Amino acid sequences of \( \alpha \)-skeletal muscle actin isoforms in two species of rattail fish, *Coryphaenoides acrolepis* and *Coryphaenoides cinereus*

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**SUMMARY:** The cDNA clones of \( \alpha \)-skeletal actins were isolated from the skeletal muscle of two species of rattail fish, *Coryphaenoides acrolepis* and *Coryphaenoides cinereus*. The complete nucleotide sequences of the cDNA and their deduced amino acid sequences were determined. Each of the two species had two \( \alpha \)-skeletal actin cDNA. The nucleotide sequences of the coding region of the two \( \alpha \)-skeletal actin isoform genes within each species had 92.0 and 91.8% homology. From the cDNA sequences of the four \( \alpha \)-skeletal actin isoforms in the two species, amino acid sequences of 377 amino acid residues were deduced. It was predicted that the two N-terminal amino acid residues of each protein are processed after translation. The amino acid sequences of \( \alpha \)-skeletal actin 1 in the two *Coryphaenoides* species were identical, as were the amino acid sequences of \( \alpha \)-skeletal actin 2 in the two species. The amino acid sequences of the two \( \alpha \)-actin isoforms, \( \alpha \)-skeletal actin 1 and \( \alpha \)-skeletal actin 2, differed by only a single amino acid, Ala/Ser at the 155th position. Northern blot analysis showed that a similar amount of each of the two \( \alpha \)-actin isoform mRNA was expressed in the skeletal muscle of the two *Coryphaenoides* species.

**KEY WORDS:** ATP binding site, \( \alpha \)-skeletal actin, cDNA cloning, *Coryphaenoides*, isoform.

**INTRODUCTION**

Actins are highly conserved proteins found in eukaryotic cells. Actins have been conserved through a very wide range of taxonomic groups, and the existence of tissue-specific isoforms of actin makes the actin genes very interesting for studying the evolution of genes and their regulatory elements.\(^1\)\(^-\)\(^4\) Actins play a central role in maintaining the cytoskeletal structure, cell motility, cell division, intracellular movements and contractile processes. On the basis of amino acid sequence, there are at least six different isoforms of actin in mammals: two striated muscle actins (\( \alpha \)-skeletal and \( \alpha \)-cardiac), two smooth muscle \( \alpha \)-actins (\( \alpha \)-aortic and \( \gamma \)-enteric) and two cytoplasmic actins (\( \beta \)- and \( \gamma \)-cytoplasmic).\(^5\)\(^,\)\(^6\) Each isoform of actin is expressed in muscle cells and non-muscle cells in a tissue-specific and time-specific manner.\(^7\)\(^-\)\(^9\) The \( \alpha \)-skeletal muscle actin, one of the four muscle actins in mammals, plays a key role in biological muscle movement. It is the major protein in muscles along with myosin and forms an actin filament. The \( \alpha \)-skeletal actin has been intensively studied and its functions include polymerization in neutral salt and binding to \( \text{Ca}^{2+}, \text{Mg}^{2+}, \) adenine nucleotides, tropomyosin and myosin.\(^10\)\(^,\)\(^11\) These multiple functions of \( \alpha \)-skeletal actin make it one of the structurally best-conserved proteins; the \( \alpha \)-skeletal actin protein in carp and that in rat have 99.4% homology at the amino acid sequence level.\(^12\)\(^,\)\(^13\) The muscles of birds,\(^14\) mammals,\(^15\)\(^-\)\(^17\) some fishes\(^12\) and amphibians\(^18\) contain one isoform of \( \alpha \)-skeletal actin. Recently, Venkatesh et al.\(^19\) reported the presence of two \( \alpha \)-skeletal actins in the muscle of pelagic fish (*pufferfish Fugu rubripes*), which have 98.7% homology at the amino acid sequence level. However, the functional difference of these two actins in the muscle of pufferfish remains to be elucidated.

The objective of the present study was to determine primary structures of \( \alpha \)-skeletal actin from the muscle of two benthic fishes, *Coryphaenoides acrolepis* and *Coryphaenoides cinereus*, to confirm the existence of two \( \alpha \)-skeletal actin isoforms in fish muscle. It was found that two \( \alpha \)-skeletal actin isoforms (i.e. \( \alpha \)-skeletal actin 1 and...
α-skeletal actin 2) exist in the skeletal muscles of both C. acrolepis and C. cinereus. The homology of the two actin isoforms within each species was 92.0% and 91.8% at the nucleotide sequence level, and 99.7% in both species at the amino acid sequence level.

MATERIALS AND METHODS

Coryphaenoides acrolepis and C. cinereus were collected using trawl nets by the RV Wakataka-maru of the Tohoku National Fisheries Research Institute. The sampling locations were 42°–04′00″N, 144°–42′00″E, 1960 m for C. acrolepis; and 38°–02′13″N, 142°–22′05″E, 997 m for C. cinereus. The fish were stored frozen after collection.

Isolation of α-skeletal actin cDNA from Coryphaenoides acrolepis

Total RNA was extracted from the dorsal skeletal muscle of C. acrolepis with RNA extraction solution according to the manufacturer's protocol (Isogen; Nippon Gene, Tokyo, Japan). Poly(A)+ mRNA was isolated from the total RNA using Oligotex-dT30 <Super>(Takara Shuzo, Otus, Japan). Five micrograms of poly(A)+ mRNA was used to synthesize double-stranded cDNA with a cDNA synthesis kit (Pharmacia Biotech, Uppsala, Sweden) with the NOT-1 d(T)₁₈ primer. A C. acrolepis muscle cDNA library was constructed using the synthesized cDNA and a phage vector ZAPII according to the manufacturer's instructions (Stratagene, La Jolla, CA, USA). The α-actin probes were prepared by polymerase chain reaction (PCR). The sequence of the 5’ primer was based on the DNA sequences of α-actins in various animals, and corresponded to positions from 372 to 392 of carp α-actin cDNA. The nucleotide sequence of the PCR primers was 5’-CATGTTTGAGACCTTGAACCT-3’ for α-actin cDNA, which involved the NOT-1 primer – end, was synthesized from 5’-AGCCGCAGACACTCACCTAA-3’, or 5’-primer-5 for α-skeletal actin 2: 5’-CCGAGCTACAACT-GAATA-3’ was used for PCR with the NOT-1 primer as the antisense primer corresponding to the 3’-end. Single-strand cDNA, which involved the NOT-1 primer sequences at the 5’-end, was synthesized from 5 μg of total RNA which had been extracted from the dorsal skeletal muscle of C. cinereus using the T-primed First-strand kit with the NOT-1 primer (Pharmacia Biotech). One-fourth of the resultant cDNA was used as a template for PCR; the PCR reaction mixture (100 μL) also contained 2 mM MgCl₂, 200 mM dNTP, 100 pmol of each primer, and 2.5 units of Taq DNA polymerase. The conditions for PCR amplification were 40 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min, using a DNA Thermal Cycler Model 2400 (Perkin Elmer, Foster City, CA, USA). The reaction mixture (100 μL) contained 2 mM MgCl₂, 200 mM dNTP, 100 pmol of each primer, 2.5 units of Taq DNA polymerase, and 50 ng of the α-actin gene. Therefore, we synthesized two specific primers that corresponded to positions from 1141 to 1160 of C. acrolepis α-actin cDNA (Fig. 1), 5’-primer-2: 5’-AACATTTGTCTCCATCATTT-3’ for α-skeletal actin 1, and 5’-primer-3: 5’-CACCCAGCCTCTGCTCTCAG-3’ for α-skeletal actin 2, according to these 3’-non-coding regions. Under the same conditions described above, PCR amplification was performed with 5’-primer-2 or 5’-primer-3 and NOT-1 primer using the C. acrolepis cDNA library. These amplified DNA fragments involved 3’-non-coding regions which considerably differ in nucleotide sequences between two isoforms. These DNA fragments were labeled with a DIG DNA labeling kit according to the manufacturer’s instructions (Boehringer Mannheim, Germany) and were used as the probes for screening the cDNA library and northern blot analysis. The DNA sequencing was performed with Dye Deoxy terminator cycle sequencing kit using a Model 373 A DNA Sequencer (PE Applied Biosystems, Foster City, CA, USA).

Isolation of α-skeletal actin cDNA from Coryphaenoides cinereus

To isolate the α-skeletal actin cDNA from C. cinereus, reverse transcription (RT)–PCR was performed with sense primers that were synthesized according to nucleotide sequences corresponding to positions from –30 to –11 of the two α-actin cDNA sequences of C. acrolepis. The 5’-primer-4 for α-skeletal actin 1: 5’-AGCCGCAGACACTCACCTAA-3’, or 5’-primer-5 for α-skeletal actin 2: 5’-CCGAGCTACAACT-GAATA-3’ was used for PCR with the NOT-1 primer as the antisense primer corresponding to the 3’-end. Single-strand cDNA, which involved the NOT-1 primer sequences at the 5’-end, was synthesized from 5 μg of total RNA which had been extracted from the dorsal skeletal muscle of C. cinereus using the T-primed First-strand kit with the NOT-1 d(T)₁₈ primer (Pharmacia Biotech). One-fourth of the resultant cDNA was used as a template for PCR; the PCR reaction mixture (100 μL) also contained 2 mM MgCl₂, 200 mM dNTP, 100 pmol of each primer, and 2.5 units of Taq DNA polymerase. The conditions for PCR were 40 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min, using a DNA Thermal Cycler Model 2400 (Perkin Elmer). The PCR product was subcloned in a T-vector (Novagen). The reaction mixture (100 μL) contained 2 mM MgCl₂, 200 mM dNTP, 100 pmol of each primer, and 2.5 units of Taq DNA polymerase. The conditions for PCR were 40 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min, using a DNA Thermal Cycler Model 2400 (Perkin Elmer). The PCR product was subcloned in a T-vector (Novagen).

Northern blot analysis

Total RNA was extracted from the dorsal skeletal muscle of C. acrolepis, and northern blot analysis was performed using the total RNA (15 μg per lane). The RNA was size-fractionated on a 1.2% agarose gel containing 50% formamide by electrophoresis, and then transferred to a nylon membrane. The probes that had been used for cDNA library screening were used as the hybridization probes. Hybridization and detection were performed according to the manufacturer’s instructions (Boehringer Mannheim).
Fig. 1  Comparison of the nucleotide sequences encoding α-skeletal actin 1 and α-skeletal actin 2 in Coryphaenoides acrolepis. A dot indicates a nucleotide residue that is identical with that in the sequence of C. acrolepis α-skeletal actin 1. The coding region is boxed. The polyadenylation signals are indicated by bold letters. The nucleotide sequences of C. acrolepis α-skeletal actin 1 and C. acrolepis α-skeletal actin 2 appear in the DDBJ/EMBL/GenBank nucleotide database with the accession numbers AB021649 and AB021650, respectively.
RESULTS

Isolation of cDNA clones encoding \(\alpha\)-skeletal actin isoforms

Ten positive clones of \(\alpha\)-skeletal actin 1 cDNA and 15 clones of \(\alpha\)-skeletal actin 2 cDNA were isolated from the 10\(^3\) phase clones of the \(C.\) acrolepis \(\lambda\)ZAPII cDNA library. The nucleotide sequence of the longest insert including the entire coding region of each isoform was determined. The cDNA sequence of \(\alpha\)-skeletal actin1 consisted of 1436 bp and a poly(A) tail of 18 bp, and that of \(\alpha\)-skeletal actin 2 consisted of 1611 bp and a poly(A) tail of 22 bp (Fig. 1). Comparison of the nucleotide sequence of the coding region of \(C.\) acrolepis \(\alpha\)-skeletal actin 1 with that of \(C.\) acrolepis \(\alpha\)-skeletal actin 2, revealed 91 nucleotide substitutions. There was 92.0% identity between the two isoforms (Table 1).

The RT-PCR strategy for cloning the entire cDNA sequence of \(C.\) cinereus \(\alpha\)-skeletal actin 1 and \(C.\) cinereus \(\alpha\)-skeletal actin 2 successfully yielded cDNA fragments of 1420 bp and 1585 bp, respectively. Comparison of the DNA sequences with those of the \(C.\) acrolepis actin cDNA revealed that the 1420 bp fragment encoded \(\alpha\)-skeletal actin 1 and the 1585 bp fragment encoded \(\alpha\)-skeletal actin 2. These nucleotide sequence data appeared in the DDBJ/EMBL/GenBank nucleotide database with the accession numbers AB021651 and AB021652, respectively. In \(C.\) cinereus, comparison of the nucleotide sequence of the coding region of \(\alpha\)-skeletal actin 1 with that of \(\alpha\)-skeletal actin 2, revealed 93 nucleotide substitutions, resulting in 91.8% homology (Table 1). Comparison of the nucleotide sequence of the coding region of \(\alpha\)-skeletal actin 1 in \(C.\) acrolepis and that of \(\alpha\)-skeletal actin 1 in \(C.\) cinereus revealed four nucleotide substitutions and 99.6% identity, while comparison of the nucleotide sequence of \(\alpha\)-skeletal actin 2 in \(C.\) acrolepis and that of \(\alpha\)-skeletal actin 2 in \(C.\) cinereus revealed six nucleotide substitutions and 99.5% identity (Table 1).

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### Table 1  Comparison of the nucleotide sequence and deduced amino acid sequence of \(\alpha\)-skeletal actin in various fish species

<table>
<thead>
<tr>
<th>(\alpha)-skeletal actin form</th>
<th>(C.) acrolepis</th>
<th>(C.) cinereus</th>
<th>Pufferfish</th>
<th>Carp</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\alpha)-skeletal actin 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actin 2</td>
<td>92.0/99.7</td>
<td>99.6/100*</td>
<td>91.8/99.7</td>
<td>94.2/99.7</td>
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<tr>
<td>Actin 2</td>
<td>92.0/99.7</td>
<td>99.5/100</td>
<td>90.6/99.5</td>
<td>89.9/98.7</td>
</tr>
<tr>
<td>Actin 1</td>
<td>91.8/99.7</td>
<td>93.0/99.5</td>
<td>89.6/98.7</td>
<td>90.7/99.2</td>
</tr>
</tbody>
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* The left and right numerals represent the percent homology of the nucleotide sequence in the coding region and amino acid sequence, respectively, of the indicated pair of proteins.

1 The accession numbers are AB021651 and AB021652 in DDBJ/EMBL/GenBank nucleotide database.
2 Data from Venkatesh et al.
3 Data from Watabe et al.
Fig. 2 Comparison of the deduced amino acid sequences of α-skeletal actin 1 and α-skeletal actin 2 of Coryphaenoides, α-skeletal actin 1 and α-skeletal actin 2 of pufferfish,\(^1\) and α-skeletal actin of carp.\(^2\) A dot indicates an identical amino acid residue with that in the sequence of Coryphaenoides α-skeletal actin 1. The 155th residues are indicated by bold letters.
forms found in the present study are expressed in completely differentiated muscle of Coryphaenoides. Therefore, the two actin isoforms found in the muscle of C. acrolepis and C. cinereus are α-skeletal actins. The muscles of various vertebrates contain a single copy of α-skeletal actin, except for the muscle of pufferfish which contains two actin isoforms. Although Xenopus laevis expresses three different α-actins, a single smooth muscle actin and two striated muscle acts (α-skeletal and α-cardiac), X. tropicalis, a more primitive species, expresses a single sarcomeric actin that is present in both the skeletal muscle and the heart. The two identical sarcomeric actin genes in X. tropicalis are considered to have arisen from gene duplication during amphibian evolution. On the other hand, teleosts such as pufferfish and the two Coryphaenoides species examined in this study have two isoforms of α-skeletal actin, which suggests that the actin genes had undergone rapid diversification during the emergence of vertebrates and that the muscle of many species of fish contains at least two α-skeletal actin isoforms. The present study showed that the fish species C. acrolepis and C. cinereus each have two α-skeletal actin isoforms, and that the two isoforms in each species differ by a single amino acid substitution at the 155th residue: Ala-155 in α-skeletal actin 1 and Ser-155 in α-skeletal actin 2. Venkatesh et al. also reported the presence of two α-skeletal actins in the muscle of pufferfish, which showed 98.7% homology at the amino acid sequence level. Comparison of the amino acid sequence of α-skeletal actin 1 and α-skeletal actin 2 in pufferfish revealed the same pattern of substitution at amino acid position 155 (i.e. Ala-155 and Ser-155), as well as the substitutions of Thr/Ser-89, Val/Ile-165, Ala/Gly-228, and Ala/Thr-278. Ala-155 is present in the amino acid sequence of the α-skeletal actin of carp and goldfish. No other α-skeletal actins of fish have been reported thus far. These findings suggest that α-skeletal actin 1 with Ala-155 is the common isoform of α-skeletal actin in various fish species. In contrast, the muscles of rabbit, chicken, mouse, human and frog contain only one α-skeletal actin protein with Ser-155.

The actin protein of various species contains two domains (historically called large and small domains, although they are nearly the same size). The small domain contains subdomains 1 [amino acid (a.a.) residues 1–32, 70–144, and 338–372] and 2 (a.a. residues 33–69), while the large domain contains subdomains 3 (a.a. residues 145–180, and 270–337) and 4 (a.a. residues 181–269). The amino acid at the 155th residue with the Ala/Ser substitution in the two isoforms of α-skeletal actin found in the present study is located in subdomain 3 and is in the vicinity of the ATP binding site at the base of the cleft in the middle of the actin sequence. The ATP forms a bridge between the small and large domains, and thereby prevents the unfolding of the protein and stabilizes the G-actin structure. The ATP is closely associated with a divalent cation in the actin

Fig. 3 Northern blot analysis of Coryphaenoides acrolepis α-skeletal actin isoform mRNA. Total RNA (15 μg/lane) that had been extracted from the dorsal skeletal muscle of C. acrolepis, was electrophoresed on a 1.2% agarose gel. The ethidium bromide-stained gel shows 28S and 18S rRNA (lower panel), and the separated RNA bands were blotted onto a nylon membrane and hybridized with the specific probe for α-skeletal actin 1 and α-skeletal actin 2 (upper panel).
protein (Mg\(^{2+}\) in native actin, Ca\(^{2+}\) in actin-DNase I crystals), and regulates the polymerization (G-F transformation) of actin following hydrolysis by actin.\(^{11,25,26}\) The divalent cation is enclosed by the charged amino acid residues Asp-11, Lys-18, Gln-137, and Asp-154, and by the \(\beta\)- and \(\gamma\)-phosphates of ATP that are involved in hydrogen bonding with the amides of residues Ser-14, Gly-15, Leu-16, Asp-157, Gly-158 and Val-159. The hydroxyl group of Ser-14 is one of six groups involved in stabilizing the position of the phosphate of ATP, and near to the 155th residue in configuration.\(^{24}\) A Ser/Ala substitution at the 14th residue results in a 40- to 60-fold decrease in the affinity of actin for ATP.\(^{25}\) The 155th-residue Ser/Ala substitution found in the present study may also influence the affinity of actin for ATP and/or the divalent cation. Sweezy and Somero\(^{28}\) reported that there are wide, interspecific structural and functional differences among skeletal actins that appear to be adaptive to its inhabitant environments, and that the heat stability of actins increases with higher average body temperature. Torigai and Konno\(^{30}\) reported that the heat denaturation of the \(\alpha\)-actin protein of carp, rainbow trout and walleye pollack is affected by the concentration of ATP, and that their ATP-bound forms are as stable as rabbit \(\alpha\)-actin. In Saccharomyces cerevisiae, the 14th-residue Ser/Ala substitution leads to the temperature-sensitive phenotype in vitro.\(^{10}\) These findings suggest that the affinity of actin to ATP influences the heat stability of actin and that the \(\alpha\)-skeletal actin protein with Ala-155, which is thought to be the common isoform in fish, has weaker affinity for ATP than the \(\alpha\)-skeletal actin with Ser-155 of birds, mammals and amphibians. The substitution, Ala/Ser-155, would result in strengthening the interaction between actin and ATP.

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