerve cord/thoracic ganglion and antennal gland. An antibody raised against a unique N-terminal sequence recognized a 62 kDa isoform in cutter claw and crusher claw closer muscles and a 68 kDa isoform in deep abdominal muscle. Ha-CalpM was distributed throughout the cytoplasm, as well as in some nuclei, of muscle fibers. Purification of Ha-CalpM showed that the 62 kDa and 68 kDa isoforms co-eluted from gel filtration and ion exchange columns at positions consistent with those of previously described $Ca^{2+}$-dependent proteinase III (CDP III; 59 kDa). Ha-CalpM mRNA and protein did not change during the moulting cycle. The muscle-specific expression of Ha-CalpM and the ability of Ha-CalpM/CDP III to degrade myofibrillar proteins suggest that it is involved in restructuring and/or maintaining contractile structures in crustacean skeletal muscle.**

Key words: calpain, calcium-dependent proteinase, muscle, atrophy, moulting, cDNA, gene expression, mRNA, lobster, *Homarus americanus*, Crustacea, Arthropoda.

## Introduction

Calpains constitute a large and diverse family of $Ca^{2+}$-dependent cysteine proteinases in animal cells (Mykles, 1998; Sorimachi and Suzuki, 2001). In mammals, there are more than a dozen calpain genes, some of which produce isoforms by alternative mRNA splicing (Sorimachi and Suzuki, 2001). cDNAs encoding invertebrate calpains have been obtained from fruit fly (Emori and Saigo, 1994; Jékely and Friedrich, 1999; Theopold et al., 1995), nematode (Sokol and Kuwabara, 2000), platyhelminth (Andresen et al., 1991) and trypanosome (Hertz-Fowler et al., 2001). All calpains have a highly conserved catalytic domain (domain II), containing a cysteine, histidine and asparagine that are essential for activity (Sorimachi and Suzuki, 2001). Domain III forms a C2-like structure with an acidic loop, which mediates $Ca^{2+}$-dependent phospholipid binding and activation (Hosfield et al., 1999, 2001; Sorimachi and Suzuki, 2001). Domain IV is a calmodulin-like domain containing five EF-hand motifs, the first three of which (EF-1, EF-2 and EF-3) bind $Ca^{2+}$ with various affinities (Dutt et al., 2000; Hosfield et al., 1999). The N-terminal sequence (domain I) is the least conserved of the four domains; it varies in length and sequence between different calpain genes (Sorimachi and Suzuki, 2001). At least in some calpains, the N terminus acts as an inhibitory domain, which is autocatalytically cleaved to activate the enzyme (Jékely and Friedrich, 1999). In addition to the $Ca^{2+}$-binding sites in domains III and IV, there are conserved aspartates in two non-EF-hand sites in domain II that bind $Ca^{2+}$ and are involved in full activation of the enzyme (Khorchid and Ikura, 2002; Moldoveanu et al., 2002).

Lobster muscle contains four $Ca^{2+}$-dependent cysteine proteinase (CDPs I, IIa, IIb and III), or calpain, activities (Mykles and Skinner, 1986). All require $Ca^{2+}$ at millimolar

levels for full activity *in vitro* and degrade myofibrillar protein (Mattson and Mykles, 1993; Mykles and Skinner, 1982, 1983, 1986). CDP IIb (195 kDa) is a homodimer, consisting of a 95 kDa subunit, which appears to be the homolog of *Drosophila* CalpA or Dm-calpain (Beyette et al., 1993, 1997; Beyette and Mykles, 1997; Emori and Saigo, 1994; Jékely and Friedrich, 1999; Theopold et al., 1995). CDP IIb undergoes an autolytic cleavage at the N terminus, but it has little effect on the $Ca^{2+}$ sensitivity of the enzyme (Beyette et al., 1993; Beyette and Mykles, 1997). CDP IIa (125 kDa) is a homodimer of a 60 kDa subunit (Beyette et al., 1997). The subunit compositions of CDP I (310 kDa) and CDP III (59 kDa) are not known, as neither has been purified to homogeneity.

Both $Ca^{2+}$-dependent and ubiquitin/proteasome-dependent proteolytic systems are stimulated during moult-induced claw muscle atrophy, when at least 40% of the muscle protein is degraded (Beyette and Mykles, 1999; Mykles, 1998, 1999; Skinner, 1966). There is a preferential degradation of thin myofilament proteins, resulting in a reduction in the thin:thick myofilament ratio and increased thick myofilament packing density (Ismail and Mykles, 1992; Mykles and Skinner, 1981, 1982). It is estimated that 11 thin myofilaments are degraded for every thick myofilament degraded (Mykles and Skinner, 1981). During postmoult, protein is resynthesized as the muscle is restored to its original mass and myofilament composition (Skinner, 1966). Thus, there is significant remodeling of the contractile apparatus as fibers undergo atrophy and restoration during the moulting cycle. Calpains probably play an important role, as they degrade the Z-line and myofibrillar proteins *in vitro* and *in situ* and have higher activities in atrophic muscle (Mattson and Mykles, 1993; Mykles, 1990; Mykles and Skinner, 1982, 1983). Moreover, the four calpain activities may have specific functions, as each calpain has unique substrate specificities when incubated with a mixture of myofibrillar proteins (Mattson and Mykles, 1993). For example, CDP IIb preferentially degrades thin myofilament proteins and thus may contribute to the loss of thin myofilaments in atrophic muscle (Mattson and Mykles, 1993). Proteasome level and ubiquitin expression and conjugation are increased in atrophic muscle, although the role of this proteolytic pathway in claw muscle remains to be established (Koenders et al., 2002; Shean and Mykles, 1995).

Here we report the cloning of a crustacean calpain gene with features that distinguish it from calpains isolated from other species. The cDNA encodes a non-EF-hand calpain with a unique N-terminal sequence and an extended acidic loop in the C2-like region of domain III. The transcript levels in various tissues and in claw muscle during the moulting cycle were quantified by northern blot analysis, relative reverse transcription–polymerase chain reaction (RT-PCR) and real-time PCR. The distribution of Ha-CalpM protein was determined by western blotting and immunocytochemistry. Since this calpain was highly expressed in skeletal muscles, we have named the gene Ha-CalpM (*Homarus americanus* muscle-specific calpain; GenBank accession no. AY124009).

## Materials and methods

### *Cloning of cDNA encoding lobster muscle calpain (Ha-CalpM)*

Cloning of a full-length cDNA sequence of Ha-CalpM from *Homarus americanus* (Milne Edwards 1837) 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. In all three steps, plasmid DNA (pcDNAII, Invitrogen) of a lobster cDNA library made from deep abdominal muscle (Cotton and Mykles, 1993; Shean and Mykles, 1995) was used as template.

For the first step, the deduced amino acid sequences of calpain catalytic subunits from diverse species were aligned to identify conserved sequences using the Clustal W method from the Lasergene biocomputing software (DNASTAR). Degenerate primer sequences were selected from regions of high amino acid sequence identity. Two pairs of primers were synthesized for nested PCR, covering a sequence segment of 553 base pairs (bp) (equivalent to bp 423–975 in *Drosophila* CalpA; accession number Z46891). 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. 1.

The external primers were used for the first PCR reaction. A Perkin-Elmer DNA thermal cycler model 9600 was used for all reactions. The composition of the reaction mixture (25 µl) was 2.5 mmol $l^{-1}$ $MgCl_2$, 10 mmol $l^{-1}$ Tris-HCl, pH 9.0, 50 mmol $l^{-1}$ KCl, 0.1% Triton X-100, 200 µmol $l^{-1}$ each of dNTPs, 1 µmol $l^{-1}$ of each primer (1F and 1B), 0.75 µg plasmid, and 2.5 units of *Taq* polymerase (Promega). Initial denaturation was at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 54.8°C for 1 min, and extension at 72°C for 1 min. The final extension was at 72°C for 6 min. A 0.1 µl sample of the PCR reaction 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.

The Expand High Fidelity PCR System (Boehringer Mannheim) was used for IPCR. Inverse primers were synthesized based on the sequence of the nested PCR product: antisense, 5′-CAA GCC TTC CAT TGT ATG TG-3′ (bp 600–619 in Fig. 1); sense, 5′-TCA TGC TTA CTC CAT CAC CC-3′ (bp 909–928 in Fig. 1). The composition of the reaction mixture (50 µl) was 2 mmol $l^{-1}$ Tris-HCl, pH 7.5, 10 mmol $l^{-1}$ KCl, 0.1 mmol $l^{-1}$ dithiothreitol (DTT), 0.01 mmol $l^{-1}$ EDTA, 0.05% Tween-20, 200 µmol $l^{-1}$ each of dNTPs, 300 nmol $l^{-1}$ of each primer, 0.75 µg plasmid, and 2.6 units of Expand High Fidelity PCR enzyme. Initial denaturation was at 94°C for 2 min, followed by 10 cycles of denaturation at 94°C for 15 s,

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      aggagcaacaacaggATGTCGCTTAGTGAGGTGGTGGAGGATCAGAGTGTGTGGAAGCCAACGGAGGACCATGACAGTCACTGCCGCTGCTACAAACGGG  100
  1                 M  S  L  S  E  V  V  E  D  Q  S  V  W  K  P  T  E  D  H  D  S  H  C  R  C  Y  K  R
      AGGGCCACGGCCACATGGTGGACGGCATAGAGTCCGTCTCCCTTCAGAAGTCTACCTACTACAGCCGCGTCAGTAATGATTACACCCAGAAGCGGATAGC  200
  29  E  G  H  G  H  M  V  D  G  I  E  S  V  S  L  Q  K  S  T  Y  Y  S  R  V  S  N  D  Y  T  Q  K  R  I  A
      CAAAGGTGGGTTGAAGATCCCCAAGAAGGGGTTCAGGACTCTGCGAGATGAGTGTCTTAAGAGCAGTAAGTTATACGAGGACCCTGAGTTCCCAGCCAAT  300
  63    K  G  G  L  K  I  P  K  K  G  F  R  T  L  R  D  E  C  L  K  S  S  K  L  Y  E  D  P  E  F  P  A  N
      GACTACTCTATCAACTTCAATGGCGTCACCCGCAGAACCTATGTTTGGAAGAGACCTCACGAGATTACAAAGAATCCACGTTTCTTCATTGACGGTGCAA  400
  96   D  Y  S  I  N  F  N  G  V  T  R  R  T  Y  V  W  K  R  P  H  E  I  T  K  N  P  R  F  F  I  D  G  A
      CACGGTTTGACATTCAACAGGGAGAACTTGGTGACTGTTGGCTCTTGGCTGCAGTATCTAATCTGACCTTGAATCCTCAAATGTTCCATGCGGTGGTACC  500
  129 T  R  F  D  I  Q  Q  G  E  L  G  D  C  W  L  L  A  A  V  S  N  L  T  L  N  P  Q  M  F  H  A  V  V  P
      AAGAGACCAAGGATTCATTGATCTTTATGCAGGCATCTTTCATTTCAAATTCTGGCAGTATGGGAAGTGGCAAGAGGTGGTGGTGGATGACAGGCTGCCC  600
  163   R  D  Q  G  F  I  D  L  Y  A  G  I  F  H  F  K  F  W  Q  Y  G  K  W  Q  E  V  V  V  D  D  R  L  P
      ACATACAATGGAAGGCTTGTATTCATGCACTCAAAGACTGAAAATGAATTTTGGTGTGCTCTTTTTGAGAAGGCATATGCTAAGTTATATGGAGGGTATG  700
  196  T  Y  N  G  R  L  V  F  M  H  S  K  T  E  N  E  F  W  C  A  L  F  E  K  A  Y  A  K  L  Y  G  G  Y
      AATCACTGCGTGGCGGCAACATTAATGAGAGTATGGTTGACCTGACAGGAGGTGTGGTGGAGATGATTGATCTTCGTAATCCACCACCCAAATTATTTTC  800
  229 E  S  L  R  G  G  N  I  N  E  S  M  V  D  L  T  G  G  V  V  E  M  I  D  L  R  N  P  P  P  K  L  F  S
      CACCTTGTGGAAGGCTTACCGTAGAGGTGCTCTCATAGGCACTGCTATAGAGCCCGACCAGTCAAACATACAGGCAGAGAGCATCCTCAGTAATGGCCTT  900
  263   T  L  W  K  A  Y  R  R  G  A  L  I  G  T  A  I  E  P  D  Q  S  N  I  Q  A  E  S  I  L  S  N  G  L
      ATAATGCGTCATGCTTACTCCATCACCCGCGTTACTACAGTAGATATTAAGTCTGTTGTCCCCAGATTGCAGGGCAAGGCTCAACTAATCCGTCTGCATA  1000
  296  I  M  R  H  A  Y  S  I  T  R  V  T  T  V  D  I  K  S  V  V  P  R  L  Q  G  K  A  Q  L  I  R  L  H
      ATCCTTGGGGCAATGAAGCTGAATGGAAAGGTTCGTGGAGTGACAAGTCTCCAGAGTGGAATTCTATCACTCCTGAGGAGAAGCAACGTCTTAAACTCAA  1100
  329 N  P  W  G  N  E  A  E  W  K  G  S  W  S  D  K  S  P  E  W  N  S  I  T  P  E  E  K  Q  R  L  K  L  N
      CTTTGAAGATGATGGGGAGTTTTGGATGAGTTTCCAGGACTTTGCATCAAATTTCACCACAGTTGAAATCTGTGATGTCACCCCAGAAGTGTTTGATCAT  1200
  363   F  E  D  D  G  E  F  W  M  S  F  Q  D  F  A  S  N  F  T  T  V  E  I  C  D  V  T  P  E  V  F  D  H
      GATGATAGTGATGATGAAAATGGTAACACAAAGATGGAGGAGTCGGCACCAAAGAGGTGGCAGATGGTGATGTACGAAGGGGCCTGGGCTGCACACCATA  1300
  396  D  D  S  D  D  E  N  G  N  T  K  M  E  E  S  A  P  K  R  W  Q  M  V  M  Y  E  G  A  W  A  A  H  H
      GTGCTGGTGGTTGCAGAAACTTTATTAACACATTTGCAAGCAACCCACAGTTTACAGTGCAGCTGGAGGATCCTGATGATGATGATGATGATGATGA  1400
  429 S  A  G  G  C  R  N  F  I  N  T  F  A  S  N  P  Q  F  T  V  Q  L  E  D  P  D  D  D  D  D  D  D  D  D
      TGATGACGATGATGATGATGATGACAGAGGTCAATGCACCATAGTTGTCTCTCTTATGCAGAAGAATGTCAGACAACTCAAACGCTATGGAGTTGACTAT  1500
  463   D  D  D  D  D  D  D  D  R  G  Q  C  T  I  V  V  S  L  M  Q  K  N  V  R  Q  L  K  R  Y  G  V  D  Y
      GTCCCAATTGGCTTCACCATATATGCGTTGCCAGCAAACATGCAGCCTGGACAAAAGCTGGACACAGAGTTCTTCAAGTACAATCCTAGCTTGGCAAAAG  1600
  496  V  P  I  G  F  T  I  Y  A  L  P  A  N  M  Q  P  G  Q  K  L  D  T  E  F  F  K  Y  N  P  S  L  A  K
      TACCATTTTTCCTCAATACTCGTGAATTAACTACTAGATTCCGTTTCCCTCCTGGTCTTTATGTCATCATCCCATCCACTTTTGAACCTGAAATGACTG  1700
  529 V  P  F  F  L  N  T  R  E  L  T  T  R  F  R  F  P  P  G  L  Y  V  I  I  P  S  T  F  E  P  E  M  T
      GAGAGTTTCTCATAAGAATCTTTACTGAGGCTAAGAGAAGGTAAattgtaattatttttaagtgacatattaagtctcaggatttagcaacaaacgcatg  1800
  562 G  E  F  L  I  R  I  F  T  E  A  K  R  R STOP
      acaaaatactactgtacactatcaaatagatttgtgttttatagaattttatcttaattatttactgtacaagtaaaagcaaatgagacagtgaaaatat  1900

      ttgtataccggtacctttaaagtgaataacttttattcaagaagacagtaattagaatgctaatgatgggctgtgcaa                        1977
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Fig. 1. The complete sequence of Ha-CalpM cDNA cloned from a lobster fast muscle library. The sequence (1977 bp) contains an open reading frame (bp 16–1743) encoding a protein of 575 amino acids (aa) with a predicted mass of 66.3 kDa. The three conserved aa (Cys 141, His 299, Asn 329) essential for hydrolase activity are indicated in bold. A stretch of 17 aspartates (aa 454–470) is underlined. The numbers on the left indicate aa positions; those on the right indicate nucleotides. The highly conserved aa sequences used for initial cloning with nested PCR are underlined and italicized. GenBank accession no. AY124009.

annealing at 59°C for 30 s, and extension at 68°C for 4 min. The reaction then underwent 20 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 30 s and extension at 68°C for 4 min. The final extension was at 72°C for 6 min.

Based on the sequence from IPCR, specific primers to the 5′ and 3′ ends of Ha-CalpM were synthesized: sense, 5′-AGG AGC AAC AAC AGG ATG-3′ (bp 1–18 in Fig. 1); antisense, 5′-TGC ACA GCC CAT CAT TAG –3′ (bp 1959–1976 in Fig. 1). The PCR conditions were the same as those used for IPCR.

For cloning PCR products, reactions were separated electrophoretically on 1% agarose gels (70 V for 1.5 h); products were excized and purified using the Qiagen Gel Purification kit. The DNA was concentrated in a Savant Speedvac and ligated into the pGEM-T Easy plasmid vector (Promega). Ligation was achieved by incubating 250 ng DNA, 50 ng vector and 3 Weiss units T4 DNA ligase in 30 mmol $l^{-1}$ Tris-HCl, pH 7.8, 10 mmol $l^{-1}$ $MgCl_2$, 10 mmol $l^{-1}$ DTT, 0.5 mmol $l^{-1}$ ATP and 5% polyethylene glycol, at room temperature for 1 h or overnight at 4°C (10 µl final volume). DH5α electro-competent *E. coli* cells were transformed with the ligation reaction by electro-transformation and plated on LB agar plates containing 100 µg $ml^{-1}$ ampicillin (Sigma). Positive colonies were selected and grown overnight; plasmid DNA was isolated using a Qiagen Plasmid Mini-Prep kit. Plasmid DNA containing inserts were sequenced in both directions using the promoter primers Sp6 and T7 and gene-specific primers (Davis Sequencing, Davis, CA, USA).

For sequence analysis, calpain sequences were retrieved from GenBank at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). Sequence homologies were determined using the NCBI BLAST program. Multiple alignment and phylogenetic analyses were done using DNASTAR sequence analysis software.

### *Analysis of Ha-CalpM mRNA by northern blot and RT-PCR*

Total RNA from lobster tissues was extracted using Trizol reagent (Gibco BRL). The procedure was essentially the same as that recommended by the manufacturer. Briefly, tissues were homogenized in 10 vol. Trizol (500 mg tissue in 5 ml) using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY, USA) for about 1 min or until the tissues were completely homogenized. Insoluble material was removed by centrifugation at 12 000 ***g*** for 10 min at 4°C. 1 ml chloroform was added to each supernatant fraction after incubation at room

temperature for 5 min. Samples were vortexed and centrifuged at 12 000 ***g*** for 15 min at 4°C. RNA in the aqueous phase was precipitated with 2.5 ml isopropyl alcohol and pelleted by centrifugation at 12 000 ***g*** for 10 min at 4°C. The precipitated RNA was washed with 75% ethanol, dissolved in 50 µl deionized formamide, and stored at –80°C.

Total RNA (20 µg) was separated electrophoretically on 1% agarose gels containing 0.5 mol $l^{-1}$ formaldehyde in 1× Mops buffer (40 mmol $l^{-1}$ Mops, pH 7.0, 10 mmol $l^{-1}$ sodium acetate and 1 mmol $l^{-1}$ EDTA) at 70 V for 3 h. RNA was transferred onto Hybond-N nylon membrane (Amersham) overnight in 20× SSC (3 mol $l^{-1}$ NaCl and 0.3 mol $l^{-1}$ sodium citrate, pH 7.0). The blot was UV cross-linked at 120 J for 30 s and hybridized in 20 ml hybridization buffer (Boehringer Mannheim) containing 50% deionized formamide, 5× SSC, 0.1% *N*-lauroylsarcosine, 0.02% SDS, 1% Blocking Reagent, and 1 µg digoxigenin (DIG)-labeled RNA probe overnight at 68°C. A DIG-RNA Labeling kit (Boehringer Mannheim) was used to synthesize the calpain probe by *in vitro* transcription from linearized plasmid containing a partial Ha-CalpM insert (bp 646–1129 in Fig. 1). Plasmid (1.8 µg) was linearized by digestion with *Nco*I (37°C for 1 h), separated electrophoretically on a 1% agarose gel, and purified with a Qiagen gel purification kit. The labeling reaction mixture (20 µl) contained 0.78 µg linearized plasmid, 1× DIG-RNA labeling mix (1 mmol $l^{-1}$ ATP, 1 mmol $l^{-1}$ CTP, 1 mmol $l^{-1}$ GTP, 0.65 mmol $l^{-1}$ UTP and 0.35 mmol $l^{-1}$ DIG-11-UTP, pH 7.5), 1× transcription buffer, pH 7.5, and 30 units Sp6 RNA polymerase; the mixture was incubated at 37°C for 2 h. The membrane was washed twice (1 h each) with 0.5× SSC and 0.1% SDS at 68°C to remove unhybridized probe. Chemiluminescent detection of probe used anti-DIG antibody conjugated to alkaline phosphatase (Boehringer Mannheim) and disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.$1^{3,7}$]decan}-4-yl)phenyl phosphate (CSPD; Roche Molecular Biochemicals) according to Boehringer Mannheim's protocol.

For first-strand cDNA synthesis, total RNA in formamide was precipitated with 0.1 vol. 4 mol $l^{-1}$ LiCl and 3 volumes of 100% ethanol. The RNA was washed in 75% ethanol and dissolved in diethyl pyrocarbonate (DEPC)-treated $H_2O$. RNA was first treated with DNase (Gibco BRL) to remove contaminating DNA, then 2 µg of RNA was reverse-transcribed using SuperScript™II RNase H– reverse transcriptase (Gibco BRL). The reaction consisted of 2.5 µg of Oligo (dT)12–18 (or 0.5 µg of random primers), 2.5 mmol $l^{-1}$ dNTP, 1× First-Strand Buffer, 5 mmol $l^{-1}$ DTT, 2.5 units RNase inhibitor and 200 units Superscript™ II RNase H– Reverse Transcriptase. The reaction mixture was incubated at 42°C for 50 min and stored at –20°C.

For the relative RT-PCR, a pair of 18S rRNA Primers and Competimers of ratio 3:7 (Ambion) were included in the PCR reaction mixture together with the calpain primer pairs directed to the 5′ end of the Ha-CalpM cDNA: sense, 5′-AGG AGC AAC AAC AGG ATG-3′ (bp 1–18 in Fig. 1); antisense, 5′-CAA GCC TTC CAT TGT ATG TG-3′ (bp 600–619 in Fig. 1). The reaction mixture contained 1× ExTaq buffer (2.5 mmol $l^{-1}$ $Mg^{2+}$, 200 µmol $l^{-1}$ dNTP, 0.5 µmol $l^{-1}$ of each primer, 1.5 units of Hot Start ExTaq polymerase (Takara). The final volume was 25 µl. After synthesis of first-strand cDNA (see above), PCR amplification followed at 92°C for 2 min, and 30 cycles of 94°C for 30 s, 60°C for 30 s and 68°C for 45 s. The final extension time was 6 min at 72°C. The conditions for amplifying lobster α-actin (Ha-actin1; accession no. AF399872) were the same as that for Ha-CalpM and used the same primers described previously (Koenders et al., 2002).

Real-time PCR quantification of Ha-CalpM transcript in lobster tissues used a SmartCycler instrument (Cepheid). A PCR master mix was assembled on ice containing 1× LightCycler FastStart Reaction Mix (SYBR Green I, dNTP mix with dUTP instead of dTTP, and FastStart Taq DNA polymerase), 3 mmol $l^{-1}$ $MgCl_2$ and 0.5 µmol $l^{-1}$ of each primer (20 µl total volume). The sense primer (RTCalp-F1) was 5′-CAA CAA CAG GAT GTC GCT TAG TGA G-3′ (bp 6–30 in Fig. 1) and the antisense primer (RT-Calp-B1) was 5′-TTC TGA AGG GAG ACG GAC TCT ATG-3′ (bp 126–149 in Fig. 1). The mixtures were placed in reaction tubes, followed by the addition of 1 µl (100 ng) of first-strand cDNA. Plasmid containing Ha-CalpM cDNA insert was serially diluted (every tenfold) at a range of 0.28 fg to 28 pg to generate a standard curve. Both the Ha-CalpM plasmid and first-strand cDNA reactions were run simultaneously in a SmartCycler instrument using the same protocol. The protocol consisted of three stages: stage 1, holding at 95°C for 10 min; stage 2, 40 cycles at 95°C for 10 s, 57°C for 15 s with Optics on, and 72°C for 10 s; and stage 3, melting curve (60°C to 95°C at 0.2°C $s^{-1}$). Cycle threshold analysis was used to quantify the absolute copy number of the Ha-CalpM transcript, as the cycle at which a specific PCR product begins to accumulate is directly proportional to the concentration of cDNA template in the reaction. A standard curve plotting the cycle threshold *versus* Ha-CalpM cDNA concentration was used to calculate the concentration of Ha-CalpM transcript in first-strand cDNA synthesized from tissue RNA.

### *Purification and N-terminal sequencing of Ha-CalpM isoforms*

Chromatographic isolation of Ha-CalpM followed the protocol described previously (Mykles, 2000). Briefly, claw and deep abdominal muscles were homogenized in Buffer A (20 mmol $l^{-1}$ Tris-acetate, pH 7.5, 20 mmol $l^{-1}$ KCl, 1 mmol $l^{-1}$ EDTA and 1 mmol $l^{-1}$ DTT) and centrifuged at 12 000 ***g*** for 15 min at 4°C. The supernatant was precipitated with 65% saturated ammonium sulfate, dialyzed overnight against 2 l Buffer A without DTT and KCl at 4°C, and chromatographed on an organomercurial-agarose (OMA) column (5 cm × 5 cm). Active fractions from the OMA column were concentrated by a stirred-cell ultrafiltration device (Model 8200, Amicon) and dialyzed against two 2 l changes of Buffer A. Protein was loaded onto a Q Sepharose column (2.5 × 18 cm) and eluted with a linear NaCl gradient from 0 to 1 mol $l^{-1}$ in Buffer A (300 ml total volume). Western blotting (see below) was used

to identify Ha-CalpM-containing fractions, which were pooled, concentrated to about 5 ml with Filtron Macrosep 50K devices, and dialyzed against 2 l Buffer B (20 mmol $l^{-1}$ Pipes-NaOH, pH 6.8, 0.1 mol $l^{-1}$ NaCl, 1 mmol $l^{-1}$ EDTA, 20% glycerol and 1 mmol $l^{-1}$ DTT). The dialyzed sample was chromatographed on a Superdex 200 (Pharmacia) gel filtration column (2.6 cm × 86 cm) equilibrated with Buffer B at 3 ml $min^{-1}$. Fractions (1.5 ml) containing Ha-CalpM (determined by western blotting) were pooled and loaded onto a Mono Q ion exchange column (Pharmacia HR 5/5) equilibrated with Buffer B. Protein was eluted from the column with a linear NaCl gradient from 0.1 to 0.5 mol $l^{-1}$ (20 ml total volume) at 1 ml $min^{-1}$. Fractions (0.6 ml) were analyzed by western blotting using Ha-CalpM antiserum.

For N-terminal sequencing, the Ha-CalpM proteins (Mono Q fractions 23–25) were separated on a 6% SDS-polyacrylamide gel and transferred to PVDF (polyvinylidene difluoride) membrane using 1× CAPS, pH 11/10% methanol transfer buffer. The protein on the blot was stained with India ink and the 62 kDa and 68 kDa proteins were excized and submitted to the Colorado State University Macromolecular Resources Facility for sequencing by Edman degradation.

### *SDS-polyacrylamide gel electrophoresis and western blot analysis*

For analysis of Ha-CalpM in lobster tissues, tissues were homogenized in 6 vol. ice-cold Buffer A containing 1× protease inhibitor cocktail {Sigma; 2 mmol $l^{-1}$ AEBSF [4-(2-aminoethyl)benzene sulfonyl fluoride], 1 mmol $l^{-1}$ EDTA, 130 μmol $l^{-1}$ bestatin, 1.4 μmol $l^{-1}$ E-64, 1 μmol $l^{-1}$ leupeptin and 0.3 μmol $l^{-1}$ aprotinin} and centrifuged at 12,000 ***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 LS-2 Filter Fluorimeter using bovine serum albumin as standard (Avruch and Wallach, 1971).

Protein samples (32 μg) were mixed with equal volumes of sample buffer (150 mmol $l^{-1}$ Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 20 mmol $l^{-1}$ DTT and 0.01% Bromophenol Blue) and incubated for 5 min at 95°C. Proteins were separated electrophoretically by discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, 1970). For the column chromatography samples, 10 μl of each fraction was analyzed. Proteins in gels were either stained with ammoniacal silver (Mykles, 1988) or electrophoretically transferred to PVDF membrane (Hybond-P, Amersham) (Towbin et al., 1979).

For immunochemical studies, a polyclonal antibody was raised against a 28-amino acid (aa) sequence (53–80) in the N-terminal region of the Ha-CalpM deduced sequence. Peptide ($NH_2$-SNDYTQKRIAKGGLKIPKKGFRTLRDEC-COOH) conjugated to keyhole limpet hemocyanin was used to raise antibody in rabbits by the Macromolecular Resource Facility at Colorado State University.

In some cases anti-Ha-CalpM IgG was affinity-purified using the Ha-CalpM peptide covalently-bonded to Immobilon-AV membrane (Millipore). Briefly, a 2 cm × 2 cm square of Immobilon-AV membrane was incubated with 1 mg peptide in 2 ml 0.5 mol $l^{-1}$ potassium phosphate, pH 7.4, at room temperature for 2 h. The membrane was washed with 1 mol $l^{-1}$ $NaHCO_3$ for 2 h, followed by two 30 min washes in PBS (phosphate-buffered saline; 80 mmol $l^{-1}$ disodium hydrogen orthophosphate, 20 mmol $l^{-1}$ sodium dihydrogen orthophosphate and 100 mmol $l^{-1}$ 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 mmol $l^{-1}$ glycine, pH 2.5, for 10 min. The solution was neutralized with 0.1 ml 1 mol $l^{-1}$ Tris-HCl, pH 8.0, and stored at 0°C with 0.02% sodium azide. The concentration of immunoglobulin G (IgG) was determined by dot-blot analysis comparing serial dilutions of affinity-purified IgG with serial dilutions of known concentrations of rabbit IgG; detection used the Biotin/Avidin System (see below).

The detection of Ha-CalpM used the Biotin/Avidin System (Vector Laboratories). The membrane was blocked with 5% non-fat milk in TBS (Tris-buffered saline; 20 mmol $l^{-1}$ Tris-HCl, pH 7.5, and 137 mmol $l^{-1}$ NaCl) at room temperature for 1 h. The primary antibody incubation was 1 h with Ha-CalpM antiserum or affinity-purified IgG (1:10,000 dilution) in 3% non-fat milk in TBS-T (0.01% Tween-20 in TBS). The secondary antibody incubation was 1 h with biotinylated goat anti-rabbit IgG (1:10,000 dilution in TBS-T). The membrane was incubated with ABC solution (10 μl avidin and 10 μl biotinylated horseradish peroxidase in 10 ml TBS-T) for 30 min. ECL Plus (Amersham Pharmacia) was used for chemiluminescent detection of peroxidase activity.

### *Immunocytochemistry*

Tissues were fixed in 4% paraformaldehyde in lobster saline (0.5 mol $l^{-1}$ NaCl, 15 mmol $l^{-1}$ KCl, 10 mmol $l^{-1}$ EDTA, 25 mmol $l^{-1}$ Hepes-NaOH, pH 7.5), dehydrated through an ethanol series, and embedded in paraffin. Immunodetection used the Vectastain ABC Elite kit (Vector laboratories) with minor modifications (Mykles et al., 2000). After hydration, sections (10 μm) were washed twice in PBS buffer containing 15 mmol $l^{-1}$ glycine (PBS/Gly) for 5 min and blocked with 1.5% normal goat serum in PBS/Gly for 1 h. Sections were incubated with Ha-CalpM antiserum or pre-immune serum (1:5000 dilutions in 1.5% normal goal serum in PBS/Gly) overnight. After washing with PBS/Gly, sections were incubated with biotinylated goat anti-rabbit IgG (1:200 dilution in 1.5% normal goat serum in PBS/Gly) for 2 h, followed by incubation with ABC reagent (avidin/biotinylated horseradish peroxidase complex) for 30 min. Antibody was detected colormetrically by incubating the sections in a solution containing 0.8 mg $ml^{-1}$ diaminobenzidine tetrahydrochloride, 0.4 mg $ml^{-1}$ NiCl, 0.01% $H_2O_2$, and 0.1 mol $l^{-1}$ Tris-HCl, pH 7.4. The sections were rinsed with deionized $H_2O$, dehydrated through an ethanol series, cleared in xylene, and mounted in Permount (Fisher Scientific). Sections were photographed under bright-field illumination.

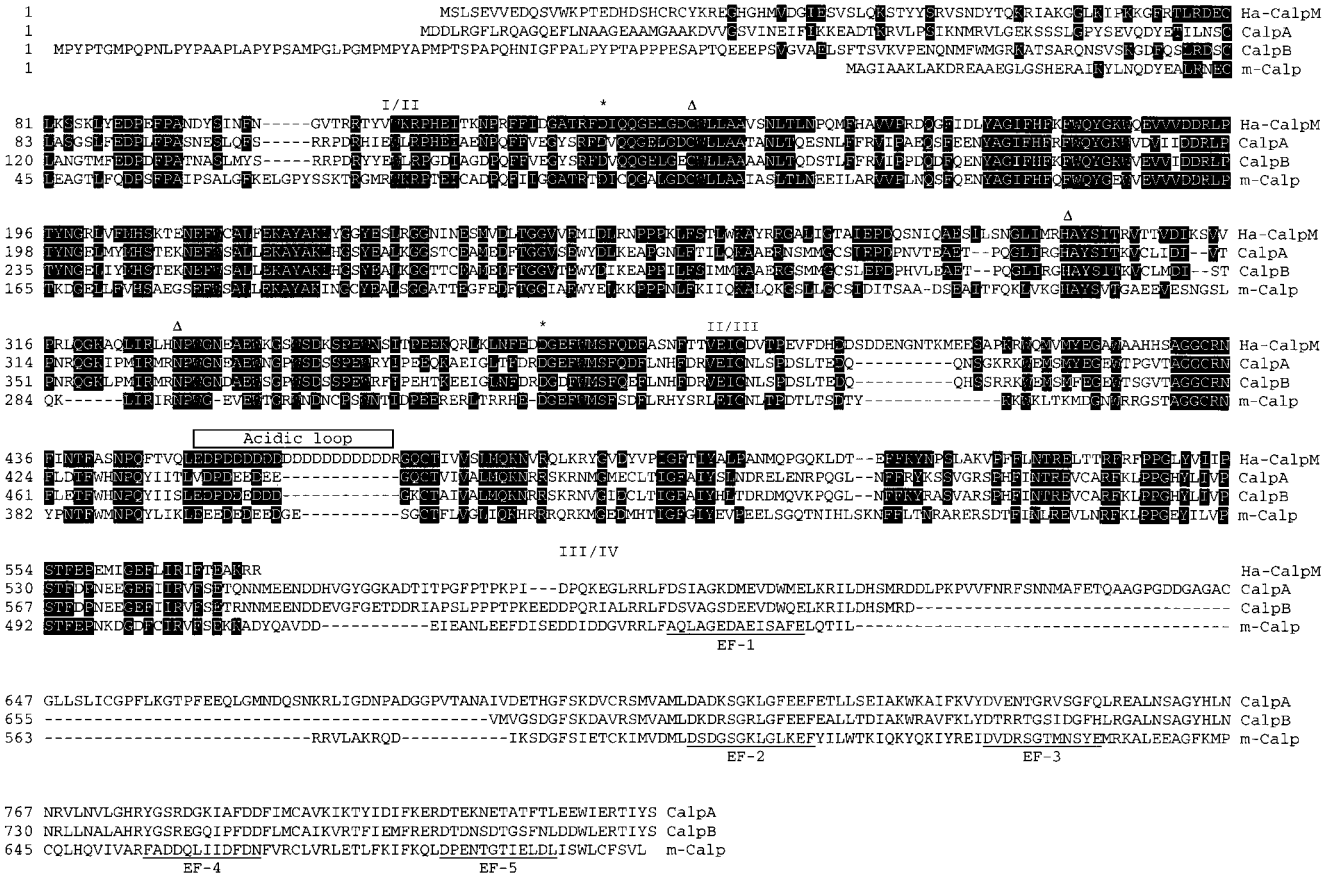


Fig. 2. Comparison of Ha-CalpM amino acid (aa) sequence with calpains from *Drosophila* and human. The deduced aa sequence of Ha-CalpM was aligned with *Drosophila* (CalpA and CalpB) and human (m-Calp) calpain sequences (see Materials and methods). Gaps (broken lines) were introduced to optimize the alignment. Aa residues that are identical to the Ha-CalpM sequence are highlighted. Ha-CalpM is highly homologous to *Drosophila* calpains (50% identity, 67% similarity). Ha-CalpM has three domains: domain I (aa 1–110) has a unique N-terminal sequence comprising the first 75 aa residues, domain II (aa 111–386) consists of a highly conserved cysteine proteinase sequence and domain III (aa 387–575) has a C2-like region that includes an acidic loop containing a stretch of 17 aspartate residues (aa 454–470). Ha-CalpM lacks a calmodulin-like domain (Domain IV) found in the human m-calpain and *Drosophila* sequences. Ha-CalpM has a short insertion sequence (aa 398–407) in domain III near the boundary with domain II that is not found in the other sequences. The boundaries between domains I and II, II and III, and III and IV are indicated by I/II, II/III and II/IV, respectively. The open triangles indicate the three amino acid residues of the catalytic triad (Cys141, His299, Asn329) in domain II. The asterisks indicate two conserved aspartate residues (Asp132 and Asp366) in domain II that bind $Ca^{2+}$. Domain IV in the *Drosophila* and human sequences contains five EF-hand motifs (EF-1, EF-2, etc.). GenBank accession numbers are Z46891 for *Drosophila* calpain A (CalpA), AF062404 for *Drosophila* calpain B (CalpB), and M23254 for human m-calpain (m-Calp).

### *Modeling of lobster calpain*

The Ha-CalpM sequence (aa residues 61–575) was modeled using the crystal structure of mammalian m-calpain as template (Hosfield et al., 1999; Strobl et al., 2000). Swiss-Model and Sybyl programs were used for initial tracing of the backbone. Further refinement of the putative secondary structure used the algorithm from Kabsch and Sander (1983) using UCSF MidasPlus software with these parameters: energy cutoff for hydrogen bonding, –0.5 kcal $mol^{-1}$; minimum helix length, 3; minimum sheet length, 3.

## Results

### *Characterization of Ha-CalpM cDNA*

Nested PCR using degenerate primers directed to four highly conserved amino acid sequences in the calpain proteinase domain yielded five partial cDNAs comprising three distinct sequences, all of which revealed high identities with members of the calpain gene family (data not shown). One of the sequences belonged to Ha-CalpM; the sequences of the other clones will be submitted once full-length sequences are obtained (X. Yu, H. W. Kim and D. L. Mykles, unpublished data). In order to obtain a full-length sequence, inverse PCR (IPCR) was used to amplify the sequences flanking the initial PCR products. Since the templates were plasmid DNA from a lobster deep abdominal muscle cDNA library, the expected size of the IPCR product would be the length of the calpain gene plus the vector (3 kb). A 5 kb product was amplified, cloned, and partially sequenced (data not shown). The end sequences of Ha-CalpM were located and primers synthesized.

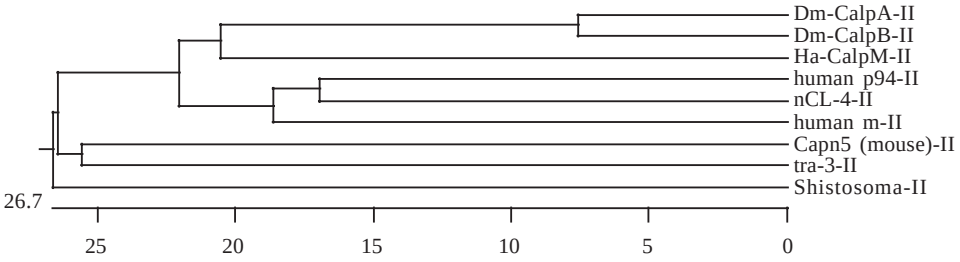


Fig. 3. Phylogenetic relationship of Ha-CalpM to other calpains. Amino acid sequences of the proteinase domain (domain II) of calpains from selected species were compared using the Clustal V method (see Materials and methods). Branch lengths are proportional to the inferred phylogenetic distances, with the *Shistosoma mansoni* domain II sequence serving as the outgroup. Ha-CalpM was grouped with *Drosophila* calpains (Dm-CalpA and Dm-CalpB), reflecting the high sequence identity among the three sequences. GenBank accession numbers for *Drosophila* calpains and human m-calpain are given in the legend of Fig. 2; the accession numbers for the other calpains are X92523 for human p94 (CAPN3), AF022799 for mouse nCL-4 (CAPN9), M74233 for *S. mansoni*, Y10656 for mouse calpain 5 (CAPN5), and U14480 for *C. elegans* tra-3.

The final full-length cDNA was PCR-amplified with the 3′ and 5′ end primers and sequenced.

The full-length Ha-CalpM cDNA consists of 1977 nucleotides (Fig. 1). The position of the initiation codon is similar to that of *Drosophila* calpains (Jékely and Friedrich, 1999; Theopold et al., 1995). The Ha-CalpM cDNA has a 5′ UTR of 15 bp and a 3′ UTR of 234 bp. The open reading frame (ORF; bp 16–1743) encodes a protein of 575 amino acids with a predicted molecular mass of 66.3 kDa.

Alignment of the deduced aa sequence of Ha-CalpM with *Drosophila* and human calpains is shown in Fig. 2. Ha-CalpM has the highest sequence identity with *Drosophila* calpains A and B (CalpA and CalpB; 50% identity, 67% similarity). In addition to *Drosophila* calpains and human m-calpain, Ha-CalpM also has significant sequence similarities to other mammalian calpains, such as digestive tract-specific calpain (CAPN9 or nCL-4; AF022799; 64% similarity) and skeletal muscle-specific calpain (CAPN3 or p94; AF127765; 62% similarity). The similarity to human m-calpain is 60%. The domain organization of Ha-CalpM was assigned according to that of the *Drosophila* calpain sequences (Sorimachi and Suzuki, 2001). Domain I (aa 1–110) has a unique N-terminal sequence (aa 1–75) that has no sequence identity with any other sequence in the NCBI database. Domain II (aa 111–396) consists of a highly-conserved cysteine proteinase sequence containing the catalytic triad (Cys141, His299 and Asn329) and two aspartates (Asp132 and Asp366) involved in $Ca^{2+}$ binding. Domain III (aa 397–575) contains a short insertion sequence (aa 408–417) and an acidic loop with an expanded stretch of an additional 11 aspartates (aa 454–470) that may bind $Ca^{2+}$ and help activate the proteinase. Ha-CalpM lacks domain IV (a calmodulin-like domain) found in EF-hand calpains.

A phylogenetic tree was generated comparing the domain II amino acid sequence of Ha-CalpM (Ha-CalpM-II) with the domain II sequences of selected calpains representing diverse phyla (Fig. 3). The *Shistosoma* calpain domain II sequence served as the outgroup in the analysis. As expected, Ha-CalpM clustered with *Drosophila* CalpA and CalpB. The human p94 (Capn3), human m-calpain (Capn2), and mouse nCL-4 (Capn9) formed a distinct, but related group; mouse Capn5 and *C. elegans* tra-3 calpains were more divergent.

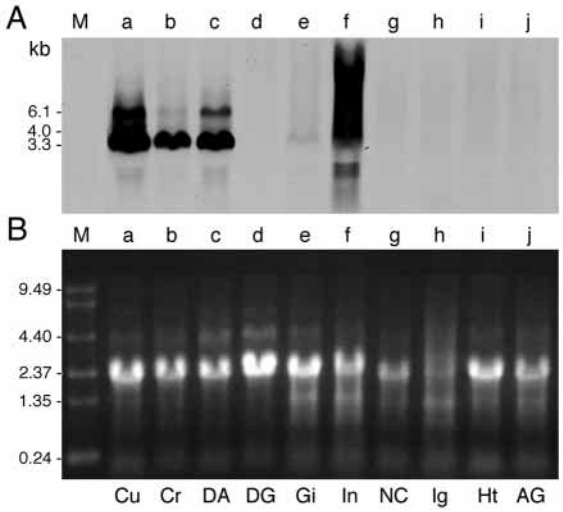


Fig. 4. Northern blot analysis of Ha-CalpM expression in lobster tissues. Total RNA (20 µg) was separated electrophoretically on a 1% agarose gel and blotted overnight. The membrane (A) was hybridized under high stringency with a DIG-labeled RNA probe synthesized from a plasmid with an insert encoding the region encompassing amino acid residues 171–332 in domain II of Ha-CalpM (Fig. 1; see Materials and methods). Three Ha-CalpM transcripts were detected (3.3, 4.0 and 6.1 kb) in cutter (Cu) and crusher (Cr) claw muscles and deep abdominal (DA) muscle (A, lanes a–c). The fast muscles expressed all three mRNAs (lanes a and c), while the slow muscle (lane b) expressed primarily the 3.3 kb mRNA. Intestine (In; lane f) showed a strong hybridization to mRNA in the 3.3–6.1 kb range, as well as to mRNAs greater than 6.1 kb. Little or no Ha-CalpM mRNA was detected in digestive gland (DG; lane d), gill (Gi; lane e), nerve cord (NC; lane g), integument (Ig; lane h), heart (Ht; lane i) or antennal gland (AG; lane j). RNA loading was determined by staining the gel with Ethidium Bromide (B). Lane M, molecular mass markers (kb) are indicated.

### *Expression of Ha-CalpM*

Northern blotting was used initially to determine the tissue distribution and sizes of Ha-CalpM mRNAs. The membrane was hybridized with a DIG-labeled RNA probe synthesized from the domain II region (equivalent to aa 171–332) of Ha-CalpM under high stringency. The results show that Ha-CalpM

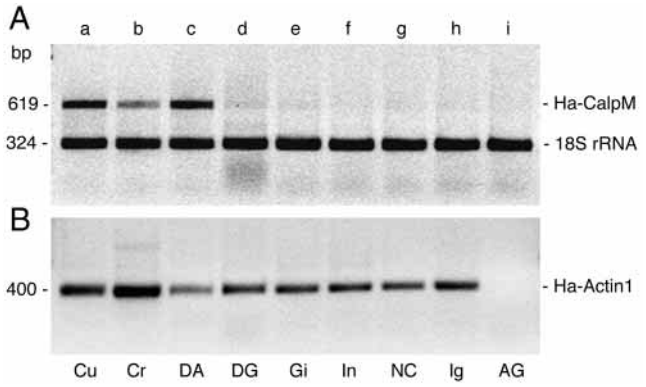


Fig. 5. RT-PCR analysis of Ha-CalpM and α-actin expression in lobster tissues. Total RNA from various tissues were DNase-treated, reverse-transcribed, and PCR-amplified using primers directed to the 5′ ends of Ha-CalpM (bp 1–18 and 600–619 in Fig. 1) and Ha-Actin1 (bp 1–23 and 379–400; see Koenders et al., 2002). PCR amplification of 18S rRNA served as an internal standard. (A) The PCR products synthesized from Ha-CalpM (619 bp) and 18S rRNA (324 bp). (B) The PCR product (400 bp) synthesized from α-actin. Shown are negative images of ethidium bromide-stained agarose gels. There were high amounts of Ha-CalpM mRNA in cutter claw, crusher claw and deep abdominal muscles (lanes a–c). Little or no Ha-CalpM product was amplified from other tissues (lane e–i). α-Actin was expressed in all tissues except antennal gland (lane i). Abbreviations as in Fig. 4.

Table 1. *Quantification of Ha-CalpM transcript in lobster tissues*

| Tissue | Number of transcripts ng$^{-1}$ total RNA | % |
|---|---|---|
| Deep abdominal muscle | 6753 | 100 |
| Cutter claw muscle | 6570 | 97 |
| Crusher claw muscle | 1612 | 24 |
| Integument | 183 | 3 |
| Gill | 154 | 2 |
| Digestive gland | 127 | 2 |
| Nerve cord/thoracic ganglion | 88 | 1 |
| Intestine | 85 | 1 |
| Antennal gland | 29 | 0.4 |

mRNA was reverse-transcribed and amplified by real-time polymerase chain reaction. Reactions containing known concentrations of lobster CalpM cDNA were used to generate a standard curve to quantify CalpM mRNA (see Materials and methods).

Ha-CalpM mRNA levels were approximately fourfold greater in fast skeletal muscle (deep abdominal and cutter claw muscles) than in slow skeletal muscle (crusher claw muscle). Other tissues expressed Ha-CalpM at much lower levels.

was highly expressed in skeletal muscle (Fig. 4, lanes a–c). Two major transcripts (6.1 kb and 3.3 kb) were detected. A minor 4 kb mRNA was also present in fast muscles (cutter claw and deep abdominal). The probe hybridized to mRNAs of varying sizes from intestine (Fig. 4, lane f).

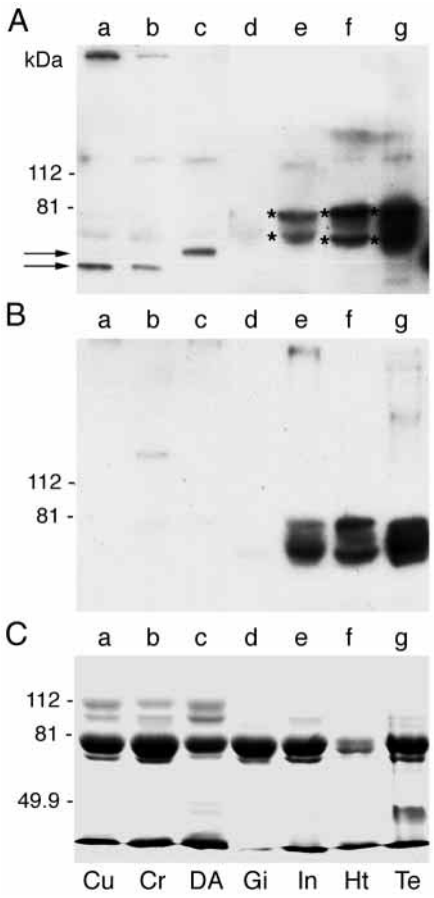


Fig. 6. SDS-PAGE and western blot analysis of Ha-CalpM in lobster tissues. Supernatant fractions (32 μg protein) from various tissues were electrophoresed on 6% SDS-polyacrylamide gels and either stained with Coomassie Blue (C) or transferred onto PVDF membrane and probed with affinity-purified anti-Ha-CalpM IgG (A) or pre-immune IgG (B). The Ha-CalpM antibody detected a 62 kDa isoform in claw muscles (lanes a and b) and a 68 kDa isoform in deep abdominal muscle (lane c). Non-specific binding of the antibody (B, lanes e–g) is indicated by asterisks in A. Hemocyanin, an extracellular 78 kDa oxygen-binding protein in the hemolymph, is a major contaminant in all tissues. Positions of molecular mass standards (kDa) are indicated. Te, testis; all other abbreviations as in Fig. 4.

RT-PCR was used to detect low levels of expression and to determine whether the hybridization of the probe to intestine RNA (Fig. 4) was specific to Ha-CalpM. Primers were synthesized to amplify the sequence encoding the unique N-terminal sequence of Ha-CalpM. As expected, a large amount of PCR product (619 bp) was synthesized from muscle RNAs (Fig. 5, lanes a–c). Little or no Ha-CalpM PCR product was obtained from digestive gland, gill, intestine, nerve cord/thoracic ganglion, integument and antennal gland (Fig. 5, lanes d–i), indicating that the hybridization in the northern blot to intestine RNA was not due to high levels of Ha-CalpM mRNA. PCR amplification of 18S rRNA (324 bp) and α-actin (Ha-Actin1; 400 bp) products indicated that each lane contained the same amount of total RNA and that the RNA

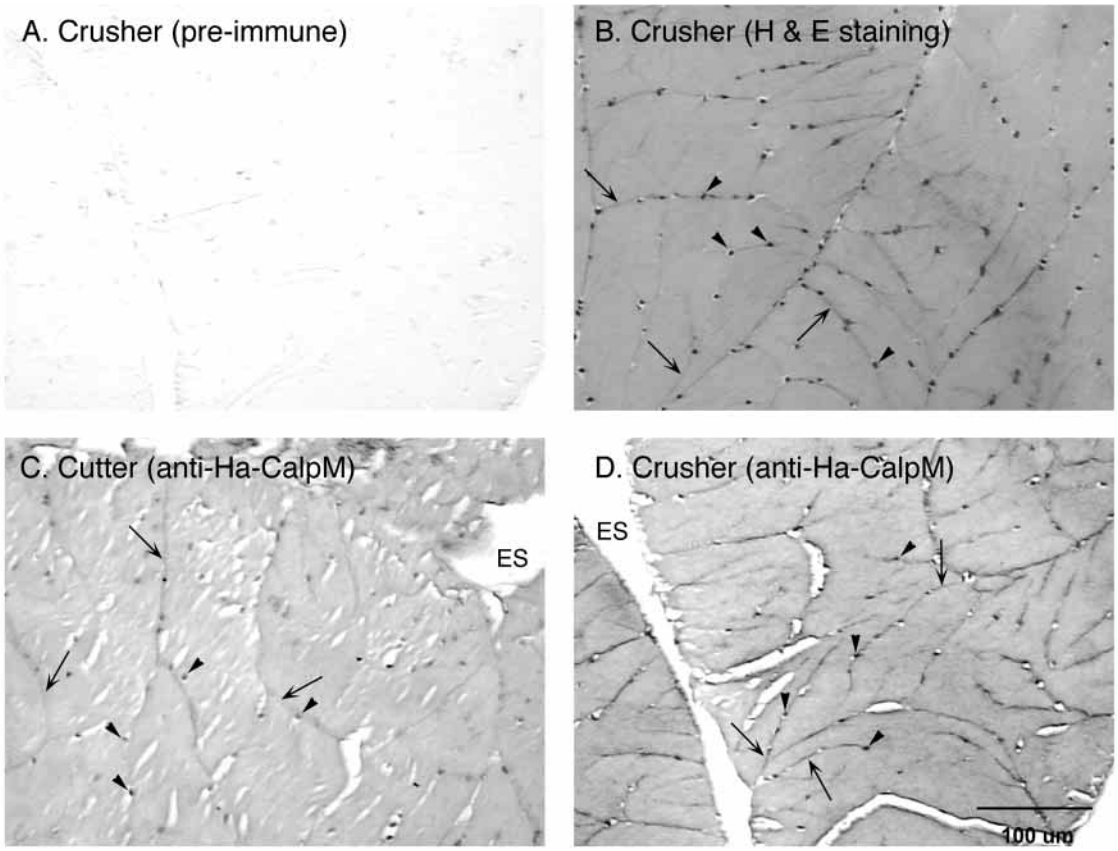


Fig. 7. Immunocytochemical localization of Ha-CalpM in claw muscles from adult lobster. Transverse sections were stained with Hematoxylin/Eosin (B) or incubated with pre-immune serum (A) or Ha-CalpM antiserum (C,D). Nuclei (arrowheads) are associated with the sub-sarcolemmal cytoplasm of sarcolemal clefts (arrows), which are large infoldings of the cell membrane. The Ha-CalpM antiserum stained the cytoplasm uniformly and some nuclei. Each field of view contains portions of two muscle fibers. ES, extracellular space. Scale bar, 100 μm.

was not degraded (Fig. 5). A negative PCR control using total RNA as template produced no products, indicating no DNA contamination (data not shown). The α-actin was expressed at a higher level in the crusher claw muscle than in the cutter claw and deep abdominal muscles, which corresponds to the higher ratio of actin to myosin in slow fibers than in fast fibers (Mykles, 1985, 1988). The same RNA was used to quantify the copy number of Ha-CalpM mRNA in the tissues by real-time PCR (Table 1). The highest copy numbers were in skeletal muscles, although the amount in slow muscle (crusher claw) was about 25% that in fast muscle (cutter claw and deep abdominal). Other tissues expressed Ha-CalpM at much lower copy numbers (0.4% to 3% that of deep abdominal muscle; Table 1).

An antibody raised against a 28-aa sequence in the N-terminal domain of Ha-CalpM (aa 53–80) was used to determine Ha-CalpM protein levels and distribution in lobster tissues. In western blots, the antibody detected two proteins in skeletal muscles: a 62 kDa isoform in cutter and crusher claw muscles and a 68 kDa isoform in deep abdominal muscle (Fig. 6, lanes a–c). The isoforms were not detected in gill, intestine, heart or testis (Fig. 6, lanes d–g). Immunocytochemistry of transverse sections of the cutter and crusher claw muscles showed that Ha-CalpM was distributed throughout the cytoplasm (Fig. 7). Ha-CalpM was also localized in some, but not all, nuclei (Fig. 7, compare the Hematoxylin staining of nuclei in B with the staining of fewer nuclei in C and D).

As calpains are involved in moult-induced claw muscle atrophy, the expression of Ha-CalpM was determined in claw muscle from lobsters at different moult stages. For real-time PCR, total RNA was isolated from crusher claw muscle at intermoult, early premoult ($D_0$), and postmoult (2–4 days post-ecdysis) stages, reverse-transcribed and PCR-amplified in the presence of SYBR Green I fluorescent dye (Fig. 8). There was no significant effect of moult stage on Ha-CalpM mRNA levels ($P$<0.28; ANOVA single factor analysis). Parallel PCR reactions using Ha-actin1 primers showed no changes in Ha-actin1 mRNA (data not shown), which was consistent with the constitutive expression of actin during the moutling cycle reported recently (Koenders et al., 2002). The 62 kDa Ha-CalpM isoform was expressed in claw muscles at all stages of the moulting cycle (Fig. 9). As levels were quite variable in intermoult muscle (Fig. 9, lanes a,b), the higher levels of Ha-CalpM in postmoult claw muscles (lanes e,f) were not significantly different.

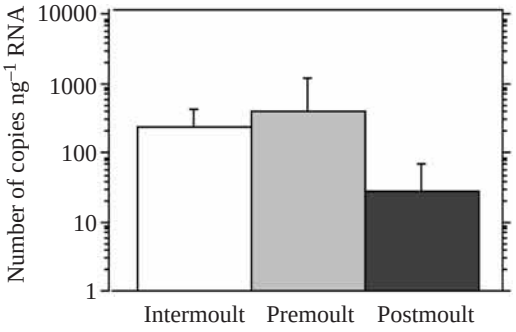


Fig. 8. Real-time PCR analysis of Ha-CalpM expression in lobster crusher claw muscles during the moulting cycle. Total RNA from intermoult, early premoult (stage $D_0$), and early postmoult (2–4 days post-ecdysis) lobsters was DNase-treated, reverse-transcribed, and PCR-amplified in reactions containing SYBR Green I fluorescent dye (see Materials and methods). Values are means ±1 S.D. ($N$=4 for each moult stage). Ha-CalpM transcript copy number did not change significantly ($P$<0.28; ANOVA single factor analysis).

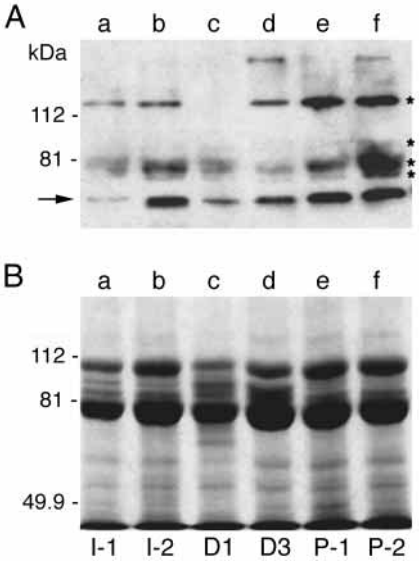


Fig. 9. Western blot analysis of Ha-CalpM in claw muscles during the moulting cycle. Supernatant fractions (32 µg protein) of claw muscles at different moulting stages were electrophoresed on 6% SDS-polyacrylamide gels and either stained with Coomassie Blue (B) or transferred onto PVDF membrane and probed with Ha-CalpM antiserum (A) or pre-immune serum (data not shown). The 62 kDa isoform (arrow) was present at all moulting stages: intermoult (lanes a,b), premoult (lanes c,d), and postmoult (lanes e,f). Proteins binding non-specifically to the antibody are indicated by asterisks in A. Positions of molecular mass standards (kDa) are indicated. I-1 and I-2, intermoult stage at 20% and 30% of the intermoult interval, respectively; D1, premoult stage $D_1$; D3, premoult stage $D_3$; P-1 and P-2, postmoult stage at 7% and 13% of the intermoult interval, respectively.

### *Purification of Ha-CalpM*

Four calpain activities (CDPs I, IIa, IIb and III) have been characterized biochemically in lobster skeletal muscles (Mykles and Skinner, 1986). The mass of the deduced Ha-CalpM sequence (66.3 kDa) and masses of the Ha-CalpM isoforms 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 intermoult lobster muscles (claws and deep abdominal) containing all four calpain activities was subjected to a series of chromatographic separations on organomercurial agarose, Q Sepharose, Superdex-200 gel filtration and Mono Q columns (Mykles, 2000). The 62 kDa and 68 kDa Ha-CalpM isoforms co-eluted from each column and the results are shown for the final two separations (Figs 10, 11). The Ha-CalpM isoforms eluted from the Superdex-200 gel filtration column at a position corresponding to the native mass of CDP III (Fig. 10, lanes h–n). The gel filtration fractions containing Ha-CalpM were concentrated and chromatographed in a Mono Q anion exchange column; the Ha-CalpM proteins eluted as a single peak (fractions 23–25; Fig. 11, lanes i,j). Edman degradation of the purified 62 kDa and 68 kDa isoforms did not yield N-terminal sequences for either polypeptide.

## Discussion

Ha-CalpM is the first crustacean calpain gene that has been cloned and sequenced. It is classified as a non-EF-hand calpain, since it lacks a calmodulin-like region (domain IV) (Huang and Wang, 2001). Other members of this group are mammalian calpains 5, 6, 7, 10 and 13 (SOLH), *Caenorhabditis elegans* tra-3, and *Drosophila* Sol (Sorimachi and Suzuki, 2001). Isoforms of *Drosophila* CalpA (Dm-CalpA′; estimated mass 64 kDa) and mammalian CAPN2 and CAPN3 lacking domain IV result from alternative mRNA splicing, which introduces a termination codon near the C-terminal end of domain III (Herasse et al., 1999; Theopold et al., 1995) or domain II (Sorimachi et al., 1993). It is unlikely that Ha-CalpM is a truncated form of a longer calpain sequence homologous to Dm-CalpA. The antibody to the N-terminal domain reacts only with the 62 kDa and 68 kDa isoforms; there is no other protein detected in soluble extracts of skeletal muscle or any other tissue (Fig. 6).

It is likely that Ha-CalpM has $Ca^{2+}$-dependent proteinase activity, even though it lacks domain IV. $Ca^{2+}$-binding regions in domains II and III in other calpains (Andresen et al., 1991; Hosfield et al., 2001; Moldoveanu et al., 2002) are highly conserved in Ha-CalpM (Fig. 2). Expressed recombinant mammalian calpains lacking domain III or IV or only containing domain II retain $Ca^{2+}$ dependency (Hata et al., 2001; Vilei et al., 1997). Tra-3, a non-EF-hand calpain involved in sex determination in *C. elegans*, is activated by $Ca^{2+}$ (Sokol and Kuwabara, 2000). These data suggest that Ha-CalpM has $Ca^{2+}$-dependent proteolytic activity, although this must be demonstrated on expressed recombinant protein using functional assays.

The Ha-CalpM cDNA appears to encode CDP III, one of four calpain activities identified in lobster skeletal muscles (Mykles and Skinner, 1986). The Ha-CalpM isoforms elute

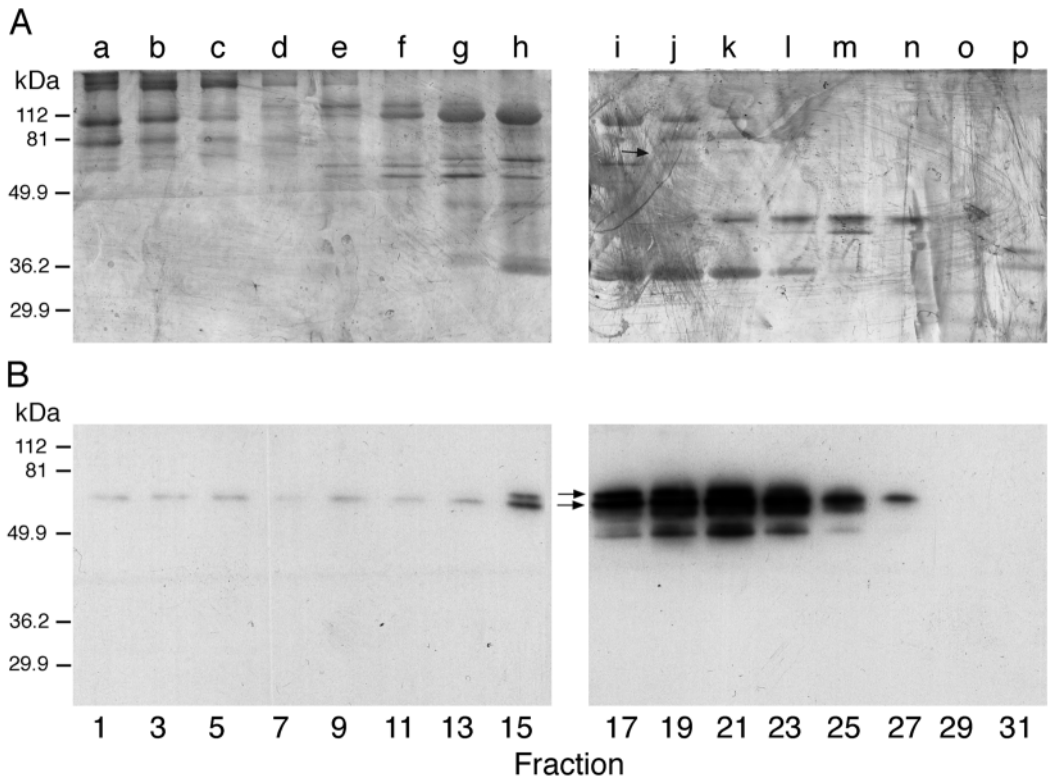


Fig. 10. Gel filtration chromatography of Ha-CalpM. A calpain fraction from Q Sepharose ion exchange chromatography was separated on a Superdex-200 gel filtration column (see Materials and methods). 10 µl samples of odd-numbered fractions were separated on 10% SDS-polyacrylamide gels and either stained with silver (A) or blotted onto PVDF membrane and probed with Ha-CalpM antiserum (B). The antibody recognized two Ha-CalpM isoforms of 62 kDa and 68 kDa (B, arrows) that co-eluted from the column. The smaller molecular mass immunoreactive polypeptides are probably degradation products. The arrow in A indicates the position of the 62 kDa isoform in the silver-stained gel. The elution position of 62 kDa and 68 kDa isoforms (maximum at fraction 21) corresponded to that of CDP III (estimated mass 59 kDa) (Mykles, 2000; Mykles and Skinner, 1986). The peak CDP IIa activity (125 kDa) elutes at fraction 15 and the peak CDP IIb activity (195 kDa) elutes at fraction 12 (Mykles, 2000). Positions of molecular mass standards (kDa) are indicated.

from a gel filtration column at the same position as CDP III (59 kDa). The other three calpains (CDP I, 310 kDa; CDP IIa, 125 kDa; and CDP IIb, 195 kDa) elute earlier from the column (Mykles, 2000). The elution position of the Ha-CalpM proteins from the Mono Q column differed from those of CDPs IIa and IIb; under identical conditions, CDP IIa elutes at fractions 17–18 and CDP IIb at fractions 27–28 (Beyette et al., 1997; Mykles, 2000). Moreover, the 68 kDa isoform 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 comprises a single polypeptide encoded by the Ha-CalpM gene. CDP IIb is a homodimer of a 95 kDa subunit, while CDP IIa is a homodimer of a 60 kDa subunit; both calpains appear to be products of distinct genes (Beyette et al., 1997). The subunit composition of CDP I is not known. However, the identity of Ha-CalpM and CDP III remains to be established. We were unable to obtain N-terminal sequences of the 62 kDa and 68 kDa isoforms by Edman degradation, suggesting that the N termini were chemically blocked.

The 62 kDa and 68 kDa isoforms are probably encoded by the same gene, since both proteins react specifically to antibody raised against the unique N-terminal sequence and co-elute through several chromatographic separations. The expression of the two isoforms is not correlated with the fiber type composition of the muscles. The 62 kDa isoform occurs in both crusher claw closer muscle, which contains only slow fibers, and cutter claw closer muscle, which contains mostly fast fibers (Mykles, 1985). The 62 kDa isoform could either be the active form of calpain produced by autolysis of the 68 kDa isoform or an isoform generated by alternative splicing or post-translational processing.

A three-dimensional model of Ha-CalpM, using the crystal structure of mammalian m-calpain (Capn2) as template, is presented in Fig. 12. The colors denote aa residues that are identical between Ha-CalpM and human m-calpain. The first 75-aa acid sequence of domain I is unique, showing no sequence identity with any other gene in the NCBI database. Consequently, the first 60 residues could not be accommodated in the model. The last 35-aa sequence of domain I has 40% and 49% identity with the same region of *Drosophila* calpains A and B, respectively. Domain II is divided into two subdomains (IIa and IIb), which form the active site (Fig. 12A) (Hosfield et al., 1999; Strobl et al., 2000). The Cys141 in domain IIa and the His299 and Asn329

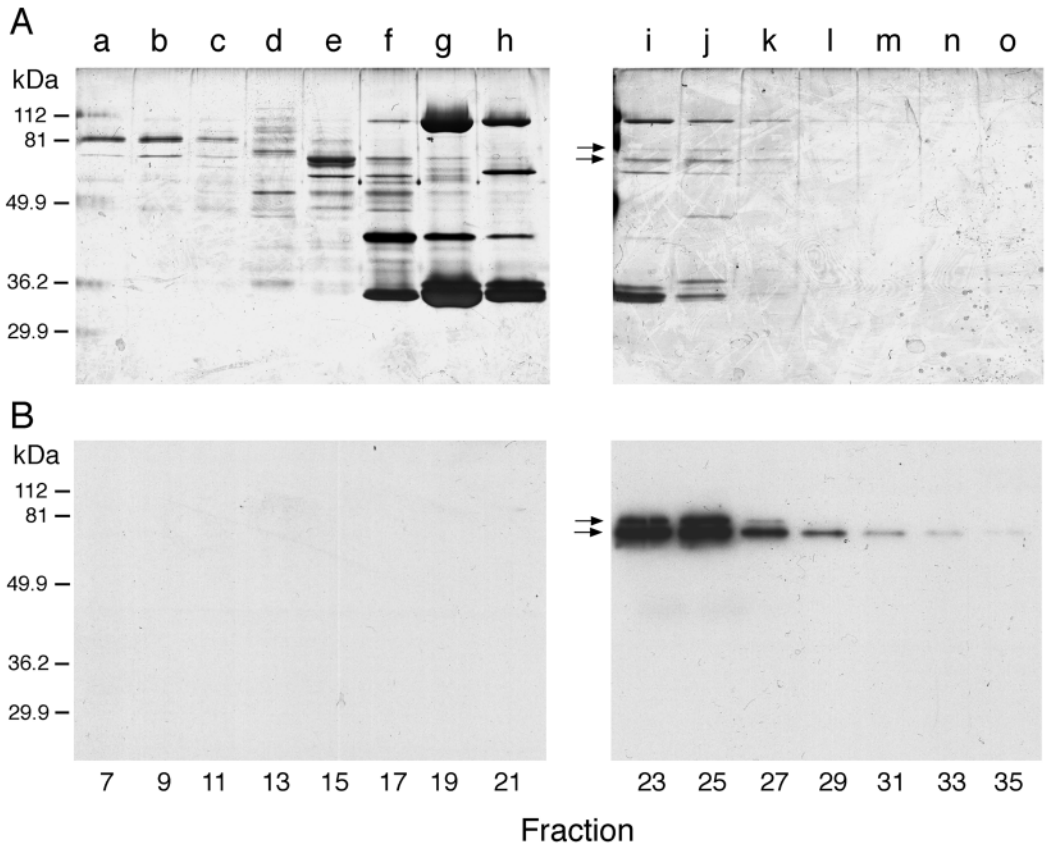


Fig. 11. Ion-exchange chromatography of Ha-CalpM. Fractions 17–23 from gel filtration chromatography (Fig. 10) were pooled and separated on a Mono Q column (see Materials and methods). 10 µl samples of odd-numbered fractions between fractions 7 and 35 were separated on 10% SDS-polyacrylamide gels and either stained with silver (A) or blotted onto PVDF membrane and probed with Ha-CalpM antiserum (B). The arrows indicate the positions of the 62 kDa and 68 kDa Ha-CalpM isoforms. The elution position of Ha-CalpM (fractions 23–25; 0.35–0.37 mol $l^{-1}$ NaCl) differed from that of CDP IIb (fractions 27–28; 0.40–0.41 mol $l^{-1}$ NaCl) and CDP IIa (fractions 17–18; 0.28 mol $l^{-1}$ NaCl) under identical conditions (Beyette et al., 1997; Mykles, 2000). Positions of molecular mass standards (kDa) are indicated.

in domain IIb form a catalytic triad essential for cysteine proteinase activity (Arthur et al., 1995). Many of the conserved residues are located in the vicinity of the catalytic site (Fig. 12A). The most striking feature of Ha-CalpM exists in domain III, which contains a highly acidic sequence consisting of a stretch of 17 aspartate residues (Fig. 12B). All calpains have an acidic loop as part of the C2-like domain, but the expansion of this acidic stretch is unique to Ha-CalpM. A repeat sequence of 44 aspartate residues has been reported in calsequestrin, a high-capacity, moderate-affinity, $Ca^{2+}$-binding protein that acts as an internal $Ca^{2+}$ store in fast muscle fibers (Treves et al., 1992). Analysis of the crystal structure of m-calpain suggests that the acidic region interacts with $Ca^{2+}$ and functions as an ‘electrostatic switch’ for regulating proteinase activity (Hosfield et al., 2001; 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., 1999; Strobl et al., 2000). These data suggest that domain III of Ha-CalpM plays a significant role for full activation of the enzyme. $Ca^{2+}$ binding would result in movement of the Asn329 and His299 on one side of the catalytic cleft toward the Cys141 on the other, thus forming of a functional catalytic site (Fig. 12A).

Calpain genes show a range of tissue expression, from ubiquitous to highly tissue-specific. The mammalian calpains 1 (µ-calpain), 2 (m-calpain), 5, 7, 10, 12 and 13, are ubiquitously expressed, although calpain 5 is highly expressed in colon, small intestine and testis and calpain 12 is highly expressed in hair follicle (Huang and Wang, 2001; Sorimachi and Suzuki, 2001). The preferential expression of Ha-CalpM in skeletal muscle is not unusual. Alternatively spliced isoforms of mammalian CAPN3 are restricted to skeletal muscle (p94 calpain), lens (Lp82 and Lp85 calpains) and retina (Rt88 calpain) (Huang and Wang, 2001; Sorimachi and Suzuki, 2001). Mammalian calpain 6 is expressed in placenta, calpain 8 in stomach mucosa, calpain 9 in digestive tract, and calpain 11 in intestine (Dear and Boehm, 1999; Lee et al., 1999). In *Drosophila*, Dm-CalpA is differentially expressed during development (Emori and Saigo, 1994; Theopold et al., 1995). Although the Dm-CalpA mRNA is ubiquitously expressed in adult flies, immunocytochemistry shows the protein is restricted to cells in the brain, ventral ganglion and blood

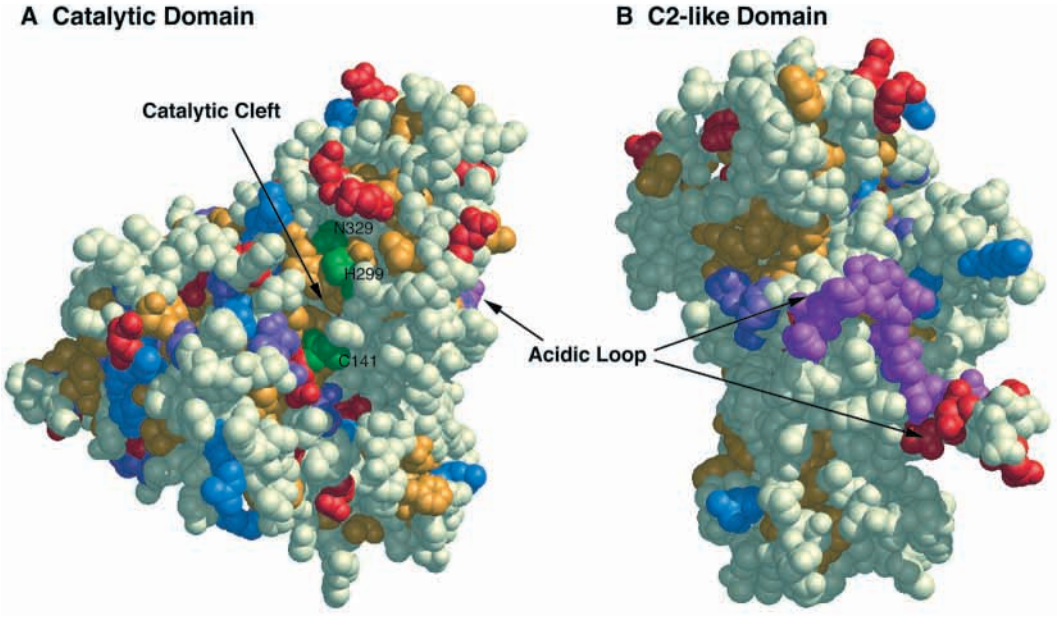


Fig. 12. Model of Ha-CalpM, based on the crystal structure of mammalian m-calpain (CAPN2) in the absence of $Ca^{2+}$. Identical residues between the lobster and human deduced amino acid sequences are colored (red, acidic residues; blue, basic; yellow, hydrophobic; lavender, uncharged polar). (A) 'Top' view of the catalytic domain (domain II) containing the three conserved residues (C141, H299 and N329, in green) forming the catalytic triad of the active site. The residues of the triad are surrounded by conserved nonpolar residues, which form a hydrophobic environment within the catalytic cleft. (B) 'Side' view showing an acidic loop (magenta and red) containing 19 acidic residues in a stretch of 20 residues (D452, D455, D457 and D458 are not colored because they did not align with identical residues in the human m-calpain; see Fig. 2). The acidic loop, which includes a stretch of 11 aspartate residues (magenta; residues 460–470) unique to the lobster sequence, is part of a putative calcium-dependent phospholipid-binding C2-like region in domain III. Binding of $Ca^{2+}$ to the acidic loop and to two aspartates (Fig. 2; D232 and D366) in domain II results in closure of the catalytic cleft to allow cooperation between C141, H299 and N329 necessary for catalytic activity (Hosfield et al., 2001; Moldoveanu et al., 2002). The first 60 residues of the N-terminal sequence were excluded, due to its high divergence from the mammalian sequence (Fig. 2). The lobster sequence lacks a calmodulin-like domain (domain IV).

(hemocytes) (Theopold et al., 1995). These data suggest that certain calpains mediate tissue-specific functions.

The size (about 2 kb) of the Ha-CalpM cDNA is smaller than the transcript sizes in northern blots. Two major transcripts of Ha-CalpM (6.1 kb and 3.3 kb) were detected at high stringency. An additional minor transcript (4 kb) was expressed in fast muscle. This suggests that the three transcripts contain the Ha-CalpM ORF but differ in the lengths of the UTRs. The 3′ UTR of the Ha-CalpM cDNA lacks a polyadenylation signal and a poly(A) tail. Given the high percentage (40%) of adenosines in the 3′ UTR, the oligo(dT) primer used for the initial cDNA synthesis may have annealed to this region, rather than to a poly(A) tail located further downstream. This suggests that the 3′ UTR of the Ha-CalpM mRNA is longer than 234 bp. The three transcript sizes may arise from alternative polyadenylation signals in the 3′ UTR.

Crustacean muscles contain four calpain activities that are involved in the degradation of myofibrillar proteins during programmed claw muscle atrophy (Mykles, 1999). Coincident with this degradation is a substantial restructuring of the myofibrils. Myofibrillar cross-sectional area decreases 78%, the thin:thick myofilament ratio decreases from 9:1 to 6:1, and thick myofilament packing increases by 51% to 72% (Ismail and Mykles, 1992; Mykles and Skinner, 1981). Although Ha-CalpM mRNA and protein levels were not elevated in premoult muscle, the muscle-specific expression of Ha-CalpM (Table 1; Figs 4–6), and the ability of Ha-CalpM/CDP III to degrade myofibrillar proteins (Mattson and Mykles, 1993), suggests that it has a role in remodeling and/or maintaining the structure of the contractile apparatus. This is analogous to the p94 isoform of CAPN3 in mammalian skeletal muscle. The activity of p94 is necessary to maintain muscle fiber viability and myofibrillar protein composition. Loss-of-function mutations in the CAPN3 gene are the underlying cause of limb-girdle muscular dystrophy type 2A in humans (Chae et al., 2001). Expression of CAPN3 responds rapidly to conditions that induce muscle atrophy or degeneration. Denervation or muscle injury result in a rapid decrease in CAPN3 mRNA in mouse skeletal muscle (Stockholm et al., 2001), whereas sepsis causes an increase in CAPN3 mRNA in rat skeletal muscle (Williams et al., 1999). The p94 appears to maintain the structural integrity of the contractile apparatus through its interaction with connectin/titin (Herasse et al., 1999).

In summary, the full-length cDNA encoding a non-EF-hand 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. The Ha-CalpM is

expressed as a 62 kDa isoform in claw muscles and a 68 kDa isoform in deep abdominal muscle; the functional significance of the two isoforms is not known. 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 remaining calpains are determined. Our data suggest that Ha-CalpM plays a role in restructuring the skeletal muscle during the moulting cycle. However, its specific role will remain undefined until endogenous substrates are identified.

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