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ORIGINAL ARTICLE

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Ubiquitin-proteasome-dependent proteolysis in rainbow trout (*Oncorhynchus mykiss*): effect of food deprivation

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Abstract The ubiquitin-proteasome proteolytic pathway is a major route of protein degradation and of particular importance in muscle proteolysis in mammals. In this study, the β proteasome subunit N3 and polyubiquitin genes of the rainbow trout, *Oncorhynchus mykiss*, were sequenced and tissue distribution of gene expression was examined. The effects of 14-day food withdrawal were assessed on the N3 subunit and polyubiquitin gene expression in terms of mRNA, 20S proteasome proteolytic activity and ubiquitin protein abundance in trout liver and muscle. Both sequences are highly conserved, and the rainbow trout ubiquitin amino acid sequence is identical to the mammalian protein. The proteasome β subunit N3 has 92% similarity to the *Xenopus* sequence. Starvation halved the polyubiquitin mRNA level in liver but had no effect on muscle levels. No significant effect of food withdrawal was observed on the proteasome mRNA in liver or muscle. Food withdrawal decreased the 20S proteasome proteolytic activity and the abundance of ubiquitin protein in both muscle and liver. Co-regulation of the proteasome and ubiquitin was indicated by the high correlation (R=0.924) between 20S activity and ubiquitin abundance. Overall, this study demonstrates that starvation down-regulates the ubiquitin-proteasome pathway, possibly highlighting differences in the regulation of protein turnover in poikilothermic and endothermic animals.

Keywords Polyubiquitin · Proteasome · Proteolysis · *Oncorhynchus mykiss* · Gene expression

Nucleotide sequence data reported in this paper have been deposited with EMBL/GenBank Data libraries under the following accession number: AF361366 (Proteasome N3), AF361365 (Polyubiquitin).

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Introduction

Protein accretion is a balance between protein synthesis and protein degradation. Mature tissues remain in a state of balance between anabolic and catabolic pathways, whereas growing tissues undergo hyperplasia or hypertrophy when anabolism exceeds catabolism. The efficiency with which an animal utilizes its dietary protein is related not only to protein consumption but is also influenced by the rate of protein turnover [5, 19]. Fish, which have lower protein turnover per unit protein consumed, convert more protein to growth than mammals [26], possibly as a result of the lower metabolic rate of ectothermic animals. Protein synthesis rates in fish appear relatively similar between individuals when protein consumption is accounted for, but the protein retention is variable and is believed to result from differing rates of protein degradation [5]. As the efficiency of retention of synthesized proteins leads to greater efficiency of food conversion, detailed knowledge of protein degradation in fish will be of benefit to the aquaculture industry.

The regulation of protein degradation is a highly controlled process [1, 18, 46]. Misfolded and damaged proteins, cell-cycle regulators, oncogenes and tumor suppressors are required to be continuously degraded. Eukaryotic cells contain two major systems for protein degradation. The first of these is the lysosomal system, where membrane-bound vacuoles contain acidic proteases, such as capthepsins and other hydrolases [9, 37]. The second major pathway of degradation is the ATP-dependent ubiquitin-proteasome pathway. Studies in mammalian cell lines with proteasome inhibitors have shown that the bulk of cellular proteins (80–90%) are hydrolysed by this route [7, 41]. Moreover, mammalian muscle proteins are hydrolysed by this route, especially during starvation [30, 55] and wasting diseases [2, 15, 48].

The ubiquitin-proteasome route of protein degradation involves two discrete steps. First, multiple ubiquitin molecules covalently attach to the protein substrate [6, 14], and second, these tagged proteins are degraded by the

proteasome [23], resulting in peptides of 7 –9 amino acid residues [52]. The core protease activity is within the 20S proteasome subunit, which is composed of four stacked rings that have outer α- and inner β-type subunits. There are seven different subunits of each type: the β subunits possess the proteolytic activity and the α subunits are required for assembly (reviewed in [17]). In eukaryotes, four of the seven β subunits are inactive, with the three active β subunits contributing chymotrypsin-like, trypsin-like and peptidylglutamyl peptide hydrolytic activity to the proteasome complex [52]. Proteins are targeted for degradation by the 20S proteasome by ligation to ubiquitin by a complex series of enzymatic reactions (reviewed in [18]). Conjugation with multiple ubiquitin molecules results in rapid degradation of the proteins in an energy-dependent fashion by the proteasome. A 19S regulatory ATPase complex caps the 20S proteasome complex to form the functional 26S proteasome [13, 52]. A subunit of the 19S complex binds ubiquitin-tagged proteins and permits their entry into the core of the 26S proteasome complex for degradation [52].

A functional ATP-dependent ubiquitin-proteasome system requires many components and is a tightly regulated process. Levels of mRNA encoding the ubiquitin-proteasome system reflect changes in proteolytic activity. The key regulatory mechanism for this process is believed to be transcriptional, with mRNAs for the different proteasome subunits and polyubiquitin changing in parallel [20, 32, 39]. In mammals there are several polyubiquitin loci that encode mRNAs that have variable numbers of ubiquitin repeats [16, 42]. Each repeat of 228 bases encodes a 76-amino-acid ubiquitin peptide, with this ubiquitin monomer highly conserved through phylogeny [53].

The pathways that are responsible for protein turnover in fish are not fully understood. There are reports of increased cathepsins both during starvation [24, 29] and following exposure to pollutants [21]. At present no information is available on the role of the ubiquitin-proteasome pathway in protein turnover in fish. In this paper, the ubiquitin-proteasome system in rainbow trout (*Oncorhynchus mykiss*) was studied by examining a proteasome β subunit and a polyubiquitin gene. Additionally, expression of the β subunit and polyubiquitin gene and the proteolytic activity of the 20S proteasome complex were determined in response to food withdrawal.

Materials and methods

Fish maintenance

Rainbow trout (*Oncorhynchus mykiss*) were maintained in fresh water at 12 °C throughout the experiment. Fish were all female stock and had an initial mean weight of (36.0 g±1.9 SEM). Each fish was individually coded using alcian blue dye on the ventral surface. Fish were fed once a day ad libitum with commercial rainbow trout diet (EWOS, protein 42%, oil 26%, 23 MJ kg^{-1}). The daily food intake did not exceed 2% body weight on any one day. The starved group was not fed for 14 days, during which time the control animals were fed as before. Both groups of animals were weighed at the end of the 14-day period. Fish from both groups were sacrificed 24 h following the feeding time; therefore, short postprandial changes were not observed.

RNA extraction

Fish were sacrificed by an overdose of benzocaine anesthetic (Sigma) followed by decapitation. Tissue for RNA extraction was frozen at –70 °C immediately after death. Total RNA was isolated by lysis in TRIzol buffer (Gibco). The RNA was resuspended in 500 µl water treated with 0.1% diethylpyrocarbonate (DEPC) and concentration was determined by spectrophotometry.

cDNA cloning and sequence analysis

For isolation of the rainbow trout proteasome subunit N3, liver total RNA (2 µg) was treated with RNase-free DNase (Gibco) to remove any contaminating genomic DNA. The RNA was reverse transcribed by using oligo dT_{17} primer (100 ng μl^{-1}) as previously described [29]. This cDNA was used as a template for PCR using primers designed to conserved regions of *Xenopus* [51], human [36] and mouse [8] proteasome N3 cDNA sequences. The primers designed were N3F2 (5' GATGGTSAYGATGAGGAGCT) and N3R2 (5' TGAGCYATDTCCCAGTTGGT), where degeneracy was as follows: S=C+G, Y=C+T, D=G+A+T. PCR conditions were 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, followed by an extension at 72 °C for 10 min. These primers produced a product of 431 bp, which was cloned into a plasmid (pCR2.1, Invitrogen). Sequence analysis confirmed this to be a homologue of proteasome N3 subunit. In order to isolate a full-length cDNA clone, this PCR product was used to screen ZAP Express cDNA library of rainbow trout kidney cDNA (10^5 plaques), as previously described [11]. For screening, plaques were transferred to a nylon membrane (Amersham-Pharmacia, Hybond-N), denatured and neutralized. DNA was fixed to the membrane by baking at 80 °C for 2 h. Membranes were prehybridized at 65 °C in "Rapid Hyb" solution (Amersham-Pharmacia) for 2 h. The membranes were hybridized overnight with ^{32}P dCTP-labelled cDNA to specific activity 1×10^8 cpm μg^{-1} (Amersham-Pharmacia rapid labelling beads). Following hybridization, membranes were washed to a stringency of 0.5×SSC, 0.1% SDS at 65 °C, and positive plaques were identified by autoradiography. Successive rounds of screening were performed until single plaques were isolated. Plasmid was then isolated from the clone by in vivo excision using helper phage Exassist (Stratagene). Sequence analysis was performed using vector primers T3 and T7 as well as the primers N3F2 and N3F2. For polyubiquitin, a partial sequence had previously been obtained by random sequencing of clones from the cDNA library; this was used to isolate a full-length clone by screening the cDNA library as described above. For full-length sequence analysis, exonuclease deletions were generated by using the Erase-a-Base System (Promega), as repeat units within the polyubiquitin cDNA did not allow internal primers to be used for sequencing.

Northern blot analysis

Total RNA (10 µg per sample) was suspended in 50% formamide and separated on a 1.5% MOPS denaturing formaldehyde agarose gel. RNA was transferred to nylon membrane (Hybond–N) by capillary blotting using 20×SSC. The RNA was then fixed to the membrane by baking at 80 °C for 2 h. The membranes were prehybridized and hybridized with either proteasome N3 cDNA or polyubiquitin cDNA. Probe labelling and washing conditions were the same as for library screening.

Gene expression studies

Total RNA isolated from liver and muscle was used as a template for first-strand cDNA synthesis as described above; this cDNA was used for the semi-quantitative PCR studies. This cDNA was diluted five-fold to 100 µl, and 2 µl was used as the template for PCR using primers designed against the rainbow trout genes of interest. Primers used were as follows: proteasome N3F2, N3R2, polyubiquitin UBF (5' ATGCAGATCTTTGTGAAGACAC 3'), UBR (5' ACCACCCCTCAGACGGAGC 3'), β Actin AF1 (5' ATGGAAGATGAAATCGCC 3'), and AR1 (5' TGCCAGATCTTCTCCATG 3'). PCR conditions were 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, followed by an extension of 72 °C for 10 min. The cycle number varied for primers sets: N3, 30 cycles; ubiquitin, 20 cycles; and β actin, 22 cycles. PCR product (10 µl) was applied to nylon membrane by dot blot under vacuum. These products were fixed to the membrane and hybridized to their corresponding probe, as described above. Individual hybridization spots were cut from the membrane, and radioactivity was determined by scintillation counting for ^{32}P. The counts obtained for proteasome N3 and polyubiquitin were normalized to the β actin signal. Confirmation that no genomic DNA was present was shown when PCR products amplified using β actin primers produced only a single band of 240 bp. The actin primers span an intron, and genomic DNA results in a product of approximately 1.1 kbp. No PCR products of this size were obtained for any cDNA samples, indicating that no genomic DNA was present in the RNA samples. Polyubiquitin characteristically has no introns.

Assay of proteasome activity in vitro

Rainbow trout tissues were homogenized in lysis buffer (50 mM Tris pH 8.0, 0.1 mM EDTA, 1.0 mM 2β-mercaptoethanol) on ice using a Dounce homogenizer (200 mg tissue to 1 ml buffer), followed by centrifugation at 20,000 *g* for 1 h at 4 °C, with the supernatant retained. The concentration of soluble proteins was determined by Bradford protein assay [4], using BSA for the standard curve. For the 20S proteasome peptidase activity, the proteasome-specific fluorogenic substrate LLVY-AMC (Alexis Corporation) was dissolved in DMSO. The final concentration of DMSO in the reaction did not exceed 4%. LLVY-AMC was incubated with tissue cytosolic proteins and SDS, as described in [43]. Briefly, 50 µg protein was incubated with 40 µM fluorogenic substrate in 100 µl of 100 mM Tris pH 8.0/0.0475% SDS. The reaction was allowed to proceed for 30 min at 15 °C. The reaction was stopped by the addition of 0.3 ml of 1% SDS and 1 ml of 0.1 M sodium borate, pH 9.1. For control reactions protein and all reagents were stopped immediately so as to determine background fluorescence. The release of the fluorogenic reagent AMC (7-amido-4-methylcoumarin) was determined with a fluorimeter with excitation 370 nm and emission 430 nm. Enzyme activity is presented as pmol AMC h^{-1} 50 µg $protein^{-1}$. A standard curve was constructed for each assay with AMC ranging from 0 to 100 pM. Proteasome-specific inhibitors (MG115 and ZLLnV, Sigma C6706) were added to reactions to a final concentration of 50 µM to confirm that LLVY-AMC degradation was proteasome-specific [25, 50].

Immuno-detection of polyubiquitin proteins

Total ubiquitin was measured in the soluble cell fraction using a ubiquitin-specific antibody (Sigma). Protein samples (5 µg), extracted as described above, were spotted onto nitrocellulose paper (pore size 0.22 µM, Sigma) using a 48-well vacuum dot-blot apparatus and allowed to dry. Before immuno-detection, the membranes were boiled for 5 min to remove secondary structure and expose epitopes, as described in [31]. After boiling, the membrane was blocked with 5% dried milk powder (Marvel) in PBS for 1 h. The membrane was incubated with ubiquitin antibody (1:1,250 in 1% dried milk) for 1 h at room temperature and then washed three times with 5% dried milk/0.05% Tween 20 for 15 min each. The second antibody was goat anti-rabbit IgG conjugated to horseradish peroxidase (PerBio) at 1:25,000 dilution. Detection was by Super Signal chemiluminescence substrate (PerBio) and exposure to X-ray film. Densitometric analysis was performed using gel image analysis (Non Linear Dynamics). Prior to dot blotting, the antibody was tested on fish protein extracts using western blotting. Protein samples then were separated on 12% polyacrylamide gels and transferred to nitrocellulose membrane using standard techniques.

Statistical analysis

Independent Student *t*-tests were used to determine statistical differences between the groups of fed and starved animals. Correlations were calculated by the Pearson method with an SPSS package.

Results

Isolation and characterization of proteasome and ubiquitin cDNAs from rainbow trout

A partial ubiquitin cDNA sequence was used to screen a rainbow trout kidney cDNA library. A full-length cDNA 1162 bp was isolated and sequenced (Fig. 1). This contained a 56-bp 5' UTR followed by an open reading frame of 915 bp, encoding 305 amino acids. Four repeat units, termed R1 to R4, were present in the sequence. R1 to R3 consisted of 228 bp coding for a 76-amino-acid ubiquitin monomer, while R4 consisted of 231 bp and coded for 77 amino acids. The first three repeats had an identical amino acid sequence, but the fourth repeat had an additional C-terminal amino acid, asparagine. The nucleotide sequences of each repeat had several differences, and these changes are non-redundant substitutions. R2 and R3 have eight changes compared to R1 (96.4% identity), with R4 having 16 nucleotide changes compared to R1 (92.9% identity). The amino acid sequences of R1 to R4 were all identical to that of ubiquitin from animals as diverse as *Drosophila*, *Xenopus*, *Gallus gallus*, and human (data not shown).

PCR products for the proteasome N3 subunit were obtained by using primers N3F1 and N3F2 of the predicted size (430 bp). Sequence analysis confirmed this to be the correct cDNA. A full-length proteasome β subunit N3 was isolated from the library as described; this clone was 930 bp in length and had a 5-bp 5' UTR and a 768-bp open reading frame encoding 256 amino acids (Fig. 2). A termination codon TGA (774–776) was followed by a 3' untranslated region of 154 bases. A eukaryotic polyadenylation signal AATAAA is 13 bases upstream on the poly A tail. The translated amino acid sequence was compared for similarity with other proteasome β N3 subunits (Fig. 3). The amino acid sequence is highly homologous to other proteasome N3 sequences, 81.6% and 92.1% similarity for human and *Xenopus,* respectively. Nucleotide identity within the open reading frame is 68.6% with the human sequence and 75.6% identity with the *Xenopus* sequence. The 3' UTR is much

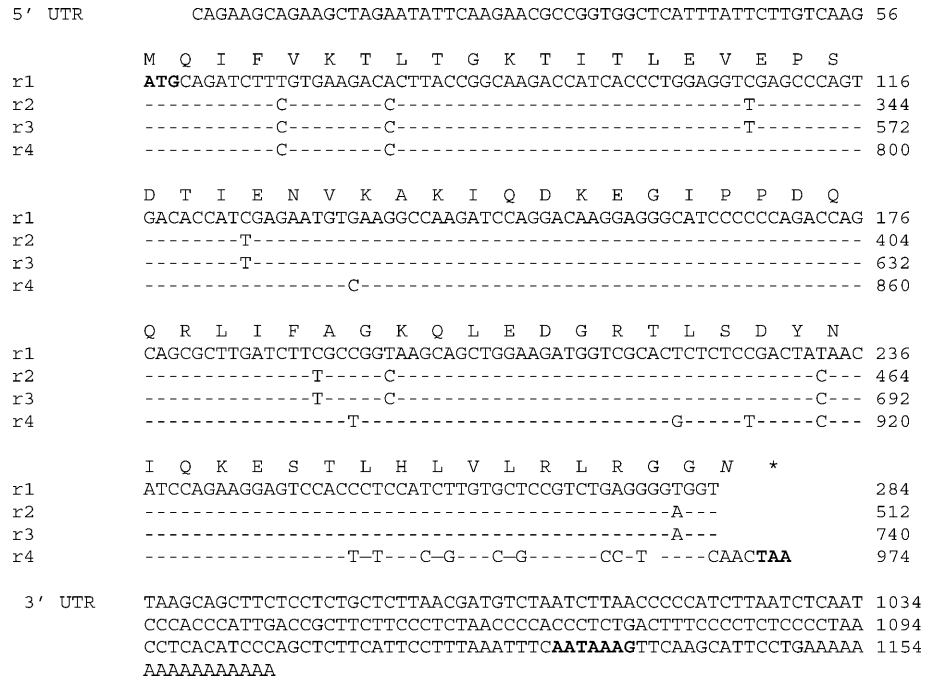

Fig. 1 Nucleotide and deduced amino acid sequence of the rainbow trout polyubiquitin mRNA. The sequence contains a 56-bp 5' untranslated region followed by four 228-bp repeats, termed R1 to R4. Identical nucleotides between repeats are indicated by a *dash*. The amino acid sequence is shown above the first repeat. The final repeat, R4, has an additional N residue before the stop codon. The stop codon is indicated by an *asterisk*, and the eukaryotic polyadenylation signal AATAAA is in *bold*

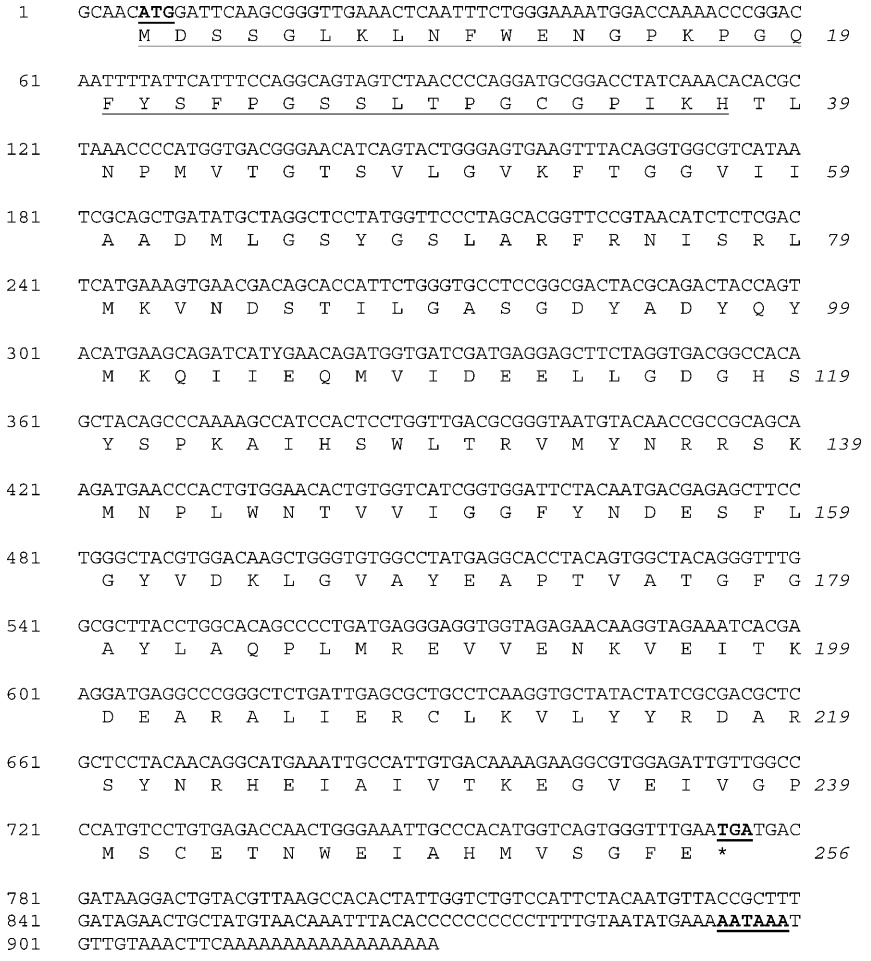

Fig. 2 Nucleotide and deduced amino acid sequence (amino acid numbers in *italics*) of the rainbow trout proteasome β subunit N3. The sequence is 930 bp in length and encodes an open reading frame of 256 amino acids. The start codon ATG, stop codon TGA, and eukaryotic polyadenylation signal AATAAA are all in *bold* and *underlined*. Amino acids 1–37 are *underlined* and are the predicted leader sequence that is cleaved to release the mature peptide

Fig. 3 ClustalW alignment of teleost, amphibian, and mammalian proteasome β subunit N3. * represents identical amino acid residues; ":" indicates conserved substitutions, and "." indicates semi-conserved substitutions. The predicted cut site is indicated. The *Xenopus* sequence is not a full-length clone

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                                                                  Cut site in
                                                                  mammals
                                                                     ▼
Rainbow Trout   --------MDSSG-LKLNFWENGPKPGQFYSFPGS-------SLTPGCGPIKHTLNPMVT
xenopus         ---------ES--------VARGTAPGELHCFPGA-------------GPVRHTLNPMVT
hum             --------MEAFLGSRSGLWAGGPAPGQFYRIPSTPDSFMDPASALYRGPITRTQNPMVT
mouse           --------MEAFWESRAGHWAGGPAPGQFYRIPATPSGLMDPASAPCEGPITRTQNPMVT
                         ::           *. **::: :*.:             **: :* *****

Rainbow trout   GTSVLGVKFTGGVIIAADMLGSYGSLARFRNISRLMKVNDSTILGASGDYADYQYMKQII
xenopus         GTSVLGVKFDGGVIIAADMLGSYGSLARFRNISRIMKVNENTILGASGDYADYQYLKQVI
hum             GTSVLGVKFEGGVVIAADMLGSYGSLARFRNISRIMRVNNSTMLGASGDYADFQYLKQVL
mouse           GTSVLGVKFDGGVVIAADMLGSYGSLARFRNISRIMRVNDSTMLGASGDYADFQYLKQVL
                ********* ***:********************:*:**:.*:*********:**:**::

Rainbow Trout   EQMVIDEELLGDGHSYSPKAIHSWLTRVMYNRRSKMNPLWNTVVIGGFYNDESFLGYVDK
xenopus         DQMVIDEELVGDGHNYSPKAIHSWLTRVMYNRRSKMNPLWNTVVIGGFYNGESFLGYVDK
hum             GQMVIDEELLGDGHSYSPRAIHSWLTRAMYSRRSKMNPLWNTMVIGGYADGESFLGYVDM
mouse           GQMVIDEELLGDGHSYSPRAIHSWLTRAMYSRRSKMNPLWNTMVIGGYADGESFLGYVDM
                 ********:****.***:********.**.***********:****: :.********

Rainbow Trout   LGVAYEAPTVATGFGAYLAQPLMREVVENKVEITKDEARALIERCLKVLYYRDARSYNRH
xenopus         LGVAYEAPTIATGFGAYLAQPLLREVTENKATLSKEEARQLVDRCMKVLYYRDARSYNRF
hum             LGVAYEAPSLATGYGAYLAQPLLREVLEKQPVLSQTEARDLVERCMRVLYYRDARSYNRF
mouse           LGVAYEAPSLATGYGAYLAQPLLREVLEKQPVLSQTEARELVERCMRVLYYRDARSYNRF
                ********::***:********:*** *::  ::: *** *::**::************.

Rainbow Trout   EIAIVTKEGVEIVGPMSCETNWEIAHMVSGFE
xenopus         EITTVTESGVEVEGPLSSETNWEIAHLISGFE
hum             QTATVTEKGVEIEGPLSTETNWDIAHMISGFE
mouse           QIATVTEKGVEIEGPLSAQTNWDIAHMISGFE
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less similar, having 44.8% and 37.2% identity for human and *Xenopus,* respectively.

Tissue distribution of mRNAs

Northern blot analysis demonstrated that polyubiquitin mRNA is expressed in all tissues studied (Fig. 4A). One major hybridization signal is observed in all tissues (1.2 kb). RNA isolated from gill had the highest level of mRNA for ubiquitin, with the lowest expression being in white muscle, although all tissues tested were positive for ubiquitin mRNA. On a long exposure for muscle mRNA, additional transcripts hybridized at 1.9, 2.5, 3.0 and 4.0 kb (Fig. 4D). For proteasome subunit N3, the probe hybridized to a single transcript of 0.9 kb. The level of expression is highest in the gill and kidney, with less in the liver and white muscle (Fig 4B). The membrane was re-probed with rainbow trout 28S ribosomal RNA probe to confirm loading between lanes (Fig 4C).

20S proteasome activity in rainbow trout tissues

The proteasome activity was measured by release of AMC from a substrate specifically degraded by the proteasome. Initial assays showed that maximum activity was obtained at 0.0475% SDS. Assays were performed at 15 °C, as this is in the optimal temperature range for rainbow trout growth [38]. Specificity of the assay for the proteasome was demonstrated by inhibition using 50 µmol of the proteasome inhibitor (MG115;Z-LLnV), which reduced 95% of the release of AMC (data not shown).

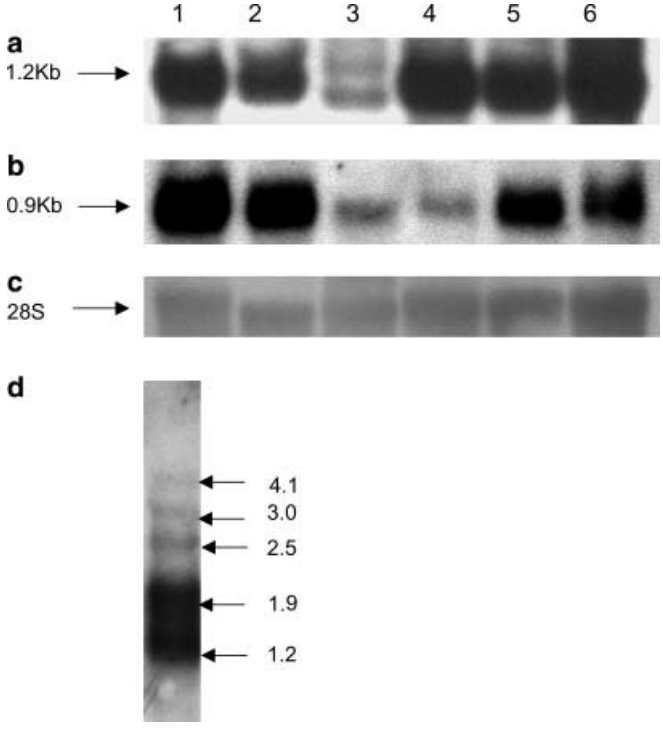

Fig. 4 Northern blot analysis showing tissue distribution of **A** the polyubiquitin mRNA and **B** proteasome β subunit N3. RNA (10 µg) was separated on 1.5% formaldehyde gel and was transferred to nylon membrane and probed with rainbow trout cDNA probes: *lane 1*, spleen; *lane 2*, heart; *lane 3*, white muscle; *lane 4*, liver; *lane 5*, kidney; *lane 6*, gill. **C** Loading of RNA on the gel was confirmed by hybridization to a 28S RNA probe. The size of major transcripts is indicated. **D** Extended exposure of Northern blot of RNA isolated from white muscle hybridized with polyubiquitin probe; additional larger transcripts can be seen at 1.9, 2.5, 3.0, and 4.1 kb

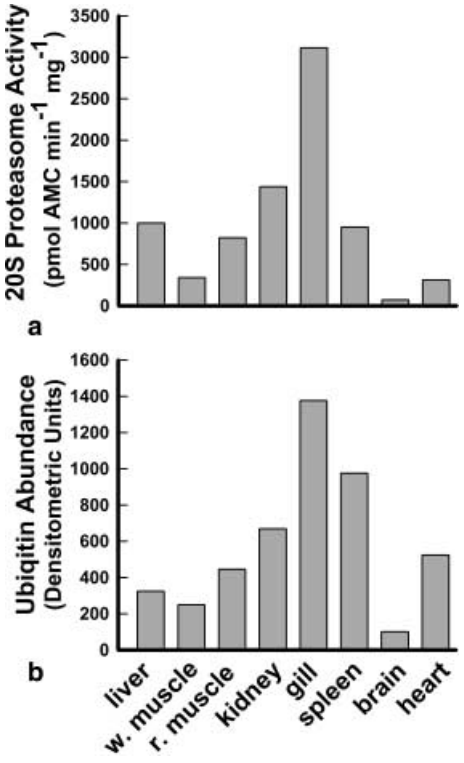

Fig. 5 a 20S proteasome peptidase activity using fluorogenic reagent LLVY-AMC in protein lysates from different tissues of rainbow trout. **b** Ubiquitin abundance in different rainbow trout tissues as determined by immuno-dot blot. Five micrograms of protein lysate was dotted onto 0.22 μm nitrocellulose membrane and ubiquitin was detected by anti-ubiquitin antisera and chemiluminescence substrate and exposure to X-ray film. The autoradiogram was scanned, and abundance of ubiquitin is expressed as arbitrary units of absorbance

Activity against the 20S proteasome-specific substrate was detected in all eight rainbow trout tissues tested, but large differences between the tissues were observed (Fig. 5a). Brain tissue had very low, but detectable, 20S-proteosome activity that was about 45-fold lower than that of gill tissue.

Ubiquitin abundance in rainbow trout tissues

Total ubiquitin protein present in the tissue was detected in dot-blot studies that detected free and conjugated ubiquitin. Boiling of the membrane was found to be essential for the antisera to detect trout ubiquitin, as has been found with mammalian ubiquitin [31]. Ubiquitin was present in all eight tissues investigated; however, as with 20S-proteasome activity, abundance between the tissues varied greatly (Fig. 5b). Brain had the lowest ubiquitin content and gill tissue the highest, with a 15-fold difference. The general pattern of 20S-proteasome activity and ubiquitin abundance in the eight tissues studied was very similar, resulting in a positive and highly significant correlation (r=0.863, P<0.01, n=8)

Effect of starvation on proteasome N3 and polyubiquitin mRNA expression

The effect of food withdrawal for a 14-day period on the expression of polyubiquitin and proteasome N3 mRNAs was studied in RNA isolated from both liver and muscle using a semi-quantitative PCR approach. The PCR reaction products for both polyubiquitin and proteasome N3 mRNAs were normalized using the signal from β actin. No significant differences in expression were found in liver or muscle proteasome mRNA levels between fed and starved groups (Table 1). There was a 2-fold (P<0.05) lower level of expression of polyubiquitin in RNA isolated from liver in starved fish, but no effect of dietary regime on muscle ubiquitin mRNA levels was observed (Table 1).

Effect of food withdrawal on 20S proteasome activity and ubiquitin abundance

Proteolytic activity against the 20S proteasome-specific substrate was determined in liver and muscle samples from fed and 14-day starved fish. Starvation significantly reduced 20S proteasome activity in the liver by 26% compared with the fed fish (P<0.05). Likewise, starvation also appeared to decrease 20S proteasome activity in muscle tissue by 33%, but this failed to attain statistical significance (P=0.15). When all the individuals were compared, irrespective of dietary regime, there was a significant positive correlation between the 20S activity in liver and muscle of individual fish (r=0.674, P<0.05). Food withdrawal for 14 days reduced the amount of ubiquitin in muscle tissues by 66% (P<0.05) and in liver by 26% (P<0.05) (Table 2). To assess whether 20S proteasome activity and ubiquitin abundance were co-regulated in these fish, both muscle and liver data for fish on both dietary regimes were pooled. There was a positive and highly significant correlation (r= 0.924, P<0.001, n=18) between 20S activity and ubiquitin abundance

Table 1 Effect of 14-day food withdrawal on mRNA levels of the proteasome N3 subunit and ubiquitin in muscle and liver tissue of rainbow trout. Data presented are mean ± SEM, n=5

Dietary regime	Proteasome N3-subunit mRNA [a]		Ubiquitin mRNA[a]	
	Muscle	Liver	Muscle	Liver
Fed	0.34±0.09	0.76±0.15	0.25±0.04	0.25±0.03
Starved	0.52±0.13	0.62±0.22	0.25±0.08	0.13±0.02*

* Means are significantly different (P<0.05) within columns from the fed group
[a] mRNA levels were determined by semi-quantitative rt-PCR and normalized to β actin

Table 2 20S proteasome activity and ubiquitin abundance in liver and muscle lysates from fed and starved rainbow trout. Data presented as means ± SEM, *n*=5

Dietary regime	20S Proteasome activity [a]		Ubiquitin abundance [b]	
	Muscle	Liver	Muscle	Liver
Fed	40.8±7.3	108.4±8.3	32.3±7.7	91.2±6.3
Starved	27.3±4.1	79.8±5.5*	10.9±3.6*	67.6±6.3*

* Means are significantly different ($P<0.05$) within columns from fed group
[a] Proteasome activity presented as pmol AMC 50 μg^{-1} protein min^{-1}
[b] Ubiquitin abundance determined by immuno-dot blot analysis followed by densitometric analysis

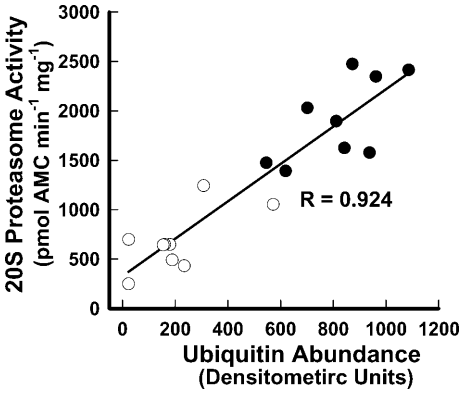

Fig. 6 Relationship between 20S proteasome activity and ubiquitin abundance in the muscle (*open circles*) and liver (*closed circle*) in individual rainbow trout. Data are from fish fed *ad libitum* and starved for 14 days

(Fig. 6). There were also correlations between 20S activity and ubiquitin abundance for muscle alone (r= 0.670, $P<0.05$, n=9) and for liver alone (r= 0.669, $P<0.05$, n=9).

Discussion

Cold-blooded animals such as fish do not require the same high continuous metabolic rate as warm-blooded mammals. In parallel, many species of fish such as salmon undergo prolonged starvation, especially during spawning migrations [33], when stored energy reserves are used. The activity and control of the ubiquitin-proteasome proteolysis system has not been studied extensively in fish.

In this study we cloned full-length cDNA sequences for the proteasome β subunit N3 and polyubiquitin mRNA and examined expression patterns of these mRNAs. In parallel, the 20S proteasome activity and abundance of ubiquitin have been assayed in cell lysates. The β proteasome subunit genes are all evolved from a single common ancestral gene and have remained markedly conserved during evolution [45]. The highly conserved structure reflects their fundamental roles in the biochemistry of the cell, with deletions of proteasomal subunit genes in yeast resulting in recessive lethality [12]. The sequence analysis has shown that the proteasome subunit has a high level of protein homology to other proteasome β N3 sequences, 84.6% amino acid identity to *Xenopus*, and 73% identity to the human sequence. This is a level of homology similar to that found for other proteasome subunits that have been isolated from fish. For example, trout LMP2 [11] has 67.2% identity to human LMP2, and goldfish subunit β6 [49] has 81% similarity to its human homologue [45]. The human N3 subunit is processed by cleavage of a leader sequence, before assembly into the 20S proteasome core [47]. In both rat and human proteins, there is a cleavage site between an arginine and threonine residue (positions 44 and 45 of the human sequence). This arginine is replaced in both *Xenopus* and trout with a histidine amino acid. The proposed function of this post-translational processing is to maintain the subunit in an inactive form until it is assembled. It has been suggested that this removal of a pro-peptide occurs for other proteasome 20S β subunits [17].

The polyubiquitin cDNA has four 228-bp repeats encoding the ubiquitin 76-amino-acid monomer, which has 100% identity to other vertebrate ubiquitin sequences. All the nucleotide changes are in second or third base positions not resulting in amino acid changes. The final repeat (R4) has more nucleotide changes, especially towards the 3' end of the repeat. The final repeat has an additional asparagine residue before the transcription stop signal, as has been shown to be the case in other polyubiquitin sequences. In mammals, this final additional amino acid is usually a tyrosine [16]. Ubiquitin genes have been cloned from various species and can be classed according to the number of repeated ubiquitin monomers as follows: nine or more, three or four, and monomeric ubiquitin. These are referred to as human UbC, UbB and UbA, respectively [54]. The sequence we report here is determined to be a homologue of UbB, as it has four repeats. Several fish ubiquitin sequences have been reported for Atlantic salmon [10] and Japanese flounder (*Paralichthys olivaceus*) [34]. However, these have been short expressed sequence tags and have not been classified, nor have the number of repeats or expression patterns been determined.

The tissue-distribution pattern of proteasome N3 shows a single transcript in all tissues. The extent of expression varies, with the highest abundance of transcript in the gill. The tissue-expression pattern of polyubiquitin shows a major transcript of approximately 1.2 kb in all tissues, with highest expression in gill. White muscle has additional hybridization bands that presumably are mRNAs encoding from other loci containing additional repeats; these would be homologues of other

UbB loci and UbC. The probe used in these experiments was a 228-bp ubiquitin sequence, which therefore will hybridize to mRNA that is encoded by other ubiquitin loci. The tissue expression pattern in rainbow trout is different to that found in mammals, where all three loci are expressed in all tissues at equal levels [16, 22].

The expression of the proteasome N3 and polyubiquitin mRNAs was examined in RNA from muscle and liver tissue of fed and starved fish by semi-quantitative PCR. The expression studies revealed that no significant difference is observed for proteasome subunit N3 gene expression in RNA isolated from either muscle or liver between fed and starved fish. Ubiquitin was not found to differ between muscle samples, but fed fish were shown to have significantly greater expression of polyubiquitin mRNA in the liver compared to starved fish. The ubiquitin expression reflects the total expression of different polyubiquitin genes, as the primers used for this analysis were within the 228-bp repeat. When PCR products were separated on 1.5 agarose gels, a ladder of products were seen to increase by 228 bp (data not shown), reflecting repeat structure of the mRNA. When all fish were grouped (fed and starved), a positive correlation was obtained between polyubiquitin and proteasome N3 expression in RNA extracted from muscle tissue (Pearson correlation 0.677, $P<0.05$, $n=10$). These results strongly indicate a similar control of gene transcription for these mRNAs.

Relative levels of ubiquitin protein were detected by immunoassay. The antibody used has been widely used for different species, and because the ubiquitin peptide sequence is completely conserved, there is no concern about recognition [31]. The sensitivity of immuno-detection of ubiquitin is increased >50-fold by boiling the nitrocellulose membrane on which the proteins are bound before detection [44]. This is believed to expose the ubiquitin epitopes that the antibody recognizes. In these experiments, both conjugated ubiquitin and free ubiquitin are measured, as the antibody does not distinguish between ubiquitin and ubiquitin-conjugated proteins. The tissue abundance of ubiquitin revealed that gill tissue had the highest concentration of ubiquitin, with liver kidney and spleen having about 50% of that found in gill. Protein lysates from liver and muscle were examined to determine whether the abundance of ubiquitin was influenced by starvation. Fed fish had significantly greater levels of ubiquitin in both liver and muscle than did starved fish. This would suggest a decrease in ubiquitination of proteins and a decrease in overall proteolysis by this pathway in starved trout.

The activity of the proteasome is measured as the release of fluorogenic substrate over a 30-min reaction time. The three main peptidase activities of the proteasome are chymotrypsin-like, trypsin-like and peptidylglutamyl peptide-hydrolysing activity [40, 46]. During in vitro assays, the 20S proteasome is activated with low concentrations of SDS. The assay developed for crude proteasome activity revealed maximum activity with 0.0475% SDS in mammals [43], and the same concentration was found to give high activity in fish. Proteasome activity was greatest in the gill, which reflects the mRNA expression pattern for the proteasome subunit N3. A positive correlation is found for proteasome activity in liver and muscle for individual animals when all the results are pooled (Pearson correlation 0.643, $P=0.045$, $n=10$).

The activity of the ubiquitin proteasome is highest in the gill. Gills are metabolically very active tissues and are the main osmoregulatory and respiratory organs of fish. Our observations from this work show the highest ubiquitin-proteasome activity and gene expression to be in the gill, which is likely to reflect the fate of synthesized proteins. In fish, liver often has the highest protein synthesis rates (reviewed in [5]), but these rates vary considerably following feeding [27, 28]. In the liver, many proteins are destined for export to the plasma, such as albumins, whereas in the gill, most of the protein synthesis is for new cell growth and rapid turnover of cellular proteins. The proteasome activity increases in parallel with an increasing abundance of ubiquitin (Fig. 6). This co-regulation agrees with findings in mammals [30]. During starvation, there is a decrease in proteasome-ubiquitin activity in both liver and muscle, which highlights the different physiology between ectothermic and endothermic animals. In mammals the proteasome-ubiquitin system is activated following 24-h starvation in rat [30, 55], indicating an increase in proteolysis. This increase reflects the much higher metabolic rate and energy requirements and general protein turnover in mammals. In rainbow trout, the relative ratio of degradation to synthesis may change, with the synthesis level decreasing more than the degradation and with the balance being shifted towards degradation in starved fish.

In fish amino acids are released by catabolism for energy production and synthesis of new proteins [3, 35]. In this report we have shown that the proteasome-ubiquitin pathway of protein degradation is decreased in fish under starvation conditions. This demonstrates altered regulation and requirements to mammals [30]. A fuller understanding of the pathways of protein degradation and the regulation of these pathways will help identify fish with a greater capacity for growth.

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