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Purification and Characterization of a Novel Isoform of Myosinase from Spear Squid Liver[1]

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A novel isoform of myosinase was purified to homogeneity from liver of spear squid by sequential chromatographies using SP Sephadex, hydroxylapatite, Zn/Co chelating affinity, and TSK-gel G2000SW columns. Myosinase activity was detected as a single peak of 45-kDa protein by gel filtration. The novel isoform of myosinase specifically hydrolyzed a rabbit skeletal muscle myosin heavy chain into products of 120 and 100 kDa in the presence of Co^{2+} ions, and the cleavage site in the myosin heavy chain was quite different from those of two known myosinase isoforms, I and II. Therefore, we named the novel isoform myosinase III. Myosinase III was also distinguishable from myosinase I by its amino-terminal sequence. The sequence showed similarity to an internal sequence of the astacin family.

Key words: amino-terminal sequence, astacin, endoproteinase, myosinase, novel isoform.

Myosinase was originally identified from a squid mantle muscle and characterized as a Zn^{2+}/Co^{2+} metal-dependent endoproteinase which specifically hydrolyzed myosin heavy chain (MyoHC) at neutral pH (*1*). Its activity is not inhibited by inhibitors of serine, cysteine, or aspartic proteinases, *e.g.*, cathepsin inhibitors, phenylmethanesulfonyl fluoride (PMSF), iodoacetate, E-64, leupeptin, and calpastatin. Because of its substrate specificity, its optimum pH for hydrolysis, its requirement for divalent actions, and the effect of inhibitors, myosinase was concluded to be a unique metal-dependent endoproteinase. Two isoforms of myosinase, I and II, were shown to exist, differing in their cleavage sites of MyoHC. The former splits chicken skeletal muscle MyoHC between Ala-1161 and Thr-1162 in subfragment 2, and produces 130- and 90-kDa fragments. The latter splits MyoHC between Glu-1381 and Thr-1382 in light mero myosin (LMM), and produces 158- and 65- kDa fragments. No other such unique metal-dependent proteinase has been reported, either from squid or from other organisms.

In the original work, we suggested that myosinases are probably related to the metabolism of myosin *in vivo*, because of their strict substrate specificity. However, our recent work revealed that the myosinase activities were identified in nonmuscle tissues rather than in mantle muscle (*2*). Therefore, in this work, we isolated myosinase from squid liver, and characterized the enzyme in comparison with myosinase from squid mantle muscle. We also determined the amino-terminal sequence of myosinase, which showed similarity to part of the internal sequence of an astacin family member. Astacin [EC 3.4.24.21], a zinc-dependent endopeptidase from the crayfish *Astacus fluviatilis* L., is the prototype of the astacin family (*3*). Zinc metalloproteinases are involved in processes as diverse as embryonic development, bone formation, and various diseases such as cancers, arthritis, and hypertension. The well-known metalloproteinases have been divided into two groups, the "gluzincins" and "metzincins," based on the third zinc ligands.

The astacin family is classified as a subgroup of the metzincins, which comprises several membrane-bound mammalian endopeptidases and developmentally implicated regulatory proteins (*4-8*). Taken together, our previous works and the results of this work suggest that myosinase is distributed in many squid tissues and plays unique roles in squid.

MATERIALS AND METHODS

Materials—Spear squid (*Loligo bleekeri*) was purchased at Tokyo Central Wholesale Fish Market. The squid was transported to our laboratory at low temperature (4°C), and the liver was dissected within a day of purchase.

Purification Procedures—Livers were excised from five squids. All the procedures were performed at 4°C.

Step 1. Extraction: Excised squid liver (approximately 40 g) was washed five times with de-ionized water, then minced gently and extracted with 200 ml of 80 mM sodium phosphate buffer, pH 7.2, containing 2 mM PMSF, 1 mM EDTA, 2 mM iodoacetic acid (MIA), 3 μg/ml pepstatin A, 3 μg/ml leupeptin, and 0.01% sodium azide. After extraction overnight with gentle stirring, the mixture was centrifuged at 10,000 × *g* for 15 min.

Step 2. Ammonium sulfate fractionation: Solid ammo-

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Abbreviations: MIA, monoiodoacetic acid; MyoHC, myosin heavy chain; PMSF, phenylmethanesulfonyl fluoride; RT-PCR, reverse transcription PCR.

nium sulfate was added to the supernatant. The precipitate which formed between 35 and 55% ammonium sulfate saturation was dissolved in SP solution (20 mM sodium phosphate, pH 6.5, 0.15 M NaCl, 1 mM EDTA, and 0.01% sodium azide) and dialyzed against the same solution.

Step 3. Cation exchange chromatography: The dialyzed solution was clarified by centrifugation at 10,000×*g* for 15 min, then applied to a SP Toyopearl 550C column (1.6×20 cm, TOSOH) previously equilibrated with SP solution, and eluted with a 200-ml linear gradient of NaCl from 150 mM to 700 mM at a flow rate of 1.4 ml/min. Fractions containing myosinase activity were collected and dialyzed overnight against HA solution (50 mM potassium phosphate, pH 6.2, and 0.01% sodium azide).

Step 4. Hydroxylapatite chromatography: The dialyzed solution was centrifuged at 10,000×*g* for 15 min, and the supernatant was applied to a Hydroxylapatite column (1.6×17 cm, Nacalai) pre-equilibrated with HA solution. Proteins were eluted with a 200-ml linear gradient of potassium phosphate, pH 6.2, from 50 mM to 500 mM at a flow rate of 0.9 ml/min. Pooled fractions containing specific proteinase activities were dialyzed overnight against CH solution (50 mM Tris-HCl, pH 7.5, 0.5 M NaCl, and 0.01% sodium azide).

Step 5. Metal-chelate chromatography: The dialyzed solution was centrifuged at 10,000×*g* for 15 min, then subjected to Co^{2+}-chelating chromatography on a Chelating Sepharose FF column (1.6×5 cm, Pharmacia) charged with $CoCl_2$ according to the Porath method (*9*) and pre-equilibrated with CH solution. The flow-through fractions were subsequently applied to Zn^{2+}-chelating column (1.6×5 cm) charged with $ZnCl_2$. Proteins which bound to the columns were then eluted with a solution containing 50 mM Tris acetic-acid, pH 4.8, and 0.5 M NaCl at a flow rate of 1.2 ml/min.

Step 6. HPLC gel filtration chromatography: Pooled fractions from both chelating columns were separately concentrated using Centriprep 30 membrane filter (Amicon), then subjected to HPLC on a TSK gel-G2000SW gel filtration column (0.75×60 cm, TOSOH). Protein was eluted with 20 mM sodium phosphate, pH 7.2, and 0.3 M NaCl at a flow rate of 0.5 ml/min.

Concentration of protein was determined according to the protein-dye binding method of Bradford (*10*), using bovine serum albumin as a standard.

Preparation of Rabbit Skeletal Muscle Myosin—Rabbit skeletal muscle myosin was prepared from skeletal muscle by the method of Perry (*11*).

Detection of Specific Proteinase Activity—The proteinase activity was detected by a previously reported method (*2*). Briefly, a 15-μl aliquot of each fraction was mixed with 35 μl of standard reaction mixture to give final concentrations of 0.6 mg/ml rabbit skeletal muscle myosin, 100 mM sodium phosphate, pH 7.2, 5 mM EDTA, 4 mM MIA, 8 mM PMSF, 20 μg/ml pepstatin A, 20 μg/ml leupeptin, and 10 mM $CoCl_2$ or $ZnCl_2$. After incubation for 30 min at 37°C, the reaction was stopped by addition of 25 μl of SDS-solution (10% glycerol, 10% 2-mercaptoethanol, 3% SDS, and 62.5 mM Tris-HCl, pH 6.7). Aliquots of the sample (20 μl) were subjected to 7.5 or 13% SDS-PAGE according to the method of Laemmli (*12*). Gels were stained with 1% Coomassie Brilliant Blue R-250, and specific proteinase activity was detected by scanning the area of bands representing rabbit skeletal muscle MyoHC.

Characterization of Myosinase—To visualize the effects of inhibitors on proteinase activity of myosinases, 15-μl portions of isolated myosinase (final concentration 6 μg/ml) were incubated with MyoHC for 30 min in the presence of various inhibitors. Other conditions were the same as in the above standard procedure for detection of myosinase activity. Time courses of hydrolysis of MyoHC by the myosinase activities were analyzed by incubation of myosinases (final concentration 6 μg/ml) with MyoHC.

Amino Acid Sequence Analysis—Amino-terminal sequences were determined from the peptides transferred onto Immobilon-PSQ polyvinylidene difluoride membranes (Millipore) by Edman degradation on an automated Shimazu PSQ-1 sequencer.

RESULTS

Purification of Myosinases—Figure 1A shows the hydrolysis pattern of rabbit skeletal muscle MyoHC for the eluate from the hydroxylapatite column. Products which were hydrolyzed by the proteinase were separated by SDS-PAGE into fragments of approximately 130, 120, 100, and 90 kDa. This result indicated the presence in squid liver tissue of several types of proteinase that were activated by Co^{2+} ions. SDS-PAGE analysis of fractions collected throughout the purification procedure indicated that the hydroxylapatite column chromatography effectively removed contaminants (lane 5 in Fig. 1B), and the enzymes were isolated as a single protein-band (45 kDa) by HPLC on a gel filtration column (lane 7 and 9 in Fig. 1B). Enzymes

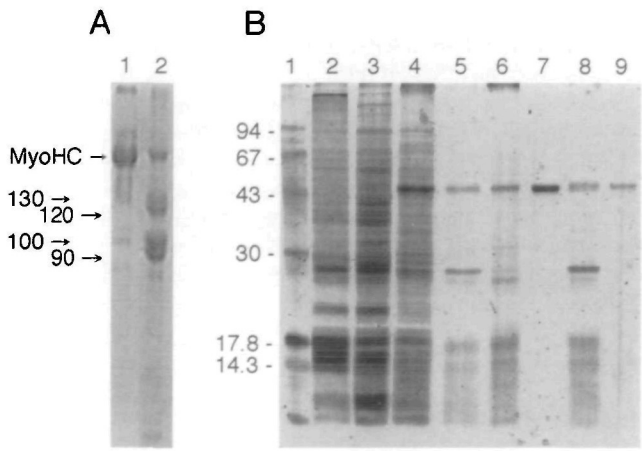

Fig. 1. **SDS-PAGE analysis of purification procedure.** (A) Hydrolysis of rabbit skeletal muscle myosin heavy chain by crude enzymes eluted from hydroxylapatite column. Lane 1, before hydrolysis; lane 2, after hydrolysis by crude enzymes activated by Co^{2+} ion. MyoHC, rabbit skeletal muscle myosin heavy chain. Numeral and arrows indicate fragments in kDa. (B) Analysis of each step of purification. Lane 1, molecular mass marker; lane 2, crude extract; lane 3, enzyme solution after ammonium sulfate precipitation; lane 4, eluate from SP Toyopearl 550C column; lane 5, eluate from hydroxylapatite column; lane 6, eluate from Zn^{2+}-chelating Sepharose FF column; lane 7, eluate from TSK gel G2000SW column (myosinase I); lane 8, eluate from Co^{2+}-Chelating Sepharose FF column; lane 9, eluate from TSK gel G2000SW column (novel isoform of myosinase). Electrophoresis was performed according to Laemmli (1970) using 13% polyacrylamide gels. Gels were stained with a silver stain kit. Ordinate, molecular size in kDa.

eluted from the hydroxylapatite column were separated into two by Zn^{2+} and Co^{2+} chelating column chromatographies, depending on their affinity with metal ions (Fig. 2). Enzymes which bound to the Zn^{2+}-chelating column showed myosinase I activity. This activity was characterized by the amino-terminal sequence of the 90-kDa fragments of rabbit skeletal muscle MyoHC (Fig. 2B). On the other hand, an enzyme which bound to the Co^{2+}-chelating column hydrolyzed MyoHC to products of 120 and 100 kDa (Fig. 2A). The amino-terminal sequence of the 100-kDa fragment of MyoHC revealed that the enzyme split the rabbit skeletal muscle MyoHC between Glu-1098 and Asp-1099 (Table I). This indicated the possible presence of a novel type of myosinase (myosinase III); therefore further purification was performed by gel filtration using a TSK-gel G2000SW column. Figure 3B shows the MyoHC hydrolysis activity of the novel enzyme eluted from the gel filtration column. A protein of approximately 45 kDa was eluted in the fractions which showed the highest hydrolysis activity. Myosinase I was also isolated using a gel filtration column; consequently 45-kDa protein was isolated (lane 7 in Fig. 1B). To ascertain whether the 45-kDa proteins isolated from the Zn^{2+}- and Co^{2+}-chelating columns corresponded to myosinase I and a novel isoform of myosinase, respectively, we determined the amino-terminal sequence of the proteins. The amino-terminal sequence of 20 amino acids of the novel enzyme shared 45% sequence similarity with that of myosinase I (Table II). Therefore, we concluded that the novel enzyme was a myosinase, myosinase III.

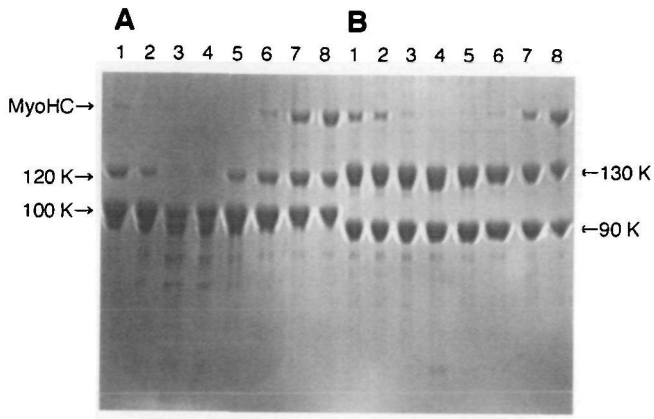

Fig. 2. **Affinity chromatography of myosinases.** The eluate from the hydroxylapatite chromatography was subjected to affinity chromatography as described in "MATERIALS AND METHODS." Proteinase activity of each 3-ml fraction was assayed using rabbit skeletal muscle MyoHC as substrate. (A) MyoHC hydrolysis activity of eluate from Co^{2+}-chelating column. (B) MyoHC hydrolysis activity of eluate from Zn^{2+}-chelating column. SDS-PAGE was carried out as indicated in "MATERIALS AND METHODS" using 7.5% acrylamide gels. Numerals show fraction number of each column. Arrows show fragments hydrolyzed by proteinase. MyoHC, rabbit skeletal muscle myosin heavy chain.

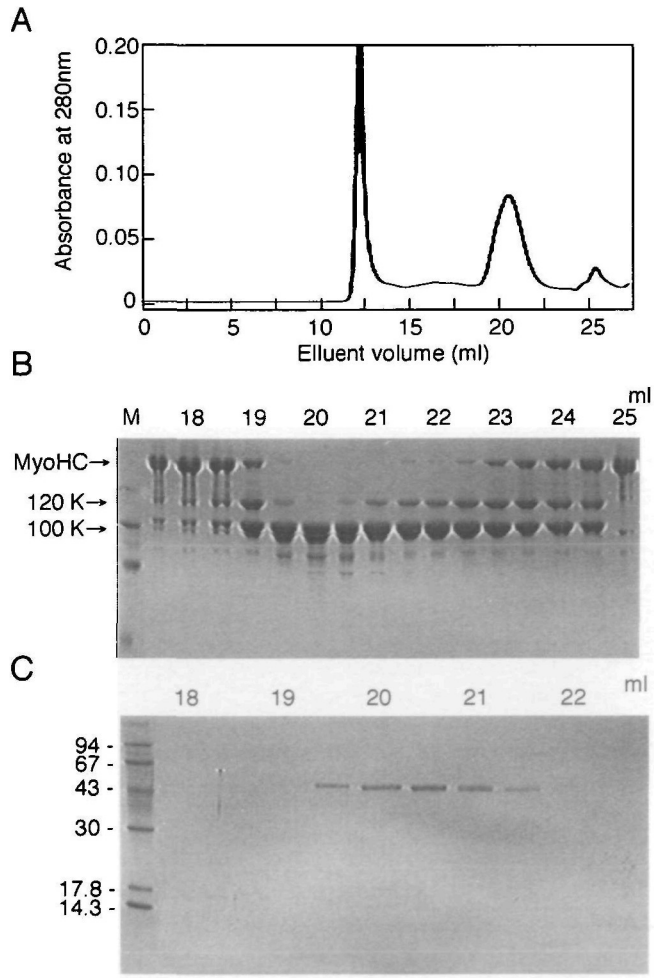

Fig. 3. **Gel filtration chromatography of the novel proteinase.** (A) Eluate from the Co^{2+}-chelating affinity chromatography was loaded onto a TSK gel G2000SW column. (B) Fractions of 0.25 ml were collected, and 15-μl aliquots were assayed for hydrolysis activity of MyoHC as described in "MATERIALS AND METHODS." Arrows show fragments hydrolyzed by the proteinase. MyoHC, rabbit skeletal muscle myosin heavy chain. (C) Aliquots (20 μl) of the same fractions were subjected to SDS-PAGE as described in "MATERIALS AND METHODS." Proteins in the gels were then visualized by staining with Coomassie Brilliant Blue R-250. Ordinate, molecular mass in kDa.

TABLE I. **The cleavage sites in myosin heavy chain for the myosinases.**

Proteinase	N-terminal sequence of hydrolysed products	Cleavage site						Molecular mass of fragments (kDa)	Reference
		P_3	P_2	P_1	P_1'	P_2'	P_3'		
Myosinase I	TAAQID*	Gly-	Gly-	Ala$_{1161}$↓	Thr$_{1162}$-	Ala-	Ala	130+90	Okamoto *et al.*, (1993)
Myosinase I	X**XAQIEMN	Gly-	Gly-	Ala$_{1159}$↓	Thr$_{1160}$-	Ser-	Ala	130+90	This paper
Myosinase II	TDAIQ*	Lys-	Thr-	Glu$_{1381}$↓	Thr$_{1382}$-	Asp-	Ala	158+65	Okamoto *et al.*, (1993)
Myosinase III	DEQXLXMQLQ	Lys-	Ile-	Glu$_{1098}$↓	Asp$_{1099}$-	Glu-	Gln	120+100	This paper

*These sequences correspond to internal sequences of chicken skeletal muscle MyoHC. **X indicates an ambiguous assignment.

Effects of Inhibitors—The effects of proteinase inhibitors on the activities of myosinase I and III were assayed by incubation with MyoHC for 30 min at 37°C (Fig. 4). The activities were not affected by inhibitors of serine, cysteine, and aspartic proteinases, but they were strongly inhibited by 10 mM 1,10-phenanthroline, EGTA, and EDTA, which are chelating agents of divalent cations. These results indicated that myosinase III was a metal-dependent endoproteinase like myosinase I. Additionally, the myosinase activities were completely inhibited under reducing conditions.

Degradation of Myosin Heavy Chain—The time courses of hydrolysis of MyoHC by the myosinase activities were monitored by SDS-PAGE analysis. Rabbit skeletal muscle MyoHC was completely hydrolyzed after 17 h of incubation with myosinase I or III. The degradation products of MyoHC hydrolyzed by myosinase I were stable for up to 17 h of incubation (Fig. 5A). On the other hand, the products hydrolyzed after a few hours incubation with myosinase III were re-hydrolyzed to products of 95-kDa peptide by 17 h of incubation (Fig. 5B). These results indicated high substrate-specificity of myosinase, though myosinase III showed slight secondary hydrolysis activity on prolonged incubation.

DISCUSSION

Myosinases are unique metal-dependent endoproteinases that were discovered in squid mantle muscle. These activities are characterized by their substrate specificity, their cleavage sites in MyoHC, their optimum pH for hydrolysis, their requirement for divalent cations, and the effects of inhibitors. In the original study, we reported that two types of myosinase, myosinase I and II, are present in the squid mantle muscle, and we suggested that they are involved in

TABLE II. **N-terminal sequences of myosinase.**

Materials	N-terminal sequence of 45-kDa peptides
Myosinase I	S V V G D P K R R W P I K K I P M H I Y S H F A Q
Myosinase III	N A I G N L K R R W P S I N I P I X*I G
C05D11.6	S I M S T Q F (114) R R W P N N E I P Y T L S S Q Y G S (138)

*X indicates an ambiguous assignment.

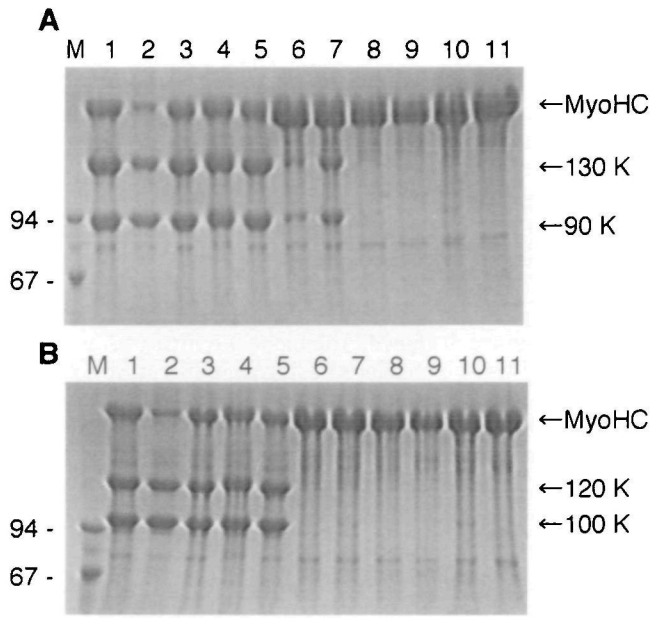

Fig. 4. **Effects of inhibitors on proteinase activity of myosinase I (A) and III (B).** Each enzyme was incubated with MyoHC at pH 7.2 for 30 min in the presence of various inhibitors. SDS-PAGE was carried out as described in "MATERIALS AND METHODS." Lane 1, no inhibitors; lane 2, 10 mM PMSF; lane 3, 20 μg/ml leupeptin; lane 4, 10 mM MIA; lane 5, 20 μg/ml pepstatin A; lane 6, 10 mM EDTA; lane 7, 10 mM EGTA; lane 8, 10 mM *o*-phenanthroline; lane 9, 2.5% 2-mercaptoethanol; lane 10, 10 mM DTT; lane 11, 2.5% SDS; M, molecular mass marker. Arrows in panel A and B show fragments hydrolyzed by myosinase I and III, respectively. MyoHC, rabbit skeletal muscle myosin heavy chain. Ordinate, molecular mass in kDa.

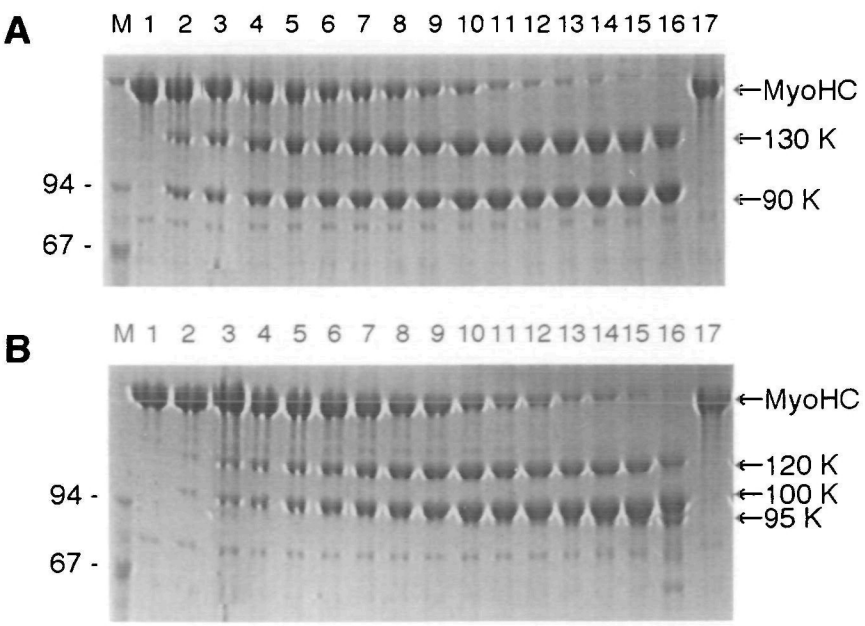

Fig. 5. **Degradation time course of myosin heavy chain.** The time course of hydrolysis of MyoHC by myosinase I (A) and III (B) was monitored by SDS-PAGE analysis. Incubation was carried out at pH 7.2 and at 37°C for 0 min (lane 1), 1 min (lane 2), 2 min (lane 3), 3 min (lane 4), 5 min (lane 5), 7 min (lane 6), 10 min (lane 7), 15 min (lane 8), 20 min (lane 9), 30 min (lane 10), 45 min (lane 11), 60 min (lane 12), 90 min (lane 13), 120 min (lane 14), 180 min (lane 15), and 17 h (lane 16). Lane 17 is a control. M, molecular mass marker. Arrows in panel A and B show fragments hydrolyzed by myosinase I and III, respectively. MyoHC, rabbit skeletal muscle MyoHC. Ordinate, molecular mass in kDa.

the metabolism of myosin *in vivo* (*1*). Recent work, however, revealed that the specific activities of myosinase were present in nonmuscle tissues rather than in muscle tissue (*2*). To elucidate the biochemical roles of myosinase, we attempted to purify the enzymes from squid liver in this work.

Myosinase I was isolated by the successive chromatographies on a cation-exchange column, a hydroxylapatite column, Zn^{2+}-chelating affinity column, and an HPLC gel filtration column. Myosinase III was also isolated in the same way, except that a Co^{2+}-chelating affinity column was used in place of the Zn^{2+}-chelating column. Both isolated proteins had molecular masses of approximately 45 kDa (Fig. 1B). In the previous work, we reported that the molecular mass of myosinase I was 16 kDa (*1*), though we now think that the protein in question was a degraded catalytic domain of myosinase I. Myosinase is easily degraded after its purification, and thus produces low molecular weight fragments (data not shown).

Rabbit skeletal muscle MyoHC was cleaved between Ala-1159 and Thr-1160 by myosinase I from spear squid liver. This cleavage site is identical to the site in chicken skeletal muscle MyoHC that can be hydrolyzed by myosinase I from mantle muscle of common Japanese squid (Table I). Such data showed that the characterization of myosinase activities by the specificity of the cleavage site in MyoHC is applicable to all myosinases in Coleoidea. The amino-terminal sequence of the 100-kDa fragment of rabbit skeletal muscle MyoHC hydrolyzed by myosinase III is different from those of products hydrolyzed by myosinase I or II. Alanyl threonine was hydrolyzed by myosinase I, glutamyl threonine by myosinase II, and glutamyl aspartic acid by myosinase III. These sequences appear many times in rabbit skeletal MyoHC, but the myosinases hydrolyze almost one of each sequences. Thus, the myosinases are thought to recognize two or more amino acids or three-dimensional structures of the myosin molecule (*1*). Analysis of the time course of MyoHC hydrolysis for the myosinases indicated a high of site recognition specificity. However, there is no common motif among the sequences of MyoHC around the cleavage sites for myosinase I, II, and III (Table I). Myosinase is therefore predicted to recognize three-dimensional structures of the myosin molecule. Additionally, it was shown by the analysis of the influence on the reducing agents that the three-dimensional structure of the myosinase molecule linked by a disulfide bond has important implications for the substrate recognition or specific activities.

We report here for the first time the amino-terminal sequences of myosinase I and III, which were similar in each other. We looked for proteins that showed similarity to the amino-terminal sequences by using the FASTA homology search algorithm (*13*). The amino-terminal sequence of myosinase I shows 35% sequence similarity with the internal sequence (S_{114}-S_{138}) of an astacin family protein, the hypothetical zinc metalloproteinase C05D11.6 from the genomic sequence of the nematoda *Caenorhabditis elegans* (Table II). By RT-PCR we determined a partial sequence of myosinase I and found that it had a signature sequence of the astacin family in the catalytic domain (data not shown). The astacins are all secreted or plasma membrane-associated proteinases, and most are involved in developmental events (*7, 8*), *e.g.*, hatching enzymes of medaka involved in degrading the egg shell of embryos (*14-16*), and human BMP-1 in bone formation (*17, 18*).

These enzymes cleave a wide variety of peptide bonds in peptides and proteins. However, myosinase I does not hydrolyze casein, which is commonly used as a protein substrate for the astacin family. Neither myosinase I nor II hydrolyzes various synthetic peptides containing the cleavage site for myosinases (*1*). In general, the astacins are neither sequence-specific nor highly specific for residues flanking the scissile bond, but proline residues in positions two and three amino acids away from the cleavage site greatly affect the recognition and hydrolytic activity (*7*). Myosinase also hydrolyzed sites with a variety of flanking amino acids in myosin heavy chain, but no proline residue was found a few amino acids away from the cleavage site. These data indicate that myosinase certainly belongs to the astacin family and is a unique protease in the family. Together with earlier studies on the distribution of myosinase, this study suggested that myosinases are ubiquitously distributed in tissues in Coleoidea, and that they might contribute to pivotal events with their unique proteinase activity.

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