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Expressions and characterization of MuRFs, Atrogin-1, F-box25 genes in tilapia, *Oreochromis niloticus*, in response to starvation

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Abstract Muscle accretion is affected by the difference between protein synthesis and its degradation. Studies on different species revealed that muscle proteolysis is mediated by different pathways including the ubiquitin-proteasome pathway in which the ubiquitin protein ligases play an important role. These muscle atrophy associated ligases were not well studied in tilapia. In this study, we characterized the ubiquitin protein ligases MuRF1/2/3, Atrogin-1 and F-box25, members of the ubiquitin-proteasome pathway in tilapia, *Oreochromis niloticus*, and their expressions in the muscle of starved, fed, refed, and control fish. Sequences of these genes revealed presence of Ring finger, B-box, and Cos domains in all MuRF genes, as well as F-box domain in Atrogin-1 and F-box25 genes. Real-time qPCR data analysis showed that expression of MuRF1/2/3, Atrogin-1, F-box25, and proteasome complex genes was significantly upregulated in starved fish compared to fed fish. Concurrently, the proteasome activity was 1.7-folds elevated in the starved fish compared to fed fish. These results confirm the important role of these genes in muscle degradation and suggest potential usage as markers of muscle accretion in tilapia.

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Introduction

Tilapia ranks as the second most cultivated fish in the world, after carp. Aquaculture of tilapia provides dietary sources of protein and minerals for millions of poor families in the world and is an important means of the economic and social improvements. Tilapia are among the most profitable and easiest fish to farm due to their fast growth, palatability, a short production interval of 6–8 months, and omnivorous low-cost dietary needs (feeds on phytoplankton, periphyton, and aquatic plants). Plant-based diets and fast growth reduce the likelihood that tilapia will accumulate environmental contaminants (Mzengereza 2016, Aanyu et al. 2017). Most tilapia that are consumed in North America and Europe come from Asia, Middle East, and South America. In America, Tilapia is considered as a new aquaculture species.

Muscle yield is an important trait affecting aquaculture profitability. Muscle yield decreases when muscle protein degradation increases compared to the protein synthesis (Bonaldo and Sandri 2013, Salem et al. 2005, Salem et al. 2006). In many cases, the rate of protein synthesis remained unchanged while the proteolysis that leads to muscle atrophy increases (Okamoto et al. 2011).

The membrane-bound lysosomal enzymes, calpain proteinases, and the ubiquitin-proteasome pathway enzymes are important proteolytic systems that are

responsible for muscle degradation in mammals and fish (Lecker et al. 2004, Paneru et al. 2018). However, some reports indicated that contribution of the proteasome to total muscle protein turnover is lower in fish muscle than that in mammals (Seiliez et al. 2014). However, a large part of the muscle degradation comes from the ubiquitin-proteasome pathway which includes the ubiquitin-activating enzymes, ubiquitin-conjugating enzymes, and ubiquitin ligases (Fareed et al. 2006; Paneru et al. 2018). During ubiquitination, E1 (ubiquitin-activating enzymes) do a series of ATP-dependent enzymatic steps. E2 (ubiquitin-conjugating enzymes) bind to E3 (ubiquitin-ligating) which bind with the substrate protein to form E2-E3_substrate complex. This complex becomes ready to be recognized and degraded by the proteasome (Seiliez et al. 2008). Muscle RING finger (MuRF) and muscle atrophy atrogin-1/F-box protein-32 (F-box32) and F-box protein-25 (F-box25) are ubiquitin-ligating enzymes (Wang et al. 2011; Bodine et al. 2001). The genes encoding for the E3 enzymes have characteristic domains which are RING-finger domain, B-box zinc finger domain and Leucine-rich coiled-coil domain in MuRF genes and F-box domain in F-box genes, as well as the motif sequences that are conserved for the enzyme activity (Wang et al. 2011). The RING finger domain and B-box zinc finger domain are evolutionarily conserved and involved in protein-protein interactions that mediate different functions as gene transcription, signal transduction, differentiation, morphogenesis, microtubule stabilization and ubiquitination (Borden 1998). In humans, one of the most important regulatory proteins in the ubiquitin-proteasome proteolysis of muscle are MuRF1 and atrogin-1 proteins (Perera et al. 2012). Atrogin-1 and F-box25 play an important role in transferring ubiquitin molecules to the substrate protein marking it for degradation (Gomes et al. 2001). Previous studies showed that MuRF1 plays an important role in muscle hypertrophy and deletion of this gene can suppress muscle atrophy in mice (Bodine et al. 2001). Wang et al., (2011) suggested that MuRF genes have important role in muscle degradation as they find the expression of these genes were up-regulated in the starved rainbow trout fish. Nebo and coworkers reported increased RNA abundance of MuRF1 and atrogin-1 in fasted tilapia (Nebo et al. 2017). Also, loss of MuRF3 gene negatively affects the mice cardiac muscle function (Fielitz et al. 2007). Atrogin-1/F-box32 was up-regulated in several types of muscle degradation (Lecker et al. 2004; Clarke et al. 2007). In fish, expression of F-box32 increases in starved rainbow trout and leads to muscle atrophy (Cleveland and Evenhuis 2010). It is noticed that the knowledge of the ubiquitin ligase enzymes in tilapia is limited. Therefore, this study was undertaken to identify and characterize genes of the ubiquitin ligases in tilapia and to examine their expression/activity under muscle catabolic conditions caused by starvation.

Material and methods

Experimental design

The experiments were approved by the MTSU institutional animal care and use committee (IACUC), protocol 17-3008. Fish fingerlings (1.4 g average weight) were obtained from Allin's company, Chianti, CT, USA and were acclimated for 15 days before the start of experiments. The study was carried out in two experiments (starved against fed and refed against control). Each experiment was conducted in triplicate tanks (50.5 × 25.5 × 31.5 cm). Each tank contained 18 fish. In the first experiment, fish were either starved for 2 weeks or manually fed a commercial fish diet (provided by Allin's company) at a ratio of 5–7% of fish body weight twice per day for 7 days a week. In the second experiment, fish were starved for 2 weeks then fed for two extra weeks as mentioned before. The control group was fed for 4 weeks. Tanks were cleaned daily to get rid of feces and remaining food particles. Water quality parameters were measured daily and were maintained at temperature = 28–30 °C, pH = 7–7.2, ammonia = 0–0.25, nitrite = 0, and nitrate = 0. At the end of each experiment, fish were euthanized using an overdose of MS222 (Syndel USA, Ferndale, WA). Muscle samples were collected in liquid nitrogen and immediately stored at − 80 °C.

RNA isolation

Total RNA was isolated from each fish (8 fish randomly selected/tank) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. The concentration of samples was measured using a Nanodrop (Thermo Scientific, Hudson, NH, USA) by measuring absorbance at 260 nm. Gel electrophoresis was performed to determine integrity of the RNAs.

cDNA library preparation

cDNA was prepared using a verso cDNA synthesis kit (Thermo Scientific, Hudson, NH, USA) according to the manufacture's instruction using a random primer. The kit contains buffer to eliminate genomic DNA contamination. Concentrations of cDNA samples were measured using a Nanodrop.

Real-time qPCR

Real-time qPCR primers for MuRFs, Atrogin-1, F-box25, and proteasome genes were designed using primer3 software (Kõressaar et al. 2018) and the cDNA sequence of each gene. The NCBI accession numbers of the cDNAs are presented in Table 1. qPCR was performed in duplicate using 80-fold diluted cDNA samples on Bio-Rad iCycler PCR detection system. Real-time qPCR reaction was performed using a SYBR green master mix (Thermo Scientific, Hudson, NH, USA) that was added to the cDNA templates at concentration of 0.006 μg/μl and the forward/reverse primers used were at a concentration of 10 μM/μl (Ali et al. 2018). The denaturation step was done at 95 °C for 7.00 min followed by 40 amplification cycles that include denaturation at 95 °C for 0.1 min, annealing at 57–64 °C according to each gene's melting temperature, and final extension at 60 °C for 5 min. Tilapia β-actin gene was used as internal control for normalization (Yang et al. 2013). The quantification of PCR data was done using ΔCt method. ΔCT was calculated by subtracting CT of β-actin from CT of the gene of interest. ΔΔCT was calculated by subtracting ΔCt of the control group (Fed or Control) from the treated group (starved or refed, respectively). Relative gene expression of the treated samples to the control samples was calculated by the formula of $2^{\wedge-\Delta\Delta CT}$ (Paneru et al. 2018).

Proteasome activity assay

Tilapia muscle samples were homogenized in phosphate buffer saline (PBS, pH 7.4) containing 1% triton then centrifuged for 20 min at 10,000 rpm, and the supernatant was retained (Fareed et al. 2006). The protein concentration was measured using a BCA assay kit according to the manufacture instructions (Thermo Scientific, Rockford, lL, USA). The proteasome activity was measured using a 20S proteasome assay kit according to the manufacture protocol (EMD Millipore Corporation, Billerica, MA, USA). A Suc-LLVY-AMC substrate was used to measure the activity (Meng et al. 1999). The fluorophore 7-amino-4-methylcoumarin (AMC) was detected after cleavage from the substrate at 380 and 460 nm as excitation and emission wavelengths, respectively. The enzyme activity was normalized to the protein concentration and used to calculate fold change in activity between the experimental groups. Data analysis and fold change of the activity was calculated and presented using Microsoft Excel.

Gene sequence and protein analyses

Clustal W2 was used in aligning the protein sequences and BOXSHADE was used for printing and shading of

Table 1 Primers used for qPCR analysis

Gene name	Forward primer	Reverse primer	NCBI accession
F-box32	ATGCCTTTTCTCGGACAGGA	GTCGTCGGCTGTTGTCTTTT	XP_003443717.1
F-box25	TCCAGCGCTATTCAGGACTT	TCCACTCAAAGACGTCAGCT	XP_005454750.1
MuRF1	CAAGCACGTGTTCTCCTTCA	TCATATTTGCTGCGTGGGTA	XP_003458184.1
MuRF2	TGTGCAGAAAATGTGCCAAT	AACGTCCACCAGAGGTCAAC	XP_003443662.1
MuRF3	CCGTGTAGTGTGTGCGATTT	CTGCGACTGATGGTCAAGAA	XP_005464863.1
Proteasome 26S	CAAGCTGCTGAGCTCTTCCT	CAGGCCTCTTGAGAGCAATC	XP_003442732.1
Proteasome alpha 5	AAGGGTTTTTGGCTGAAGGT	CCTGGAACAATCTTCCCTCA	XP_003441568.1
Proteasome beta 3	ATGCTGAATGCAGTTGATCG	CTAGTCCATCCTGGCCTTCA	XP_003448021.2
Polyubiquitin	GCTGTTGTGTGAGCGTCATT	TTGGCTGTAGTCTTGCGATG	XP_019221483.1
β-actin	TCTCGGCTGTGGTGGTGAA	GACCCACACAGTGCCCATCT	XM_003455949

Table 2 Significant difference in body weight between groups in response to starvation and refeeding

Group	Average final body weight	Number of fishes
Starved	0.839* ± 0.154	18
Fed	5.621 ± 0.942	18
Refed	3.236* ± 0.332	18
Control	7.879 ± 1.039	18

*Represent the significant difference between the group with its respective control (starved vs fed and refed vs control. Data presented as mean ±SD ($n = 18$). The average weight for all fish at the beginning of the experiment was 1.64 ± 0.064, fish were randomly distributed between groups

the multiple-alignments (Sievers et al. 2011). Functional domains were predicted by aligning each gene with the same gene predicted in trout (Wang et al. 2011; Cleveland and Evenhuis 2010).

Results and discussion

Changes in body weight with starvation and refeeding

After 2 weeks of starvation, fish average body weigh was significantly less than that of the fed fish, 0.839* ± 0.154 g versus 5.621 ± 0.942 g (Table 2). In the second experiment where fish were starved for 2 weeks then fed for two extra weeks, fish body weight was significantly less (3.236* ± 0.332 g) compared to the control group (7.879 ± 1.039 g) which was fed for 4 weeks (7.879 ± 1.039 g).

Relative gene expression of MuRF1/2/3, Atrogin-1 and F-box25

The results of qPCR revealed that expression of all tested MuRFs, Atrogin-1, and F-box25 genes were

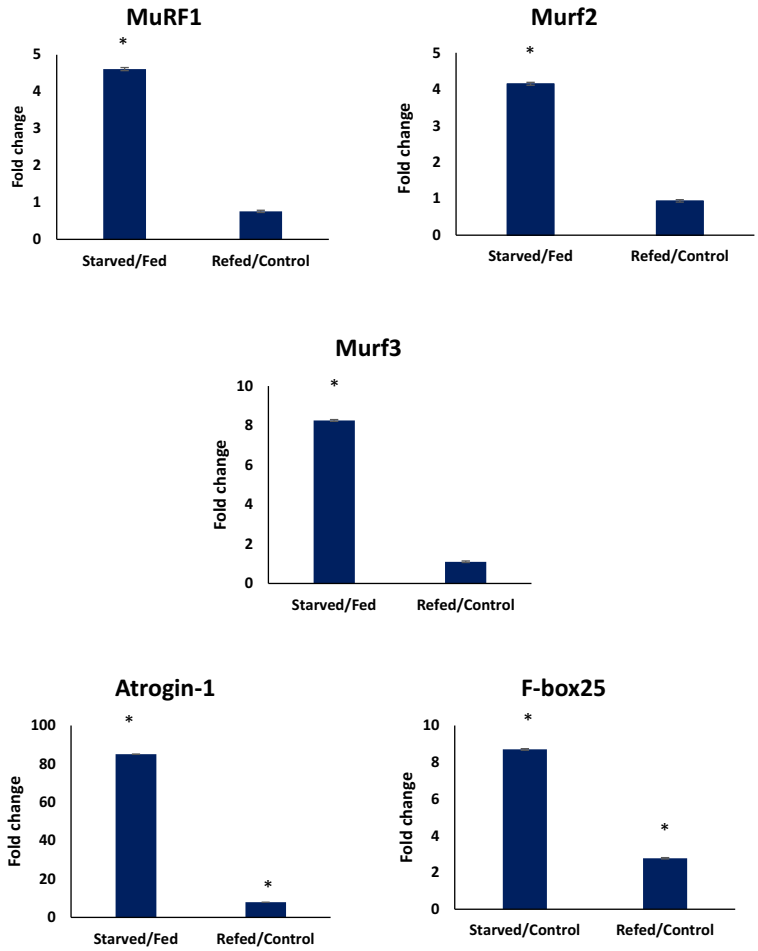

Fig. 1 Differential gene expression MuRF1, MuRF2, MuRF3, Atrogin-1, and F-box25 genes in starved versus fed and refed versus control groups. Gene expressions were determined by qPCR. The data were normalized using the β-actin gene. The expression of genes was presented using fold change between starved and fed groups and refed and control group ± standard deviation where ($n = 7$) and ($p \leq 0.05$)

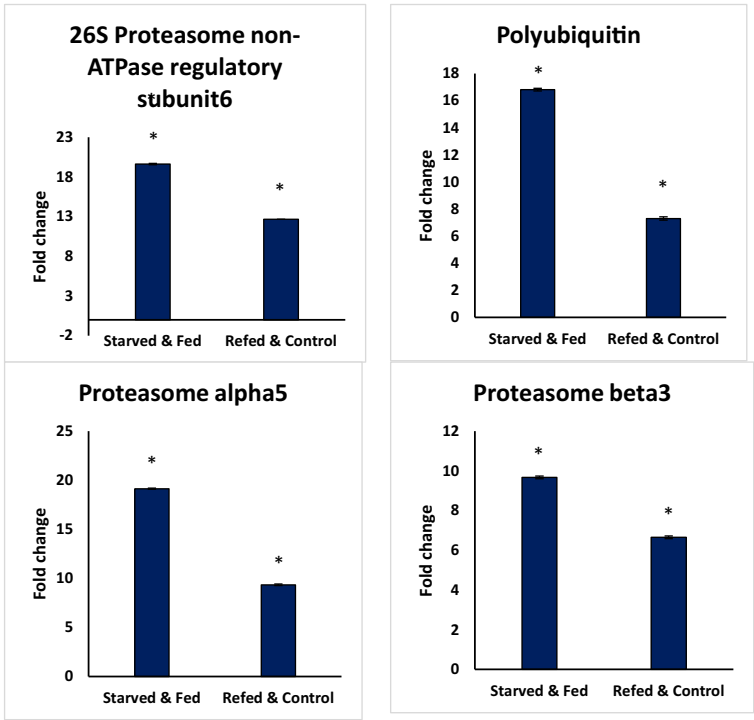

Fig. 2 Differential gene expression of the 26S proteasome non-ATPase regulatory subunit 6, proteasome alpha5, proteasome beta3, and polyubiquitin genes in starved versus fed, refed, versus control groups. Gene expressions were determined by qPCR. The data were normalized using the β-actin gene. The expression of genes was presented using fold change between starved and fed group and refed and control group ± standard deviation where $n = 7$) and $p \leq 0.05$

significantly increased in the starved group compared to the fed group (respective control). The expression values of MuRF1/2/3 genes were upregulated by 4.6-, 4.2-, and 8.3-folds, respectively, in the starved fish compared to the fed fish (respective control, $p \leq 0.05$). Meanwhile, the refed fish showed no significant difference in MuRF1/2/3, compared to the control as depicted in Fig. 1. Similarly, expression of Atrogin-1 and F-box25 genes were drastically increased under starvation conditions as described in Fig. 1**.** Atrogin-1 was 85-fold upregulated in the starved group compared to the fed group and 8-fold in the refed group compared to the control group ($p \leq 0.05$). F-box25 showed 8.7-fold increase in the starved group compared to the control group and 2.8-fold increase in the refed group compared to the control group ($p \leq 0.05$).

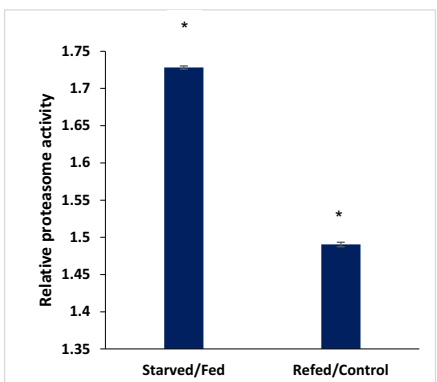

Fig. 3 Effect of starvation on the proteasome enzyme activity in starved versus fed, and refed versus the control groups ($P \leq 0.05$). The activity was expressed as relative fold change ± SD

Consistent with our data, previous studies on rainbow trout showed upregulation of MuRF genes in starved fish compared to the control fish (Wang et al. 2011). A recent study showed that tilapia exposed to starvation had more than tenfold expression increase in Atrogin-1 after 1, 2, and 3 weeks of starvation. Similarly, MuRF1 had 2–4-fold increases in starved fish compared to control group (Nebo et al. 2017). In Atlantic salmon, starved fish showed 47-, 8-, and 5-fold increases in Atrogin-1, MuRF1, and Fbox25, respectively (Tacchi et al. 2010). In mammals, starving rats for 51 h led to an increase in the Atrogin-1 level by 5.8-folds (Dehoux et al. 2004). Also, Atrogin-1 in human had 8.2- and 9.8-folds increase in expression levels after 1 and 2 days of starvation, respectively (Jagoe et al. 2002). The conserved role of these genes in muscle atrophy suggests that these genes could be used to develop genetic markers for muscle accretion in tilapia (Salem et al. 2006, Macqueen et al. 2014, de Boer et al. 2007).

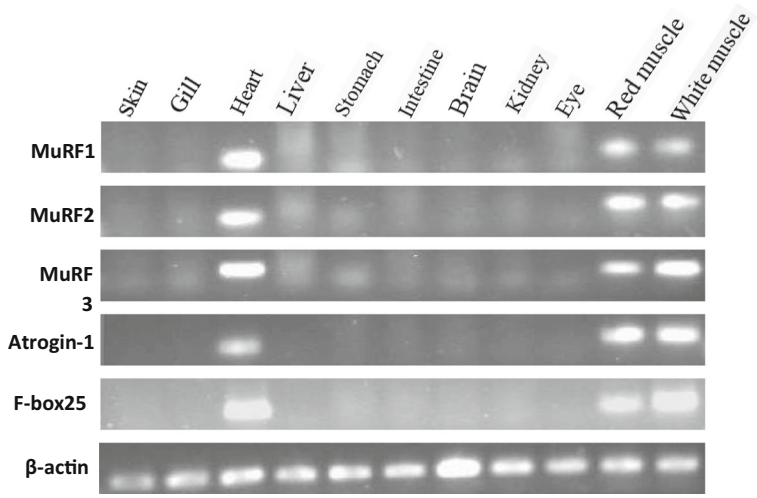

Fig. 4 Predominant expression of MuRF 1,2,3, Atrogin-1 and F-box25 genes in muscle tissues. Three to four cDNA samples were used in RT-PCR amplification and one set of genes was presented

Several studies investigated the role of Atrogin and MURFs in regulating muscle growth and atrophy. Atrogin and MURFs target specific proteins with critical roles in muscle growth including MyoD and eIF3-f and ATP generation especially glycolysis. In addition, Atrogin and MURFs can target myofibrillar proteins including myosin-binding protein C (MyBP-C) and myosin light chains 1 and 2 (MyLC1 and MyLC2), and myosin heavy chain (MyHC). For review, see Bodine and Baehr 2014 (Bodine and Baehr 2014) who suggested that Atrogins controls protein synthesis, whereas MuRFs control protein degradation.

Relative gene expression of proteasome genes

Four genes chosen to represent the proteasome pathway showed concurrent upregulated expression in the starved and refed fish compared to the control groups (Fig. 2, $P \leq 0.05$). Expression of the 26S proteasome non-ATPase regulatory subunit 6, proteasome alpha5, proteasome beta3, and polyubiquitin genes were 19.6-, 19.1-, 9.7-, and 16.8-folds upregulated, respectively, in the starved group compared to the fed group**.** The positive correlation in expression of MuRF genes, Atrogin, and F-box25 with the proteasome genes indicates active ubiquitin-proteasome proteolysis in muscle during starvation (Fig. 2). The ubiquitin-proteasome pathway is considered a major system responsible for the bulk proteolysis during muscle atrophy (Paneru et al. 2018).

Proteasome activity

Consistent with the starvation effect in increasing mRNA abundance of the ubiquitin-proteasome pathway

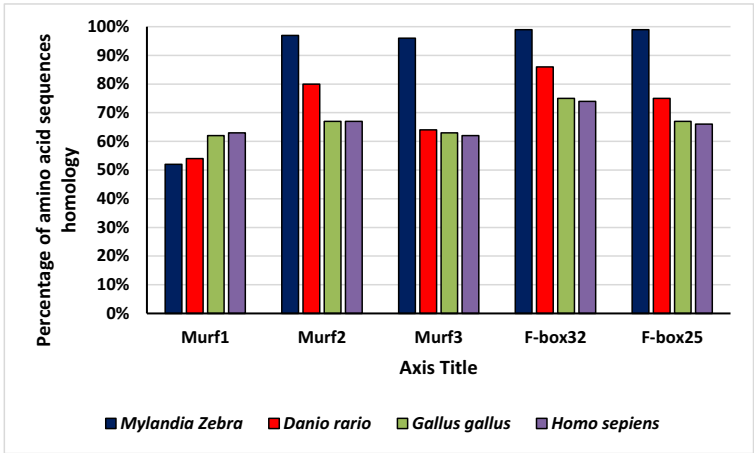

Fig. 5 Percentage of amino acid sequences homology of MuRF1,2,3, Atrogin-1, and F-box25 genes in tilapia compared to different species

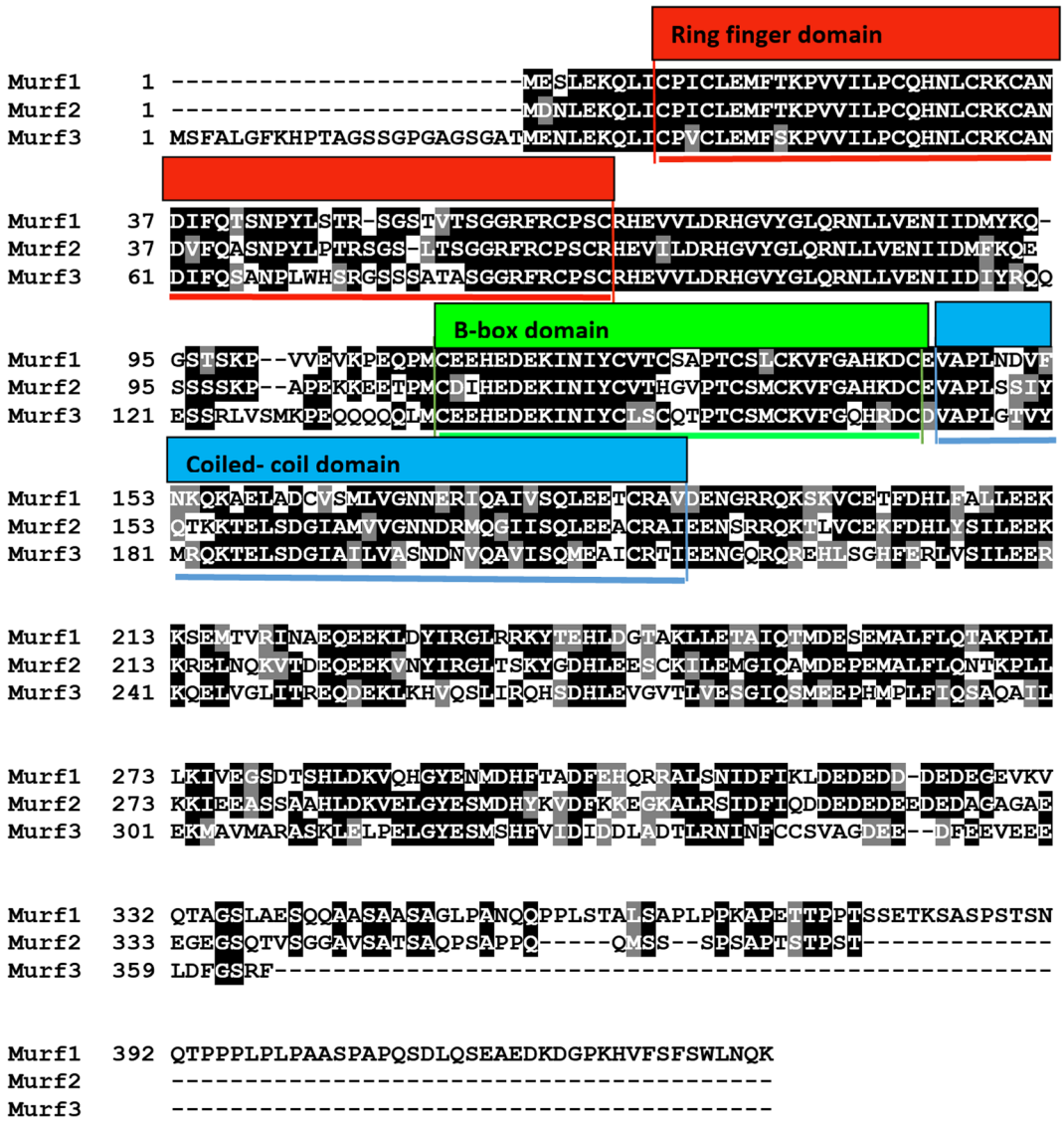

Fig. 6 Multiple alignments of MuRF1,2, and 3 amino acids sequences of tilapia (*Oreochromis niloticus*). Dark background indicates identical amino acids, gray background indicates same properties of different amino acids, and amino acids with no background indicates different name and properties. The red box indicated the amino acid sequences of zinc finger-ring type domain. The green box indicates the B-box type zinc finger domain, and the blue box indicates cos domain (coiled coil)

genes, results of this study also showed that the proteasome enzyme activity was significantly higher in the starved fish muscle by 1.7 folds compared to the fed fish as illustrated in Fig. 3**.** The activity was also elevated in the refed group by 1.5 folds compared to the control group. Similar results were observed in rainbow trout fish that exhibited slight but significant increase in the hepatic proteasome activity after 3 weeks of starvation (Salem et al. 2007).

MuRFs and Atrogin tissue distribution

Real-time qPCR was used to determine expression of the MuRF 1,2,3, Atrogin-1, and F-box25 in 10 tissues/organs; white muscle, red muscle, heart, skin, gill, eye, kidney, liver, stomach, and intestine. All genes revealed predominant expression in muscle tissues, white muscle, red muscle, and heart as shown in Fig. 4. Similar results were reported in rainbow trout fish (Wang et al.

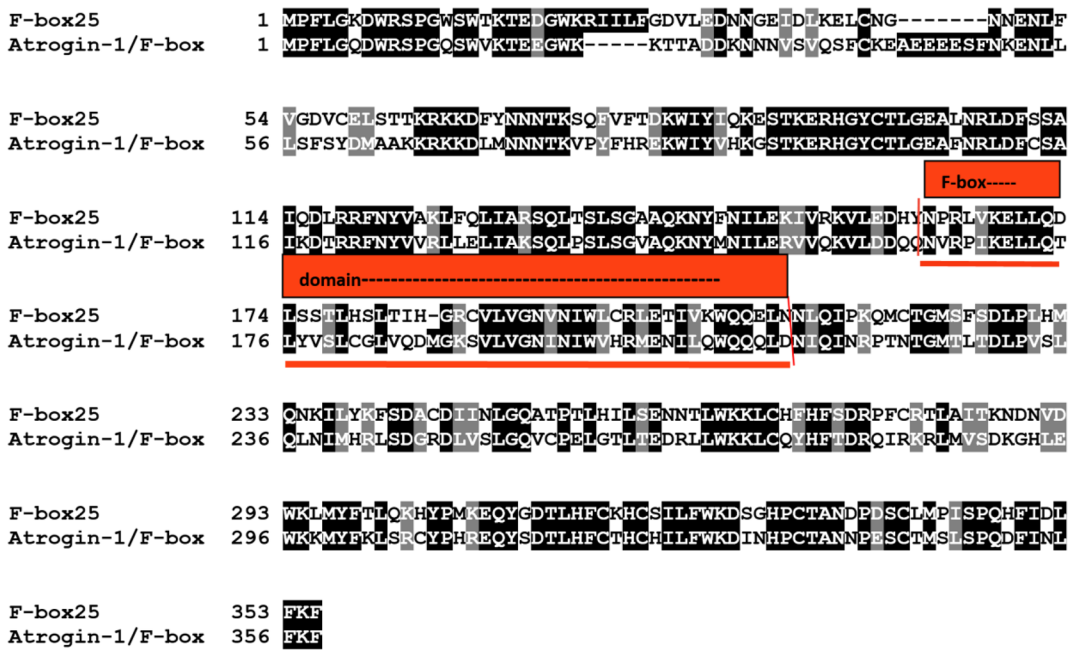

Fig. 7 Multiple alignments of F-box25 and Atrogin-1 amino acids sequences of tilapia (*Oreochromis niloticus*). Dark background indicates identical amino acids, gray background indicates same properties of different amino acids, and amino acids with no background indicates different name and properties. The red box indicates the amino acid sequences of F-box domain

2011, Cleveland and Evenhuis 2010) indicating important role of these genes in muscle physiology of fish.

Molecular characterization of MuRF1/2/3, Atrogin-1, and F-box25

Searching the GENBANK database, muscle degradation genes, such as MuRF genes, Atrogin-1, and F-box25 were identified. Table 1 shows the accession numbers for MuRF 1, 2, and 3 cDNAs, where they were found to comprise of 2264, 2258, and 2335 nucleotide (nt) sequences, respectively. The longest ORFs were 432, 372, and 365 amino acids for MuRF1, 2, and 3, respectively. Amino acid sequence of tilapia MuRF1 shared variable homology percentages with different species including 52% with *Maylandia zebra*, 54% with *Danio rario*, 62% with *Gallus gallus*, and 63% with *Homo sapiens*. The amino acid identity similarities were higher for MuRF2 and MuRF3; 97% and 96% identical to *Maylandia zebra*, 80% and 64% identical to *Danio rario*, 67% and 63% identical to *Gallus gallus*, and 67% and 62% identical to *Homo sapiens*, respectively (Fig. 5).

Tilapia Atrogin-1/F-box32 and F-box25 cDNAs had 2459 and 3596 nt sequence. The longest ORFs were 358 and 355 amino acids for F-box32 and F-box25, respectively. Amino acid sequences of F-box32 and F-box25 were 99% identical to *Maylandia zebra*, 86% and 75%, identical to *Danio rario*, 75% and 67% identical to *Gallus gallus*, and 74% and 66% identical to *Homo sapiens*, respectively (Fig. 5).

Protein functional domains for these genes were predicted. MuRF1/2/3 protein sequences contain functional Zinc finger domain comprising amino acids 9–65 in Murf1/2, and 34–90 in Murf3 as shown in Fig. 6. Zinc finger-Ring type domain is characterized by the presence of zinc atoms and finger-like protrusions that strongly bind enzymes with their substrates in the degradation process. This action affects the protein transcription, transduction, differentiation, and ubiquitination, that leads to substrate protein degradation (Wang et al. 2011; Witt et al. 2005). B-box Zinc finger domain contains amino acids 110–143 in MuRF1/2 and 138–171 in MuRF3 as presented in Fig. 6. B-box domain also is a protein-protein interaction domain (Borden 1998). Moreover, coiled-coil domain contains amino acids 145–187 in MuRF1/2 and 173–215 in MuRF3 as cleared in Fig. 6. The predicted coiled-coil domains are involved in association with microtubules (Spencer et al. 2000). On the other hand, Atrogin-1/F-box32 and F-box25 contain F-box domain that contain amino acids from 163 to 211 and 165 to

214, respectively, as indicated in Fig. 7. The predicted F-box domain can act with Leucine-rich repeats in protein-substrate interaction (Cleveland and Evenhuis 2010; Gomes et al. 2001).

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