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Cloning and Biochemical Characterization of Astacin-Like Squid Metalloprotease[1]

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We have cloned four cDNAs encoding astacin-like squid metalloproteases (ALSMs)–I and –II from the Japanese common squid and ALSMs-I and -III from the spear squid. Analysis of the deduced amino acid sequences revealed that ALSMs possess a signal peptide and a pro-sequence followed by an astacin-like catalytic domain and an MAM (meprin, A5 protein, receptor protein-tyrosine phosphatase μ) domain. Phylogenetic analysis revealed that ALSM corresponds to a new cluster of astacins. To analyze the function of the MAM domain, wild-type ALSM and an MAM-truncated mutant were expressed in a baculovirus expression system. The expressed protein encoding full-length ALSM hydrolyzed myosin heavy chain as effectively as native ALSM, whereas the MAM-truncated mutant possessed no protease activity, suggesting that the MAM domain contributes to substrate recognition. ALSM has been isolated from squid liver and mantle muscle. However, analysis with a specific antibody generated against ALSM indicated the presence of ALSM in a wide variety of tissues. ALSM was located in the extracellular matrix of mantle muscle cells. Thus, ALSM is a secreted protease, as are other members of the astacin family. The extracellular localization raises the possibility of substrates other than myosin. The physiological role of ALSM remains unknown, at this time.

Key words: astacin, MAM domain, metalloprotease, myosin, zinc binding.

ALSM was originally isolated from squid mantle muscle as a metalloprotease and was shown to hydrolyze myosin heavy chain (MyHC) with high specificity (*1*). Our recent studies showed that three distinct isoforms exist in squid, each with a similar molecular mass (45 kDa) (*2*). Two ALSM-I isoforms were isolated from both spear squid and the Japanese common squid, while types II and III isoforms were restricted to the Japanese common squid and spear squid, respectively. These isoforms were classified according to the specificity of their hydrolysis sites in rabbit skeletal muscle MyHC. ALSM-I hydrolyzes Ala^{1159}-Thr^{1160} to produce peptides of 130 and 90 kDa. ALSMs-II and -III hydrolyze Glu^{1379}-Thr^{1380} and Glu^{1098}-Asp^{1099}, respectively. Under conditions appropriate for ALSM activity, preferential hydrolysis of MyHC was observed in a crude extract of mantle muscle. This led us to propose that ALSM plays a role in muscle protein metabolism, namely in myosin turnover. Therefore, the enzyme was designated as myosinase (*1*). However, our recent findings revealed ALSM activity in a wide variety of tissues in addition to mantle muscle (*3*). Furthermore, the N-terminal amino acid sequence of ALSM shows high similarity to that of astacin family members (*2*). Thus, we changed the name of the enzyme to ALSM.

The astacin family was named after the digestive enzyme astacin [EC 3.4.24.21], isolated from the stomach of the freshwater crayfish *Astacus astacus* (*4, 5*). Astacins are referred to as multifunctional proteases and may have various physiological functions. All members of the astacin family can be classified roughly into three categories. Meprins are found in mammalian kidney and intestinal tissues and play a crucial role in the processing of biologically active peptides and extracellular-matrix proteins (*5, 6*). BMP-1 (*7, 8*) or tolloid (*9–11*) comprises the second class and contributes to morphogenesis or embryonic pattern formation. The third class is composed of hatching-related enzymes that are implicated in the breakdown of the egg envelope during the hatching of *Xenopus* (*12*), fish [HCE and LCE; (*13*)] and birds [CAM; (*14*)]. Members of the astacin family are either secreted or are plasma membrane–bound proteases (*5*). Our results suggest that ALSM is also a secreted astacin-like protease and that MyHC may not be the native substrate for ALSM. To investigate the similarities between ALSM and astacin proteases, we cloned full-length cDNAs encoding the ALSM isoforms and compared the deduced amino acid sequences with those of astacins. To obtain further information on functional aspects of ALSM, full-length ALSM and a truncated mutant were expressed in a baculovirus system. In addition, the tissue and cellular distributions of ALSM were examined using

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Abbreviations: ALSM, astacin-like squid metalloprotease; BMP, bone morphogenetic protein; CAM, chorioallantoic membrane; FITC, fluorescein isothiocyanate; HCE, high choriolytic enzyme; HMP, hydra metalloprotease; LCE, low choriolytic enzyme; MAM, meprin, A5 protein, receptor protein-tyrosine phosphatase μ; MyHC, myosin heavy chain; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; TFA, trifluoroacetic acid; TLD, tolloid; XLD, *Xenopus* tolloid.

an ALSM-specific antibody.

EXPERIMENTAL PROCEDURES

Materials—Spear squid (*Loligo bleekeri*) and the Japanese common squid (*Todarodes pacificus*) were purchased at the Tokyo Central Wholesale Fish Market.

Amino Acid Sequence Analysis—ALSMs-I and -III were purified from the liver of *L. bleekeri*, and ALSM-II was purified from the liver of *T. pacificus*, as previously described (*2*). Amino-terminal sequences were determined from the purified ALSMs transferred to Immobilon-PSQ polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA) by Edman degradation on an automated Shimadzu PSQ-1 sequencer (Shimadzu, Kyoto). Internal sequences were determined by digestion of the purified proteins with a lysyl endopeptidase according to the method of Iwamatsu (*15*), which was followed by peptide separation by reversed-phase chromatography on a 5C18-P column (Nacalai Tesque, Kyoto) equilibrated with 0.1% trifluoroacetic acid (TFA) in distilled water. The peptides were eluted with a linear gradient of 0–100% of eluent (0.1% TFA, 80% acetonitrile) at a flow rate of 0.5 ml/min. Peaks defined by absorbance at 230 nm were collected and stored at –20°C until peptide sequence analysis.

Cloning and Sequencing of ALSMs-I and -II from the Japanese Common Squid—Degenerate oligonucleotide primers for reverse transcription–PCR (RT-PCR) were designed from the consensus sequence of astacin family members (*5*). The forward primer, 5′-CA(C/T)GA(A/G)CT(G/C)(A/G)ACCA(C/T)GC(C/T)CTGGG(G/C)TT-3′, corresponded to the consensus amino-acid sequence HELXHALGF. The reverse primer, 5′-TA(A/G)TGCAT(G/T/C)A(C/T)(G/T)GA(A/G)G(A/C)(A/G)TAGTC(A/G)TA-3′, corresponded to the consensus amino-acid sequence YDYXSXMHY. PCR amplification was performed with Ex Taq DNA polymerase (TaKaRa, Osaka) with liver cDNA from the Japanese common squid. Following an initial melting for 1 min at 94°C, 35 cycles of denaturing (1 min at 94°C), annealing (1 min at 60°C), and extension (1 min at 72°C) were performed. The PCR products were subcloned into pGEM-T easy vector (Promega, Madison, WI, USA) and sequenced in both directions with a Big-Dye Terminator reaction (Amersham Pharmacia Biotech, Little Chalfont, UK) and an automated ABI 377 DNA sequencer (Perkin-Elmer, Norwalk, CT, USA). An insert of the PCR clones was too short to use as a probe for screening; therefore, a longer extended cDNA clone was obtained by additional RT-PCR with a forward primer, 5′-GACAGAGACCAATTCGTAAAAG-3′, designed from the sequence of the PCR clone, and a reverse primer, 5′-CTGATCTAGAGGTACCGGATCC-3′, a complementary sequence of oligo-dT-adapted primer sequence. A total of 5×10^4 phage plaques of a liver cDNA library from the Japanese common squid, prepared from poly(A)-enriched RNA by insertion of cDNA into λ-ZAPII (Stratagene, La Jolla, CA, USA), was screened with the insert labeled by an ECL direct labeling system (Amersham Pharmacia Biotech). Sequence analysis revealed one positive plasmid clone for

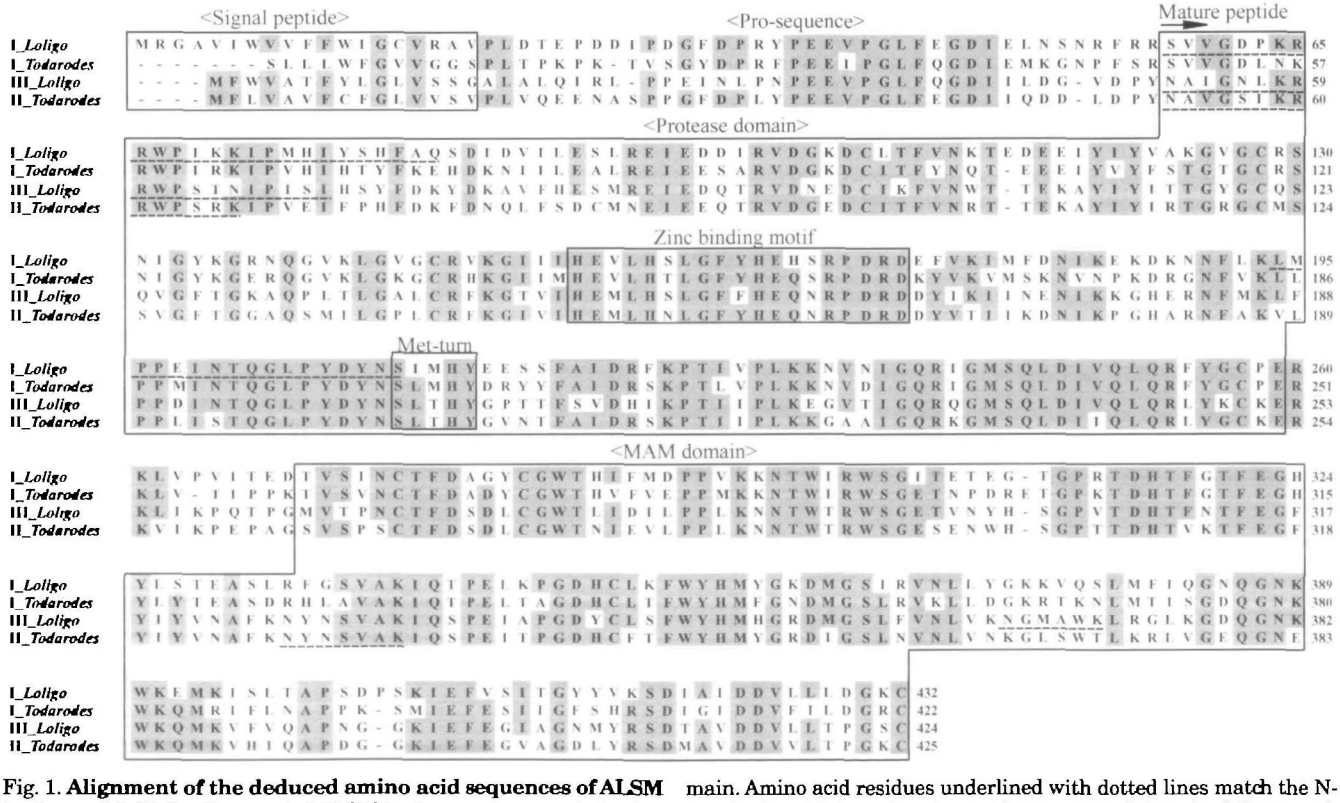

Fig. 1. **Alignment of the deduced amino acid sequences of ALSM isoforms.** Multiple alignment of ALSM isoforms was generated using the Clustal X program (*16*). The dark shading indicates greater than 50% amino acid identity. ALSM has a signal peptide and a pro-sequence followed by an astacin-like protease domain and an MAM domain. Amino acid residues underlined with dotted lines match the N-terminal and internal amino acid sequences determined from purified proteins. I_*Loligo*, *Loligo* ALSM-I; I_*Todarodes*, *Todarodes* ALSM-I; II_*Todarodes*, *Todarodes* ALSM-II; III_*Loligo*, *Loligo* ALSM-III.

ALSM-I and four positive plasmid clones for ALSM-II obtained from the phage plaques.

Cloning and Sequencing of ALSMs-I and -III from Spear Squid—To clone ALSM-I cDNA from spear squid, degenerate RT-PCR oligonucleotide primers were designed from the amino acid sequence of the purified enzyme as follows. The forward primer, 5′-GG(I)GA(C/T)CC(I)AA(A/G)(A/C)G-(I)(A/C)G(I)TGGCC-3′, corresponding to amino-acid sequence GDPKRRWP, was derived from the N-terminal sequence of spear squid ALSM-I (*2*). The reverse primer, 5′-CC(A/G)TT(A/G)TA(A/G)TT(A/G/T)AT(C/T)TC(I)GG(I)G-GC-3′, corresponded to internal amino-acid sequence PPEINTQG (indicated in Fig. 1). PCR amplification was carried out as above with liver cDNA from spear squid, with the exception of an annealing temperature of 54°C. Inserts were subsequently sequenced and used as probes for screening. A total of 5×10^4 phage plaques of a spear squid cDNA library, constructed in λ-ZAPII, were screened. Nine positive clones were excised from the phage plaques and sequenced as described above.

For the cloning of spear squid ALSM-III, a forward primer, 5′-AA(C/T)GC(I)AT(A/C/T)GGGAA(C/T)(C/T)T(I)A-AAAGACG-3′, was designed from the sequence NAIGNL-KRR derived from the N-terminus of ALSM-III (*2*). Homologous residues in both ALSM-I and ALSM-II were used as a template to design the reverse primer, 5′-TG(A/G)CTCA-TTCC(A/G)A(A/T)(A/G)CG(C/T)TGACC-3′. PCR amplification was carried out as above at an annealing temperature of 54°C, and the products were subcloned into pGEM-T easy vector, and then sequenced and labeled with ECL for screening. From a total of 5×10^4 phage plaques, four positive clones were obtained and sequenced as described above.

Phylogenetic Analysis of the Deduced Amino Acid Sequences—Multiple alignments of ALSM isoforms and the protease domain sequences of astacin family members were generated using a Clustal program (*16*). Phylogenetic analysis of the astacin family was performed by the maximum parsimony method with PHYLIP version 3.6 (*17*). Bootstrap sampling (1,000 trials) was used to estimate degrees of confidence in the branching order.

Expression of Wild-Type ALSM-I and a Truncated Mutants with a Bac-to-Bac Baculovirus Expression System—The cDNA encoding spear squid ALSM-I was digested with *Not*I and subcloned into the *Not*I sites of the pFastBacI donor plasmid vector (Life Technologies, Rockville, MD, USA). MAM-truncated ALSM-I (ΔMAM) was constructed by deleting residues after Arg^{260} by PCR with primers T7 and MaseI-23 (5′-AAGCTTACGGACATCCGTAGAATCT-3′). The amplified cDNA encoding truncated ALSM was subcloned into the pGEM-T easy vector and further excised with *Not*I/*Hin*dIII and ligated into pFastBacI. Recombinant baculovirus was obtained for each cDNA construct according to the protocols recommended by the manufacturer (Invitrogen, Carlsbad, CA, USA). SF9 cells were infected according to the manufacturer's instructions. The expression of recombinant proteins was verified by immunoblotting, as described below.

SDS-PAGE and Immunoblotting—SDS-PAGE was performed according to the method of Laemmli (*18*). Gels were either stained with Coomassie Brilliant Blue or blotted to Immobilon membranes. The membranes were pretreated with 2% H_2O_2 in PBS for 20 min and blocked with 5% non-fat dry milk in TPBS (0.05% Tween-20 in PBS) for 1 h at room temperature. Primary antibody TY-1D, an affinity purified polyclonal antibody raised against an 11–amino acid peptide corresponding to the deduced amino-acid sequence LREIEDDIRVD of ALSM-I, was used at a dilution of 1:100. Horseradish peroxidase–conjugated secondary antibody (HRP-conjugated anti–rabbit IgG; DAKO Japan, Kyoto) was used at a dilution of 1:2,000. Detection was performed by chemiluminescence (ECL; Amersham Pharmacia Biotech).

Immunohistochemistry—Samples of mantle muscle were dissected from a fresh squid and embedded in OCT compound (Tissue-Tec, Miles Scientific, Naperville, IL). Frozen sections (14-μm thickness) were fixed with –20°C methanol for 5 min and permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature. After a rinse with PBS, nonspecific binding sites were blocked with 2% normal goat serum and 2% BSA in TPBS for 30 min. Purified TY-1D was used as the primary antibody (diluted 1:50), and the secondary antibody was FITC-conjugated anti–rabbit IgG antibody (diluted 1:150) (Kirkegaard and Perry Lab.). Specimens were examined under a confocal scanning microscope (LSM 410; Carl Zeiss, Oberkochen, Germany).

RESULTS

cDNA Cloning and Sequence Analysis of ALSMs-I, -II, and -III—We previously reported the amino-terminal sequences of ALSM isoforms (*2*). To obtain cDNA clones for each isoform, we analyzed additional internal amino acid sequences of ALSM, and the sequences were used as templates to design degenerate RT-PCR primers. A 1,415-bp clone containing a 1,296-bp open reading frame (ORF) (GenBank accession number AB081634) was predicted to encode a 432-residue polypeptide. This ORF was flanked by 5′ and 3′ untranslated regions (UTRs). The deduced amino acid sequence of this clone matched the sequences determined from purified spear squid ALSM-I (see Fig. 1), indicating that the clone encoded full-length spear squid ALSM-I. Likewise, the deduced amino acid sequences of a 1,329-bp clone with a 1,275-bp ORF (GenBank accession number AB081636) and a 1,352-bp clone with a 1,272-bp ORF (GenBank accession number AB081637), both flanked by 5′ and 3′ UTRs, included sequences that matched those determined from purified ALSM-II and ALSM-III, respec-

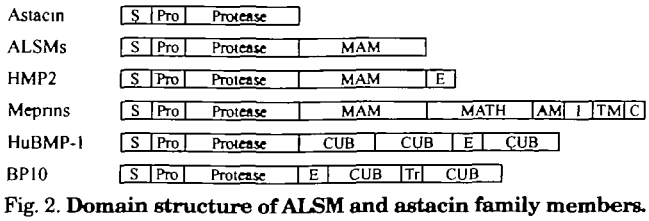

Fig. 2. **Domain structure of ALSM and astacin family members.** The domain structure of ALSM is shown with those of astacin family members. There is a putative signal peptide (S) at the N-terminus of all astacin family proteases, followed by a conserved pro-sequence (Pro). In ALSMs, HMP2, and meprins, an MAM domain (MAM) is located next to an astacin-like protease domain (Protease). No transmembrane (TM) or cytoplasmic (C) domains are found in astacin, ALSMs, or HMP2. E, epidermal growth factor-like domain; MATH, meprin and TRAF homology domain; AM, after MATH domain; I, inserted domain; CUB, Clr/s complement-like domain; Tr, Thr-rich domain.

tively (Fig. 1). However, one clone containing a 1,268-bp ORF with a 3′ UTR (GenBank accession number AB-081635) lacked the 5′ UTR and the translation-initiation site. Sequence alignment of the deduced amino acid sequences revealed that the sequence of this clone was 66% identical to that of spear squid ALSM-I, 52% identical to that of ALSM-II, and 51% identical to that of ALSM-III. On the basis of these data, this clone was designated a partial cDNA of ALSM-I from the Japanese common squid.

The putative cleavage site of the signal peptide and the functional protein domains were analyzed by the SMART (Simple Modular Architecture Research Tool) program (*19, 20*), as indicated in Fig. 1. Consistent with this analysis, the N-terminal amino acid sequences determined from purified ALSMs were not initiated from the first methionine (see arrow in Fig. 1), supporting the idea that ALSMs have a pro-sequence that is proteolytically cleaved, similar to that of other astacin family members (*5*). A homology search for functional protein domains was also performed by BLAST analysis against the Prodom protein domain database (*21*) and revealed that ALSMs possess a conserved astacin protease domain including a zinc-binding motif (HEXXHXXGFXHEXXRXDRD). Schematic diagrams of the domain structures of the astacin family members are shown in Fig. 2. Sequence comparison of ALSMs with astacin family members revealed that the protease domain of ALSM has sequence identity (>40%) with BMP-1 (Fig. 3A). ALSM-I has a Met-turn motif (SXMHY) that is essential for the structural integrity of the zinc-binding site. The conserved Met in this motif is, however, replaced by Thr in ALSMs-II and -III. Only ALSM has a 6-residue insert within the protease domain. Astacin family members possess various C-terminal domains, including CUB, EG, and MAM domains (*5*). ALSM also possesses a unique C-terminal domain, showing sequence identity with the MAM domains of meprins and HMP2 (Fig. 2). Comparisons of the protease domain sequences have been done to determine classes within the astacin family (*5*). The protease domain

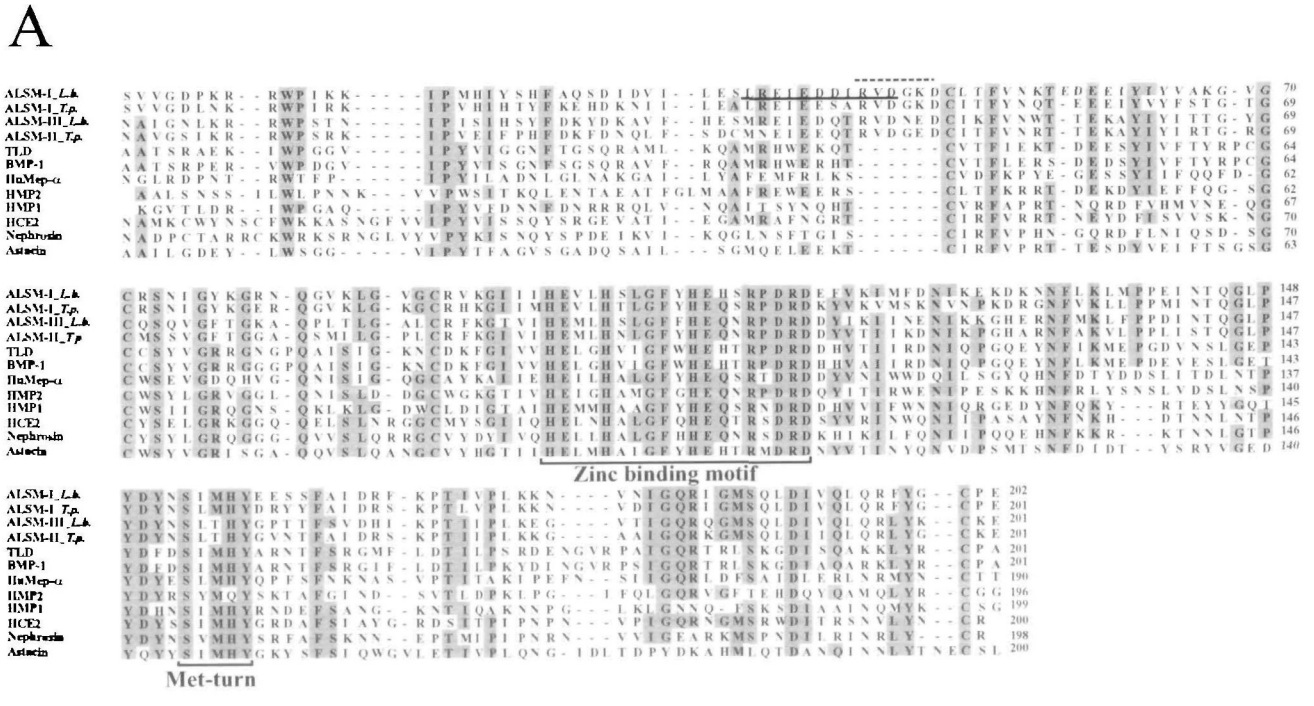

Fig. 3. **Sequence comparison and phylogenetic analysis of the protease domains.** A: Protease-domain sequences of astacin family members were aligned as described in "EXPERIMENTAL PROCEDURES." ALSM isoforms possess a conserved 6-residue insert as indicated by the dotted line. The zinc-binding motif and methionine turn (Met-turn) are indicated under the sequences. Underlined sequences were synthesized *in vitro*, and used to generate a polyclonal antibody. B: Phylogenetic analysis of the astacin family based on protease-domain sequences was performed by the maximum parsimony method. The percentage of the time a node occurred out of 1,000 bootstrap trials is indicated. ALSM-I_*L.b.*, *Loligo* ALSM-I; ALSM-I_*T.p.*, *Todarodes* ALSM-I; ALSM-III_*T.p.*, *Todarodes* ALSM-III; ALSM-II-_*L.b.*, *Loligo* ALSM-II.

sequence of ALSM was compared with those of astacin family members with the use of Clustal X and PHYLIP programs to generate an unrooted phylogenetic tree (Fig. 3B). Most of the astacin family members analyzed could be assigned to one of four main clusters, one of which includes ALSM isoforms.

Immunoblotting and Histologic Analysis—The activity of ALSM toward MyHC has been reported (*3*). However, the distribution of ALSM at the protein or mRNA level has not been determined. Therefore, a specific antibody generated against a synthetic peptide of ALSM-I was raised in rabbits and used to detect the distribution of ALSM in spear squid. In a liver extract, the antibody recognized a major band at approximately 45 kDa and minor band at approximately 30 kDa (Fig. 4). In other tissues (mantle, stomach, pancreas, and testis), the 45-kDa band was present, but other bands were frequently detected (Fig. 4). The cellular localization of ALSM in mantle muscle was visualized by indirect immunofluorescence staining and confocal microscopy. Staining was evident not in the cytoplasm but in the extracellular matrix (Fig. 5), where myosin is not present, indicating that myosin and ALSM do not colocalize.

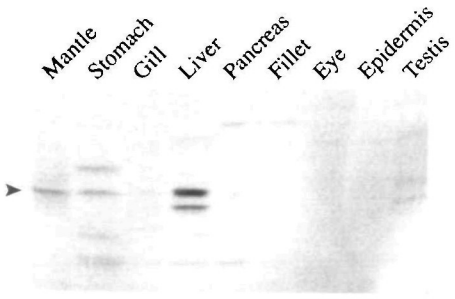

Fig. 4. **Tissue distribution of *Loligo* ALSM-I as revealed by Western blot.** Proteins in various tissue extracts (15 μg) were electrophoresed in an SDS–polyacrylamide gel and subjected to immunoblotting with an affinity purified polyclonal antibody, TY-1D. The arrowhead indicates the predicted molecular mass (45 kDa) of ALSM.

Expression of ALSMs in a Bac-to-Bac Baculovirus Expression System—To analyze the ALSM functional domain, recombinant wild-type and truncated ALSMs were expressed in a baculovirus system. Expression of the recombinants, illustrated in Fig. 6A, was confirmed by Western blotting with an affinity-purified polyclonal antibody (Fig. 6B). The expressed proteins (in SF9 cell culture medium) were concentrated by TCA precipitation followed by Western blotting. Faint bands were detected at the appropriate molecular masses for recombinant ALSM-I (45 kDa) and ΔMAM (30 kDa). A 30-kDa peptide was also detected in the recombinant ALSM preparation (Fig. 6B). This band increased in intensity as the 45-kDa band decreased in intensity and therefore is likely a proteolytic product derived from the 45-kDa form; generation of the 30-kDa form was suppressed by the addition of a protease inhibitor cocktail (data not shown). Expression of active expressed protease was confirmed by assay with MyHC as a substrate (Fig. 6C). Like purified native ALSM-I, recombinant ALSM-I digested MyHC to produce 130- and 90-kDa fragments. No MyHC hydrolysis was identified in the ΔMAM-incubated sample. The data suggest that recombinant ALSM-I func-

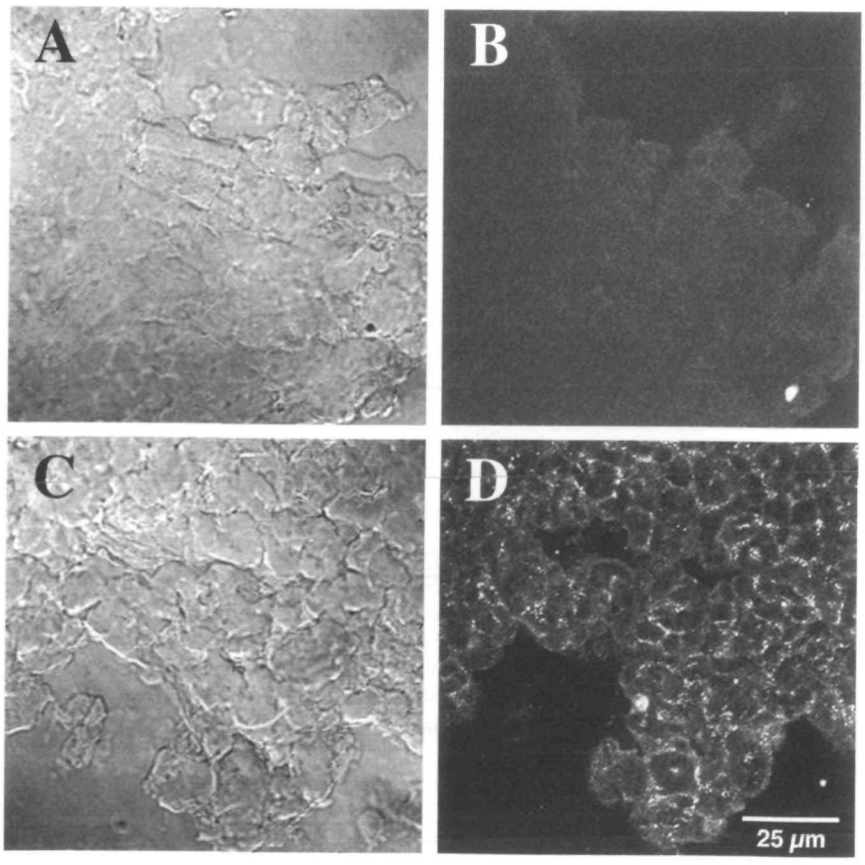

Fig. 5. **Immunohistochemical localization of ALSM-I protein in *Loligo* mantle muscle.** A and C: Bright-field images of 14-μm sections obtained from *Loligo* mantle muscle. B and D: Indirect immunofluorescence images of the sections. The sections were incubated with primary (TY-1D) and secondary (FITC-conjugated) antibodies (C and D). In control experiments, the primary antibody was omitted (A and B).

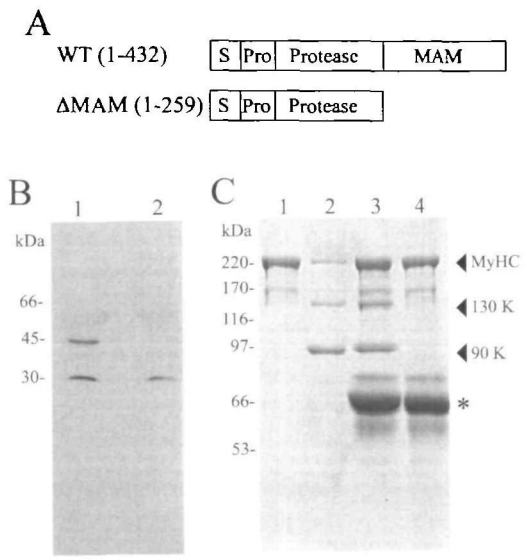

Fig. 6. **Expression of *Loligo* ALSM-I and an MAM-truncated mutant (ΔMAM) using a Bac-to-Bac baculovirus expression system.** A: Schematic diagram of the domain structure and mutant used in this study. WT (1–432), full-length of ALSM-I; ΔMAM (1–259), truncated ALSM expressed from the first methionine to residue number 259. The domain structure and the names of each domain are as indicated in the legend to Fig. 2. B: Immunodetection of the recombinant proteins. Expressed proteins for ALSM-I (lane 1) and ΔMAM (lane 2) were electrophoresed in an SDS–polyacrylamide gel and analyzed by immunoblotting with antibody TY-1D. C: The enzyme activity of expressed proteins against myosin heavy chain was examined. Rabbit skeletal myosin (lane 1) was incubated with native ALSM-1 (lane 2), expressed wild-type ALSM-1 (lane 3) or MAM-truncated mutant (lane 4). The asterisk indicates the protein band derived from the cell culture medium. MyHC, myosin heavy chain; 130 K and 90 K, 130- and 90-kDa fragments of MyHC, produced by ALSM hydrolysis. Ordinates in (B) and (C), molecular mass in kDa.

tions similarly to purified ALSM-I and that the MAM domain of ALSM plays a role in substrate recognition, at least under these *in vitro* conditions.

DISCUSSION

In this study, we cloned cDNAs encoding full-length ALSMs-I and -III from spear squid and ALSM-II from the Japanese common squid, plus a partial cDNA of ALSM-I from the Japanese common squid. The amino acid sequences determined from the purified proteins were reproduced in the deduced amino acid sequence of the clones, confirming that the cDNA clones encoded ALSMs. Between the individual ALSMs, there is high sequence identity (56–72%), and the ALSMs also show similarity (30–40% identity) to the protease domains of members of the astacin family. BLAST analysis revealed that ALSM possesses a unique functional domain structure comprising an astacin-like protease domain followed by an MAM domain. ALSM possesses a well-conserved zinc-binding motif (HEXXHXXGFXHEXXRXDRD) and a unique 6-amino-acid insert within the protease domain. A methionine-turn motif (SXMHY), which is essential for the structural integrity of ion-binding sites (*22*), is found not only in astacin family proteases but also in serralysin, snake venom, and matrix metalloprotease (*23, 24*). ALSM-I has a conserved methionine in this motif, but neither ALSM-II nor -III have this residue. Interestingly, we reported that ALSM-I has a higher affinity for cobalt than zinc, whereas ALSMs-II and -III have a higher affinity for zinc (*1–3*). This suggests that the ion preference of each isoform and the reflected protease activity is dependent on the difference in the methionine-turn motif. Taken together, these data indicate that ALSM is a novel astacin family member. Notably, phylogenetic analyses based on the protease domain sequences indicate that the ALSM isoforms form a class that is distinct from astacin family members.

The astacin family is assigned to the M12A group of proteases by MEROPS (*25*), and includes over 20 members from hydra to human (*5*). Members of the group are characterized as secreted or membrane-bound proteases and have various functions, such as digestion (*26*), hatching (*12, 14, 27–29*), hormone activation (*30*), and embryonic development (*10, 31*). However, only a few substrates specific for each protease have been reported (*5*). The sequence similarity of ALSM to astacins led us to propose that ALSM is a secreted protease and, therefore, that it is unlikely that cellular myosin is its native substrate. This was confirmed by the finding that the distribution of ALSM is mainly in liver tissue as well as other internal organs, and that the cellular distribution of ALSM in mantle muscle is confined to the extracellular matrix.

The protease domains of ALSM isoforms are highly conserved and show obvious sequence identity with BMP-1 and its homologs. The latter are reported to contribute to the biosynthesis of the extracellular matrix and to pattern formation during embryogenesis (*7, 32*). Among astacin members, BMP-1 is structurally most similar to *Drosophila* tolloid (TLD) (*33*) and its homolog *Xenopus* tolloid (XLD) (*34*), although TLD and XLD contain additional EGF and CUB domains (*35*). TLD and XLD are thought to play a role in dorsal-ventral patterning by releasing active morphogens from latent complexes as a result of hydrolysis of their inhibitors (*36*). BMP-1 is expressed highly in bone and placenta; however, this expression is restricted to embryonic development (*10*). Such patterns may not be evident for the expression and distribution of ALSM, and ALSM may not be involved in squid embryonic morphogenesis.

Domain analysis showed that ALSMs have an MAM domain, similar to meprins (*5*) and HMP2 (*37*). Enterokinase, rat apical-endosomal glycoprotein, *Xenopus* thyroid hormone–induced protein B, and pig zonadhesin are also known to possess MAM domains (*38–41*). Except for meprins, the physiological function(s) of this domain is not established. Meprins are expressed abundantly in the brush border membranes of mammalian kidney and intestinal epithelial cells (*6, 42, 43*). In meprins, the MAM domain, containing 170 amino acids, is believed to be involved in oligomerization (*44, 45*). This proposed function was revealed by analysis of MAM-truncated meprin mutants that result in misfolding of the expressed protein followed by proteasome-mediated proteolysis (*46*). The data suggest this domain is required for molecular folding and also for oligomerization. Unlike meprins, ALSMs are believed to be monomeric, as indicated by gel column chromatography (*2*). To study the function of the MAM domain, recombinant wild-type ALSM and an MAM-truncated mutant were expressed in a baculovirus expression system.

ALSM was originally identified as a protease with the ability to hydrolyze MyHC at a specific site. However, as mentioned above, MyHC is not likely to be the native substrate of ALSM. In addition, we previously reported that 23 synthetic peptides, as well as casein, are not effective substrates (*in vitro*) for ALSM (*1*). In this study, the protease activity of the recombinant proteins was evaluated using MyHC as a substrate. The expressed full-length protein was able to hydrolyze MyHC and produced 130- and 90-kDa fragments, similar to native ALSM, confirming successful expression and refolding of the recombinant protein. MyHC was not hydrolyzed by the MAM-truncated ALSM, suggesting that the MAM domain plays a role in substrate recognition.

In conclusion, this study documents the first characterization of a novel astacin family member, ALSM. ALSM is a secreted metalloprotease with an astacin-like protease domain and an MAM domain. ALSMs are expressed in a wide variety of tissues, and are extracellular in the mantle muscle. Expression of the MAM-truncated mutant resulted in a lack of protease activity, indicating that the MAM domain contributes to substrate recognition. Further studies are required to determine the native substrate(s) for ALSM and to elucidate its physiological function.

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