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Post-mortem Changes in Gene Expression of the Muscle Tissue of Rainbow Trout, *Oncorhynchus mykiss*

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A subtracted cDNA library was used to identify specific genes that increase in post-mortem muscle of rainbow trout (*Oncorhynchus mykiss*) during on-ice storage. Of the 200 cDNAs analyzed, 82 had significant homologies to previously identified genes from salmonids and other species such as homologues of troponin I, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and so on, whereas 40 had no significant homologies and were designated as unknown. Comparison of gene expression profiles by dot blot hybridization confirmed an increase or induction of mRNA in the muscle after 3 h of on-ice storage compared to that at 0 h after death. Real-time reverse transcriptase−Polymerase Chain Reaction analysis showed that troponin I and GAPDH mRNAs were increased by 24 h and, in particular, that the change in troponin I mRNA was greater than that of GAPDH mRNA. These results suggest that the increased mRNAs in rainbow trout muscle occurred by transcription immediately after death.

KEYWORDS: Post-mortem; rainbow trout; muscle tissue; subtracted cDNA library; GAPDH cDNA; troponin I cDNA

INTRODUCTION

Research on the post-mortem changes in fish tissues is important in the field of food science. Studies have been conducted on changes of various components and physical properties occurring during the storage and processing of fish (*1*−*3*). Recently, with the enhanced quality of people's diets in Japan, people prefer eating raw fish such as sashimi and sushi more than ever; thus, studies for developing techniques to maintain the freshness and to control the texture of raw fish meat, which are important factors in the taste, have been actively and extensively pursued (*4*−*8*).

After capture, fish generally die due to neurologic dysfunction and respiratory distress. Upon death, most cells retain their life-sustaining activities, and as time passes, cells are gradually converted into dead cells. Cells deprived of a supply of oxygen and external energy supply become the site of various reactions, such as a decline of pH, an accumulation of lactic acid, a depletion of ATP, a reduction of phosphocreatine, an increase of intracellular Ca^{2+} concentration, changes in development of intra- and extracellular ion imbalance, an abnormal decomposition of biocomponents without regulation, and damage to cellular membranes (*9*−*11*). These processes result in the death of all cells. On the other hand, muscle tissues gradually shrink, harden, and remain in this state, called rigor mortis, for a while. Then, the hardened tissues become soft in a process called softening (*2*, *3*).

Previous studies on those phenomena investigated using chemical, physical, and histological analytical techniques by focusing on indicators, such as pH, lactic acid, and ATP levels and the level of rigidity, from the food science point of view (*1*); thus, few studies include genetic investigation. It is possible that the gene expression in the tissue is retained until cell functions stop, which is similar to apoptosis or necrosis. Genetic analysis of post-mortem changes of fish muscle tissues not only has biological significance but is also expected to provide new observations for practical application, such as in understanding post-mortem changes in fish from the food science point of view and in the development of techniques to maintain freshness.

The present study aimed to elucidate a novel phenomenon of post-mortem changes in tissues by tracing changes in gene expression of rainbow trout, *Oncorhynchus mykiss*, which is generally easy to obtain and has a large amount of genetic information available. Expression of mRNA and specific cDNA in the muscle tissues after death was analyzed by a differential screening method using a subtraction technique.

MATERIALS AND METHODS

Fish Sample. Several cultured fishes of rainbow trout, *Oncorhynchus mykiss*, (25−33 cm in length and 250−380 g in weight) were commercially bought at the Tokyo central wholesale market in April

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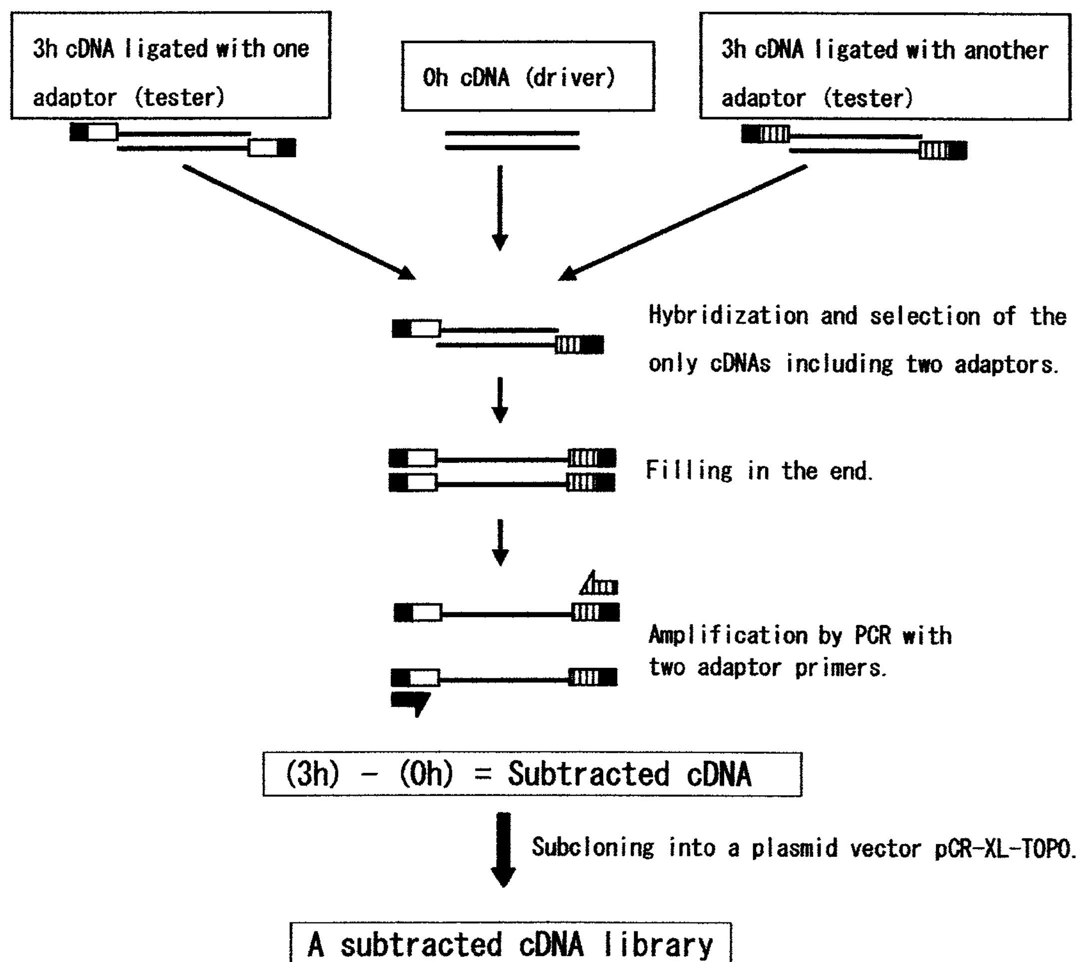

Figure 1. Preparation of a subtracted cDNA library from rainbow trout muscle during on-ice storage. Tester and driver cDNAs were prepared from 3 and 0 h mRNAs, respectively. Two tester cDNA populations (3 h) were ligated to different adaptors after *Rsa* I digestion. The cDNA templates for PCR amplification were generated from differentially expressed sequences.

and were carried live to our laboratory. After fishes were immediately killed by hitting the head with a hammer, the dorsal muscle was separated and stored at 0 °C. At 0, 3, 24, 48, 72, 120, and 168 h during on-ice storage, 0.1 g of the muscle was dissected for RNA isolation.

Selection of Differentially Expressed Genes. mRNA from fish muscle was isolated with a QuickPrep Micro mRNA purification kit (Amersham Biosciences Corp., Piscataway, NJ). As shown in **Figure 1**, the muscles from 0 and 3 h were used to prepare control (driver) and experimental (tester) cDNAs, respectively. Two micrograms of each mRNA extract was subjected to subtractive hybridization and selective PCR amplification of differentially expressed sequences using a PCR-Select cDNA Subtraction Kit (Clontech Laboratories, Inc., Mountain View, CA) according to the manufacturer's instructions. PCR products from the subtracted libraries were screened after insertion into a plasmid vector pCR-XL-TOPO by using a TOPO XL PCR Cloning Kit (Invitrogen Corp., Carlsbad, CA). Two hundred randomly selected clones were sequenced with an ABI model 373A sequencer using a Taq Dye Deoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, CA). DNA sequences were compared to those in the GenBank DNA and protein databases by using the Blast-N and Blast-X at the DNA analysis web site maintained by the National Center for Biotechnology Information.

Differential Expression Screening by Dot Blot Hybridization. The obtained plasmid DNAs (0.1 μg) were spotted on nylon filters, Hybond N^+, (Amersham Biosciences Corp.) with an Immunodot AE-6190 (ATTO Corp., Tokyo, Japan) and then were denatured, neutralized, and affixed to the filters by baking at 80 °C for 2 h. Probes for the dot blot hybridization were derived from the subtractive and unsubtractive PCR products as described in the PCR-Select cDNA Subtraction Kit (Clontech Laboratories, Inc.). The dot blot hybridization and detection were carried out with an ECL direct nucleic acid labeling and detection system (Amersham Biosciences Corp.). The signal intensity on the dot blot hybridization analysis was measured by a Luminescent image analyzer system LAS-1000plus (Fujifilm, Tokyo, Japan), which can capture chemiluminescent images.

Real-Time Reverse Transcriptase−Polymerase Chain Reaction (Real-Time RT-PCR). Real-time RT-PCR was performed with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) using SYBER Green I. A 1.5 μg amount of total RNA was reverse transcribed using an Omniscript Reverse Transcriptase (QIAGEN GmbH, Hilden, Germany), and PCR amplification was performed in a 25 μL reaction containing 0.3 μL of cDNA sample, 0.3 μM of each primer, and 2× QuantiTect SYBR Green PCR Master Mix (QIAGEN). The thermal cycling profile consisted of three stages: 94 °C for 30 s, 45 °C for 30 s, and 72 °C for 1 min, which were repeated for 40 cycles. All standards and samples were assayed in triplicate. Thermal cycling was initiated with an initial activation of DNA polymerase at 95 °C for 15 min. After this initial step, 40 cycles of PCR (94 °C for 30 s, 45 °C for 30 s, and 72 °C for 1 min) were performed. A standard curve was generated by amplifying serial dilutions of a known quantity of each PCR product. The standards and cDNA samples were amplified in the same reaction plate. The amount of product in a particular sample was determined by interpolation from a standard curve of cycle threshold (C_T) values generated from the PCR product dilution series (1:1, 1:10, 1:100, 1:1000, and 1:10000). The cDNA (169 bp) encoding rainbow trout glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was PCR-amplified by using two primers, the 5′-primer (5′-CAACAGGTGTTTTCAC-CACC-3′) and the 3′-primer (5′-GTTGTGCATGACGCATTGCT-3′) (*12*). Troponin I cDNA (413 bp) was PCR-amplified by using two primers, 5′-TGATGCTCCAGATTGCAGCCA-3′ and 5′-GTTCT-TACGCCAGTCACCAAC-3′. All measurements were performed in triplicate.

Table 1. cDNA Clones Identified in the Subtracted cDNA Library Prepared from Rainbow Trout Muscle

identification	species	accession no.	length of the longest cDNA clone (bp) and no. of similar clones	*E* value identity *x*/*y* nucleotides or amino acids (%)
troponin I	*Salmo salar*	gb|U83878	114M13R (505), $n = 25$	0, 406/428 (94%)
glyceraldehyde-3-phosphate dehydrogenase	*Oncorhynchus mykiss*	dbj|AB066373	116M13R (456), $n = 13$	0, 414/429 (96%)
phosphorylase B kinase α regulatory chain	*Gallus gallus*	ref|XM_420177	113T7 (529), $n = 7$	0.000005, 45/50 (90%)
myosin regulatory light chain 2	*Salmo salar*	emb|AJ557151	71T7 (416), $n = 5$	e-132, 270/277 (97%)
myosin light chain 3	*Oncorhynchus mykiss*	gb|AF330141	90M13 (396), $n = 4$	e-168, 381/394 (96%)
40S ribosomal protein	*Oncorhynchus mykiss*	emb|AJ312336	130M13R (391), $n = 4$	e-134, 262/265 (98%)
poly(A) binding protein	*Danio rerio*	ref|NM_201296	117T7 (492), $n = 3$	7e-70, 284/333 (85%)
18S rRNA	*Salmo salar*	emb|AJ427629	59T7 (429), $n = 2$	0, 404/409 (98%)
actin	*Oncorhynchus keta*	dbj|AB032464	125T7 (433), $n = 2$	6e-67, 161/168 (95%)
cytochrome *c* oxidase subunit I	*Oncorhynchus mykiss*	ref|NP_008292	137T7 (362), $n = 2$	1e-42, AA89/94 (94%)
cytochrome *c* oxidase subunit III	*Oncorhynchus masou*	dbj|D63410	123T7 (495), $n = 2$	e-153, 309/320 (96%)
ribosomal protein L19	*Mus musculus*	ref|NP_033104	141T7 (419), $n = 2$	1e-40, AA81/84 (96%)
ribosomal protein S3A	*Danio rerio*	gb|AC016642	516T7 (348), $n = 2$	e-104, 306/339 (90%)
28S rRNA	*Oncorhynchus mykiss*	gb|U34341	121T7 (554), $n = 1$	e-175, 316/317 (99%)
adenylate kinase	*Gallus gallus*	dbj|D00251	128M13R (372), $n = 1$	2e-08, 64/74 (86%)
cytochrome *b*	*Oncorhynchus mykiss*	ref|NP_008302	53T7 (378), $n = 1$	9e-66, AA121/124 (97%)
cytochrome *c* oxidase subunit II	*Oncorhynchus mykiss*	gb|L29771	30M13 (280), $n = 1$	e-153, 279/280 (99%)
cytochrome *c* oxidase subunit Vb precursor	*Oncorhynchus mykiss*	gb|AF255351	601M (381), $n = 1$	e-178, 328/330 (99%)
fatty acid binding protein H-FABP	*Oncorhynchus mykiss*	gb|U95296	617M (409), $n = 1$	0, 400/406 (98%)
myomesin	*Gallus gallus*	ref|NM_204959	151T7 (402), $n = 1$	0.0003, 39/43 (90%)
ribosomal protein L37a	*Ictalurus punctatus*	gb|AF401594	520T7 (295), $n = 1$	2e-44,154/174 (88%)
troponin C	*Danio rerio*	ref|NM_131563	135M13R (383), $n = 1$	5e-64, 196/219 (89%)
unknown			(48–447), $n = 40$	

RESULTS

A total of 82 cDNA clones, accounting for 41% of all samples, had >85% identity with sequences in the database and some showed identity in regions longer than 100 bases in nucleic acid sequences. Thus, as indicated in **Table 1**, they were identified as coding for a particular protein molecule. On the other hand, 20% of the clones were classified into an unknown group. Among the putative substances, 12.5% were muscle troponin and 6.5% were GAPDH.

The possibility that nonspecifically expressed genes were included among the group of clones was examined by confirming whether the obtained clones were specifically transcribed after death. Dot blot hybridization was conducted, and the intensity of black-stained dots was compared to estimate the differences in mRNA levels, as shown in **Figure 2**. The membrane filter stained with the cDNA probe from the tissue sample kept on ice for 3 h was more intense (darker) overall than that using the cDNA probe from the tissue sample of just-killed trout; thus, the specificity of mRNA expression was indicated. Furthermore, the intensity of dots was determined optically, and the values were compared. Significant differences were observed in some genes, including 28S rRNA, cytochrome *b*, fatty acid binding protein H-FABP, myosin regulatory light chain 2, and troponin I (**Table 2**). Therefore, it was suggested that such indigenous proteins in the muscle tissues are specifically expressed after death.

However, because the results above were obtained only from the tissues stored on ice for 3 h, and no quantitative determination of mRNA was conducted, further information was required to assess specific synthesis and changes. Thus, real-time PCR analysis was used to track chronological changes in the absolute level of mRNA for 1 μg of total RNA in troponin I and GAPDH, which were more frequently detected as listed in **Table 1**, in tissues collected from the fish immediately after being killed and after 168 h on ice (**Figure 3**). As the whole nucleic acid sequence of rainbow trout troponin I was not available, RT-PCR was performed after cloning the full-length cDNA and determining the nucleic acid sequence as indicated in **Figure 4**. Although the data are not shown in this paper,

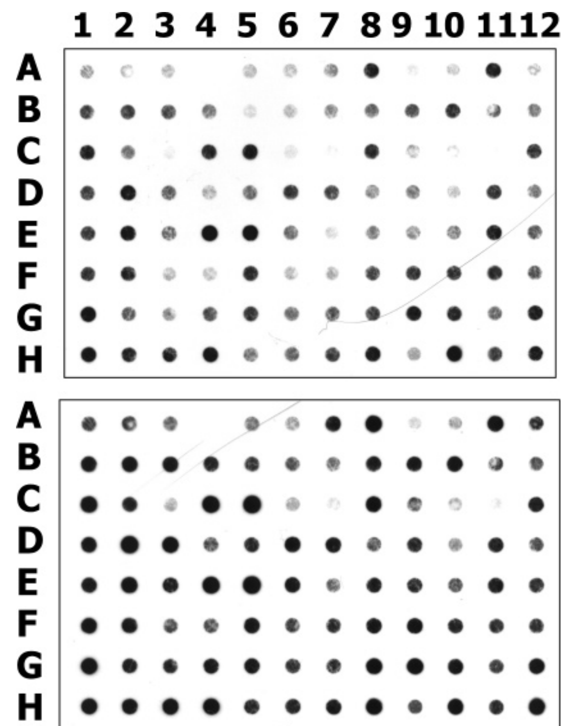

Figure 2. Array of 96 putatively on-ice storage-expressed clones, shown after hybridization to probes derived from rainbow trout muscle mRNAs during on-ice storage for 0 h (top) and 3 h (bottom). Dark signals in the filter membranes indicate cloned loci that were expressed at high levels in rainbow trout muscles during on-ice storage for 3 h: A1, cytochrome *c* oxidase subunit Vb precursor; A3, fatty acid binding protein H-FABP; A6, myosin regulatory light chain 2; A8, myosin light chain 3; A9, ribosomal protein S3A; A11, glyceraldehyde-3-phosphate dehydrogenase; B1, troponin I; B12, poly(A) binding protein; C1, 18S rRNA; C3, phosphorylase B kinase α regulatory chain; C6, 40S ribosomal protein; C11, cytochrome *b*; E9, 28S rRNA; F1, actin; F3, adenylate kinase; F9, troponin C; G2, ribosomal protein L19; G10, myomesin.

quantitative determination was conducted by synthesizing cDNA of known concentration and creating a standard curve for the

Table 2. Genes with Differential Expression between 0 and 3 h of Rainbow Trout Muscle

protein	ratio[a]	*n*
28S rRNA	3.6	2
cytochrome *b*	3.3	2
fatty acid binding protein H-FABP	2.7	2
myosin regulatory light chain 2	2.6	4
troponin I	2.4	13
40S ribosomal protein	2.3	4
troponin C	2.2	2
poly(A) binding protein	2.2	3
adenylate kinase	2.1	2
myosin light chain 3	2.1	3
18S rRNA	1.9	2
ribosomal protein L19	1.9	2
glyceraldehyde-3-phosphate dehydrogenase	1.8	11
phosphorylase B kinase α regulatory chain	1.8	4
actin	1.8	2
ribosomal protein S3A	1.7	2
cytochrome *c* oxidase subunit Vb precursor	1.4	6
myomesin	1.4	2

[a] Normalized ratio is calculated with the mean value of signal intensity on the dot blot hybridization analysis.

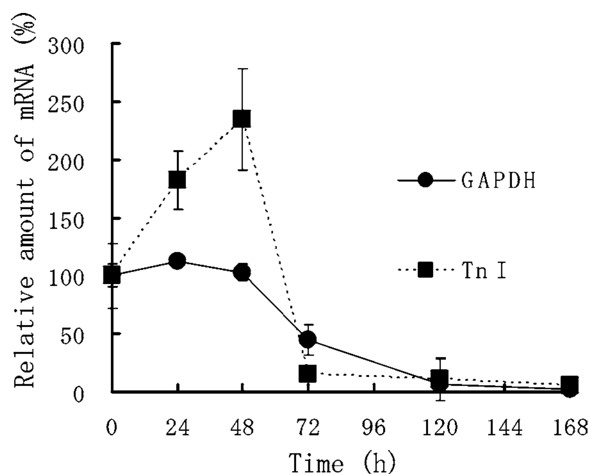

Figure 3. Relative amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and troponin I (TnI) mRNAs from rainbow trout during on-ice storage. RNA extracts from rainbow trout muscles were subjected to real-time RT-PCR. The results present the mean of at least three fishes per point.

quantitative determination of mRNA. **Figure 3** shows that the level of troponin I mRNA increases as time passes, and after 48 h, it reaches a level of more than twice that observed immediately after the fish had been killed. An insignificant increase (12%) of GAPDH mRNA was found for 24 h, and thereafter the level immediately after slaughter was mostly maintained for 48 h. The levels of troponin mRNA and GAPDH mRNA rapidly decreased after 72 h. These results indicated that both mRNAs may be still synthesized in the muscle tissues kept on ice for at least 24 h, or up to 48 h, suggesting that the changes in mRNA levels are somehow involved in the phenomena of rigor mortis and muscle softening.

DISCUSSION

In the analysis for specific expression in the fish tissues kept on ice for 3 h, an especially large number of troponins was detected, mostly troponin I (*24*) and troponin C (*1*) as listed in **Table 1**. Troponin is composed of three distinct subunits: Ca^{2+} binding (TnC), tropomyosin binding (TnT), and inhibition of the actin−myosin interaction (TnI) (*13*−*15*). It is well-known that troponin I binds to actin and suppresses the contraction in relaxed muscle. Recently, it has been reported that troponin I is useful for myocardial injury as an early and sensitive marker and that marked increases of troponin I can be detected within 2−6 h from the onset of exercise-induced muscle injury (*16*, *17*). After individual death, Ca^{2+} is abnormally released from endoplasmic reticulum in the muscle and starts to cause muscle contraction with cellular damage and death. Therefore, it is assumed that troponin I mRNA was specifically transcribed in muscle cells to relax the muscle contracted by inhibition of the interaction between actin and myosin and/or to compensate for the rapid loss of troponin I during cellular injury. On the other hand, the expression of troponin C, which has Ca^{2+} binding ability (*18*−*21*), is thought to reduce the increased intracellular concentration of Ca^{2+}. In addition, the transcriptions of myosin regulatory light chain 2, myosin light chain 3, actin, myomesin, and so on may be also related to regain equilibrium from a state of high intracellular Ca^{2+} concentration and to muscle contraction. However, because the information regarding translation products from these troponin mRNAs has not been obtained yet, it is necessary to clarify whether specific post-mortem expression is involved only in transcription or also in translation. We have tried to measure the protein amounts of rainbow trout muscle GAPDH and troponin I by immunoblot analysis with antibodies against mammalian counterparts (data not shown). Unfortunately, we have not succeeded in detecting them quantitatively because of accompanying proteolysis in post-mortem changes and different recognition of antibodies between lower vertebrates such as fish and higher vertebrates.

As for troponin I in various organisms, the partial or whole primary structure and the expression of mRNA have been determined (*22*−*24*). It has been reported that there are slight differences among fish species, and some isoforms were identified (*22*, *23*). Recent studies on the fish troponin I gene based on at least four fish species are available; however, there is no report on rainbow trout troponin I. Therefore, the primary structure of the protein, which is coded by rainbow trout troponin I cDNA obtained in the present study, was analyzed as shown in **Figure 4**. It was found that rainbow trout troponin I cDNA codes a polypeptide with 171 amino acid residues, including the signal peptides, and the molecular weight was estimated to be approximately 19600. Rainbow trout troponin I exhibited the highest level of amino acid sequence identity (95%) to Atlantic salmon, *Salmo salar*. On the other hand, rainbow trout troponin I had 51% identity to human fast skeletal muscle troponin I. On the basis of the information of the primary protein structure, a search for translation products from the mRNA is currently being conducted.

The results of the present study have indicated the possibility that the cells of muscle tissues synthesize troponin I and GAPDH mRNAs for at least 24 h, or up to 48 h, in fish kept on ice. Because rainbow trout stored on ice for 48 h reached the stage of muscle softening exhibiting deteriorated appearance and texture, the mRNA levels corresponded well with such changes in the fish after 48 h (*1*−*3*). These results suggest that the transcription profile at cellular level can be a sensitive indicator of freshness during on-ice storage. It is known that various factors of rigor mortis, such as the initiation timing, the level of rigidity, the duration, and the initiation timing of the softening, vary not only by species and size of the animal but also with age, nutrition condition, type of death (instant or prolonged death), and environmental temperature after death (*1*, *2*). However, details of these factors and mechanisms are

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Rainbow trout     1 MSEKRMTSSRKHHLKSLMLQIAATLIEEEKKQIKEEKANFMAKISALDLSGDQAALMEML  60
                    *********************** *******::*:** **** * ********* *****
Atlantic salmon   1 MSEKRMTSSRKHHLKSLMLQIAANLIEEEKKEMKQEKVNFMANIPALDLSGDQAMLMEML  60

Rainbow trout    61 KKLSQTIDKVDEERYDAEAKVKKTEKEIEDLKMKVIEVQGIKKPALKKVRMSADAMLAAL 120
                    ************************************************************
Atlantic salmon  61 KKLSQTIDKVDEERYDAEAKVKKTEKEIEDLKMKVIEVQGIKKPALKKVRMSADAMLAAL 120

Rainbow trout   121 LGTKHKASMDFRANLKEVKKEVKEEEEVGDWRKNVDEQAGMDGRKKKFEST          171
                    **********************************************
Atlantic salmon 121 LGTKHKASMDFRANLKEVKKEVKEEEEVGDWRKNVDEQAGMDGRKKKLSPHK         172
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Figure 4. Alignment of the deduced amino acid sequences of troponin I from rainbow trout (accession no. AB256537) and Atlantic salmon muscles (accession no. AAC23580). Asterisks and colons signify amino acid identities and similarities, respectively. The inhibitory regions of actin–myosin interaction are underlined.

not yet known. Therefore, further genetic investigation on postmortem changes will elucidate the various phenomena after the death of fish and, additionally, contribute to the development of technology for retaining functionality of cells and tissues.

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