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Characterization of calpastatin gene in fish: Its potential role in muscle growth and fillet quality

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Abstract

Calpastatin (CAST), the specific inhibitor of the calpain proteases, plays a role in muscle growth and meat quality. In rainbow trout (RBT), we identified cDNAs coding for two CAST isoforms, a long (CAST-L) and a short isoform (CAST-S), apparently derived from two different genes. Zebrafish and pufferfish CAST cDNA and genomic sequences were retrieved from GenBank and their exon/intron structures were characterized. Fish CASTs are novel in that they have fewer repetitive inhibitory domains as compared to their mammalian counterparts (one or two vs. four). The expressions of CAST mRNAs were measured in three RBT strains with different growth rates and fillet firmness that were fed either high energy or control diets. CAST-L and S expressions were significantly lower ($p<0.01$) in the strain that has the slowest growth rate and yielded the softest fillet. Strain or diet did not affect level of calpain mRNAs. However, the decrease in the CAST/calpain ratio at the mRNA level did not lead to a corresponding change in the calpain catalytic activity. Further investigation should reveal a potential use of the CAST gene as a tool to monitor fish muscle growth and fillet firmness.

Keywords: Calpastatin; Gene expression; Muscle growth; Fillet quality; Rainbow trout

1. Introduction

Calpastatin (CAST) is a specific, endogenous inhibitor of the calcium-dependent neutral proteases, calpains. In mammals, calpains play an important role in myofibrillar protein degradation that leads to muscle protein turnover during animal growth (Goll et al., 1992; Huang and Forsberg, 1998). Calpains are involved in the disassembly of myofibrils during early stages of turnover (Goll et al., 1992) by mediating the degradation of various myofibrillar proteins (Goll et al., 1989; Koohmaraie, 1992a). Degradation of these proteins results in loss of the Z disk (Busch et al., 1972). Proteolysis by these proteases in the early postmortem period impacts muscle texture with an inverse relationship between CAST levels and meat tenderization (Koohmaraie, 1992b,c; Geesink and Koohmaraie, 1999; Duckett et al., 2000). The role of CAST in muscle growth and meat quality is well-studied in livestock. CAST activity is associated with muscle hypertrophy in sheep carrying the callipyge gene (Duckett et al., 2000); β-adrenargic agonist administration decreased protein degradation through increased expression of the CAST gene (Pringle et al., 1993). CAST was used to slow muscle wastage in experimental animals (Tidball and Spencer, 2002). Recently, studies in beef animals have been completed using CAST-based, DNA marker-test (GeneSTAR® Tenderness, Genetic solutions, Lafayette, CO, USA). This technology will allow cattle breeders to make significant genetic progress in tenderness selection and thus eating quality of beef.

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Our knowledge of the calpain/CAST system in fish is limited. Fish calpains are able to digest the myosin heavy chain (Muramoto et al., 1989). Geesink et al. (2000) showed that incubation of salmon myofibrils with salmon m-calpain produced titin-like degradation products. Previously, we found that softening of postmortem trout muscle could be accelerated by activation of calpains with exogenous calcium (Salem et al., 2004a). Furthermore, we found a net increase in the calpain/CAST mRNA ratio with a corresponding increase in calpain catalytic activity under conditions of muscle breakdown induced by starvation (Salem et al., 2005). These studies implicate calpains/CAST as an important proteases system controlling fish muscle protein turnover during fish growth and textural changes postmortem. This study was undertaken to identify and characterize the CAST gene in fish and to examine the relationship between CAST expression/activity and growth performance and fillet firmness (shear forces) in rainbow trout.

2. Materials and methods

2.1. CAST cDNAs and genomic sequences

Nucleotide and amino acid BLAST searches (http://www.ncbi.nlm.nih.gov/) were used to examine rainbow trout (*Oncorhynchus mykiss*) expressed sequence tags to identify CAST-like clones. Two CAST-like cDNA clones (GenBank accession Nos.: CA367610 and CA359304) with homology to mammalian CASTs were identified. Both clones were completely sequenced and aligned to generate the full-length sequences for RBT CAST-L and CAST-S V1, respectively. CAST-S V2 was retrieved by aligning CAST-S V1 against a cDNA sequence with GenBank accession No.: BX880124. CLUSTAL W version 1.81 algorithm (www.cmbi.kun.nl/cgi-bin) was used for protein alignments. The characteristic domains of fish CASTs were assigned by blasting their sequences against the Protein Families Database of Alignment (Pfam, http://www.sanger.ac.uk/Software/Pfam/search.shtml) and according to a previous report by Goll et al. (2003). An EST cluster (TC291402) from zebrafish (*Danio rerio*) was retrieved from the TIGR Gene Indices (http://tigrblast.tigr.org/tgi/) using RBT CASTs. The exon/intron structure of the zebrafish CAST was identified by aligning the retrieved mRNA with the zebrafish genomic sequence BX927307 using the NCBI Spidey program (http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/index.html). Similarly, the pufferfish (*Takifugu rubripes*) exon/intron structure was obtained by aligning the retrieved mRNA sequence (CAF89862.1) with the genomic sequence CAAE01007190.1.

2.2. Northern blot

Total RNA was isolated from trout heart, muscle, stomach, gill, brain, kidney, and immature and mature ovary using the Tri-reagent (Sigma-Aldrich, St. Louis, MO, USA) according to Salem et al. (2005). mRNA was isolated from 250 μg of total RNA using the PolyATract mRNA isolation system III as described by the manufacturer (Promega, Madison, WI, USA). mRNA was separated on a 1% agarose gel using a formaldehyde RNA gel protocol (Ausubel et al., 1994). Subsequently, mRNA was transferred to a nylon membrane (Amersham Pharmacia Biotech) using a PosiBlot 30–30 pressure blotter and pressure control station (Stratagene); transferred RNA was UV-crosslinked to the membrane. Trout CASTs were digoxigenin (DIG)-labeled using primers designed based on conserved regions of the CAST-L and S sequences. Forward primer 5′-AGTCACGGAGGGCAGGACAT-3′ and reverse primer 5′-CCAGGGCATCCAAGGCAAAGTCA-3′ were used to PCR amplify the trout CAST probe from the CAST-S specific plasmid. The probe was 270 bp in length and has enough nucleotide identity (89%) to cross-react the CAST-L transcripts. The CAST probe was used according to the manufacturer's protocol (Roche, Indianapolis, IN, USA). The membrane was washed using high stringency conditions (5 min with 2× SSC at room temperature then 10 min with 2× SSC at 68 °C followed by 10 min at 0.2× SSC at 68 °C) before being photographed using the FluorChem system. The membrane was also hybridized to a DIG-labeled RBT GAPDH probe generated by PCR using gene specific primers (5′-TGGGGAGATGCTGGTGCC-3′ and 5′-CGGCGTGAACGGTGCTC-3′) for RNA loading control.

2.3. Fish and sample collection

Three strains of RBT, *O. mykiss* were used. According to Silverstein and Rexroad (2003), strain A was developed in the foothills of Southern Idaho, strain B originated from alpine lakes in Washington State and strain C was developed from crosses between Puget Sound Steelhead and Canadian Kamloops. RBT fish, 20 to 23 cm in length, were obtained from the National Center for Cool and Cold Water Aquaculture (Kearneysville, WV, USA). RBT were stocked into a three-level doubled raceway system, six units total, 9 × 0.9 × 0.9 m each. Approximately 1000 RBT fish were

Table 1
Primers used for real-time RT–PCR analysis of calpain/CASTs genes

CAST-S For	5-ATGACAGAGCAGCTGTCCAATC-3
CAST-S Rev	5-5-TFTTGAAGCAACATCACTGCAA-3
CAST-L	5-ACGGCACCTTTCCTTTCCATTACCA-3
CAST-L Rev	5-CGGGGGGGAGCAGGAGACTTGGT-3
B-Actin For	5-GCCGGCCGCGACCTCACAGACTAC-3
B-Actin Rev	5-CGGCCGTGGTGGTGAAGCTGTAAC-3
Capn1 For	5-GCCAAAACATTGCCTGTTATCTTAG-3
Capn1 Rev	5-ATAGGAGGCCGTATCAAAATTCC-3
Capn2 For	5-GATTCATCCAGAACGTGTAGG-3
Capn2 Rev	5-GGTTAAACACTGGAGCGTGTC-3
cpns For	5-GCTGCCTTCAAATCTGCATGT-3
cpns Rev	5-TGTACCTGCGAGCGATCAACT-3