



Proteome analysis of rainbow trout (*Oncorhynchus mykiss*) liver proteins during short term starvation

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Abstract

Basal rates of protein synthesis in the liver are similar in fed and starved trout; during starvation protein degradation must increase as the animal loses weight and the liver decreases in size. Little is known about how protein degradation is controlled in fish under various physiological circumstances. In this study proteome analysis has been used to identify proteins that are changed in abundance that may be involved in increased protein degradation in the liver of rainbow trout following a period of 14 days without food. Protein extracts from whole liver were analysed on high resolution two dimensional gels. The protein profiles from individual fish were digitised and computer software used to construct a composite reference gel. In total 780 protein spots were identified and their abundance monitored for fed and starved groups of fish. All protein spots were recorded in terms of their isoelectric point (pI), molecular weight and abundance. Twenty four proteins were found to have differences in abundance between the two groups, 8 were increased in fed fish with 16 increased in abundance as a result of food withdrawal. Twenty two protein spots were excised from gels and subjected to trypsin digestion followed by peptide separation by MALDI-TOF spectrometry. Peptide masses were used to search the GenBank data base for protein identification. Twelve of the proteins were identified on the basis of the homology of their peptide profiles to existing protein sequences. One protein, which increased in abundance under starvation conditions, was identified as cathepsin D, a lysosomal endopeptidase involved in protein degradation. Northern blot analysis of RNA isolated from liver of rainbow trout showed an increase in expression of cathepsin D reflecting either increased transcription or stability of the mRNA in starved fish, supporting the proteome evidence. Thus in starved trout an increase in lysosomal proteases may play a part in the loss of proteins.

Introduction

The conversion of ingested protein to protein growth varies between individuals and between species. The efficiency of protein growth is related to the balance between protein synthesis and protein degradation, for positive growth rates, synthesis of protein needs to exceed degradation. In fish, a high proportion of the synthesised protein is retained as protein growth (Houlihan et al. 1995). To further understand the mechanism that controls protein retention it is necessary to identify the protein degradation pathways that

may be involved in the control of protein deposition during protein accretion.

Proteolytic systems are involved in various major biological events, including the control and degradation of specific proteins during the cell cycle, growth and development (Raj et al. 2000; Schmidt et al. 2000) and during the immune response (Pamer and Cresswell 1998). Until recently protein degradation was viewed as a method of eliminating abnormal, misfolded or damaged proteins and for the regulation of some key enzymes by undefined processes. It is now clear that protein degradation is a highly regulated cellular process (Attaix et al. 1999; Cuervo and Dice

1998). The two main routes of protein degradation are the lysosomal and non-lysosomal or ubiquitin dependent pathways which are highly conserved amongst eukaryotes. In mammals, pathways of protein degradation vary in importance between tissues, with the lysosomal enzymes being particularly active in the liver (Scornik and Botbol 1987), and the ubiquitin-proteasome route being more significant in the muscle (Mitch and Goldberg 1996). The identification of proteins by the cell for degradation is under strict control with the entry of protein into lysosomes prior to degradation is highly specific and that degradation rates are highly variable between proteins (Waterlow et al. 1978). Selection for ubiquitination of a limited number of proteins may depend on the N terminal amino acid of a protein (Solomon et al. 1998). The main process responsible for hepatic protein degradation is macro autophagy and subsequent degradation by lysosomal enzymes (Meijer et al. 1999), accounting for approximately 80% of starvation induced proteolysis.

The liver responds early to the withdrawal of food, in mammals; loss of protein is extremely rapid, with rat liver losing 20–25% of cellular protein after 1 day of starvation (Seglen and Bohley 1992). This process is inhibited by insulin and amino acids, but is stimulated by glucagon (Blommaert et al. 1997), resulting in the protein content of the liver fluctuating with nutritional intake, with proteolysis being more pronounced during fasting (Botbol and Scornik 1991).

Few studies have been performed to determine if fish undergo similar mechanisms of proteolysis as mammals and if these processes are regulated in a similar manner. Starvation in fish results in low levels of protein synthesis in liver and higher rates of protein degradation (McMillan and Houlihan 1998; Houlihan 1991). During starvation protein stores are depleted to release free amino acids for both energy and synthesis of new proteins (Navarro et al. 1997). In the current report we have used the techniques of proteomics to identify proteins that are expressed in a differential manner in fed and starved trout to obtain an insight into the mechanism leading to protein degradation. Liver protein homogenates from fed and starved rainbow trout were analysed by two dimensional protein gel electrophoresis (2DE) and those proteins showing differences between the two groups were subjected to peptide mass mapping. One protein that was found to be dramatically upregulated in fish that had food withdrawn was identified as cathepsin D. In addition northern blot analysis showed the

mRNA encoding cathepsin D was also increased under starvation conditions.

Materials and methods

Fish

Rainbow trout were maintained in fresh water at 12 °C throughout the experiment. Fish were all female stock and had a mean weight of 185.5 g (± 10.9 S.E.M $n = 30$) at the beginning of the experiment. Fish were fed once a day *ad libitum*, this did not exceed 2% body weight on any one day. The experimental group was starved 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. The growth rates G_w , % body weight per day of the groups was calculated from the equation $G_w = ([\ln W_2 - \ln W_1]/t) \times 100$ where W_2 is the final weight and W_1 is the initial weight and t is time in days (Ricker 1979).

Protein extraction and 2D gel electrophoresis

Fish were sacrificed by an overdose of anaesthetic (benzocaine, Sigma) followed by decapitation; the livers tissue were removed immediately after death and spilt into two roughly equal pieces and stored at -70 °C. Frozen tissue was homogenised in lysis buffer (9 M urea, 2% (w/v) CHAPS, 25 mM Tris-HCl pH 7.5, 3 mM EDTA, 50 mM KCl, 50 mM DTT, 2% (w/v) Resolyte™ [Merck], 40 μ M leupeptin) at room temperature, using a Dounce teflon homogeniser. Following homogenisation the tissue lysates were centrifuged at 50,000 g for 20 min at 15 °C to remove any insoluble particles. The supernatant was then stored at -70 °C until gel electrophoresis was performed. Soluble trout liver proteins (10 μ l) were mixed with 115 μ l re-swelling buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.3% DTT), and then added to a 7 cm pH 4–7 immobilised pH Gradient strip (IPG) (Amersham-Pharmacia Biotech). Isoelectric focussing was performed in three stages with a ramped voltage change between each step, 200 V for 1 min, 3500 V for 1 h 30 min, (all stages at 2 mA and 5 W). For the second dimension electrophoresis the IPG strip was laid onto a 10–15% gradient polyacrylamide slab gel (8 \times 7 cm) and the proteins electrophoresed at 150 V for 450 Volthours (Cash et al. 1999). The resolved proteins were detected using Colloidal Coomassie Blue G250 staining (Anderson

et al. 1991). Molecular masses of the proteins were determined by coelectrophoresis with standard protein markers. Iso-electric points were determined based on the linearity of the IPG strip.

Computer analysis

The 2-D protein profiles were used to construct a 2-D data base for trout liver proteins, the gels were scanned at a resolution of 200 dpi using a Hewlett Packard Scanjet 3p flat bed scanner and stored as TIF files. Subsequent analysis of the gel images was performed using the software package *Phoretix 2-D* version 5.01 (NonLinear Dynamics, Gateshead, UK). Protein spots were detected using automated routines from the software combined with manual editing to remove artefacts. One gel from a fed fish was selected as the basis for the construction of a reference gel against which the remaining gels were matched using standard routines from within the software. Each spot within the reference gel was assigned a spot number which is used in the subsequent description to refer to individual spots. The amount of protein present in a spot is taken as the area of the spot multiplied by the density and referred to as the volume. Following removal of background the spot volumes were normalised to the total protein detected for each gel. The normalised spot volume is referred to as abundance. A 2-fold difference in protein abundance was taken as showing a difference between two gels. Those proteins found to be consistently different between the groups were analysed for significance using a student's *t*-test.

Protein identification by peptide mass mapping

Proteins of interest were excised from the stained gel and subjected to in-gel trypsin digestion (Jensen et al. 1999). Briefly excised spots were washed, reduced, S-alkylated, and digested within the gel using trypsin (sequencing grade modified trypsin; Promega) as described elsewhere (Shevchenko et al. 1996; Wilm et al. 1996). An aliquot of the peptide extract produced by this process was passed through a GELoader tip containing a small volume of POROS R2 sorbent (PerSeptive BioSystems, USA) as previously described (Wilm et al. 1996). The adsorbed peptides were eluted in 0.5 μ l saturated solution of α -cyanol-4-hydroxycinnamic acid in 50% acetonitrile/5% formic acid. The mass spectra of the peptide fragments were obtained on a PerSeptive Biosystems Voyager-DE STR MALDI -TOF mass spectrometer. The instrument was operated in the reflection delayed extraction

mode. Spectra were internally calibrated using trypsin auto-digestion products.

Molecular masses of the tryptic peptide profiles produced were used to search the National Center for Biotechnology Information (NCBI) nucleotide data base using the MASCOT program (Perkins et al. 1999). The peptide masses were compared to the theoretical peptide masses of all available proteins and predicted proteins from DNA sequences. Unmatched peptides were not considered in the analysis. All peptide fragments that were obtained for each digest were submitted for searching. Search parameters for the program were as follows: maximum allowed error of peptide mass 250 ppm; cysteine as S-carbamidomethyl-derivative and oxidation of methionine allowed.

RNA extraction and probe preparation

Liver tissue for RNA extraction was frozen at -70°C immediately after death. Total RNA was isolated using TRIzol reagent (Gibco). Briefly, liver (approximately 500 mg) was homogenised in the lysis buffer (5 ml), 200 μ l chloroform was added to each one ml of lysis buffer, vortexed and centrifuged for 10 min at 14,000 *g*. The aqueous phase containing the RNA was retained and RNA precipitated with an equal volume of isopropanol. The RNA pellet was washed with 80% ethanol, dried and resuspended in 500 μ l DEPC treated water. The concentration of RNA was determined by spectrophotometry.

For probe preparation, total RNA from liver was reverse transcribed using oligo dT₁₇ primer (100 ng μ l⁻¹). RNA was denatured (65 $^{\circ}\text{C}$ 10 min) and then cooled slowly to allow annealing of the primer to the template. To this 4 μ l 5 \times reverse transcriptase buffer, 2 μ l dNTPs (final concentration 200 μ M each), and 15 U reverse transcriptase enzyme (RNase H⁻ Gibco BRL) was added, the reaction was incubated at 42 $^{\circ}\text{C}$ for 1 h and stopped by incubation for 10 min 65 $^{\circ}\text{C}$. This cDNA was used as a template for PCR using primers designed against the rainbow trout cathepsin D sequence (forward 5'ACCTCAGCCAGACACATGC 3'; reverse 5'CAATGAATACATCTCCCAGG 3') (Brooks et al. 1997). PCR conditions were 30 cycles 94 $^{\circ}\text{C}$ 1 min, 55 $^{\circ}\text{C}$ 1 min, 72 $^{\circ}\text{C}$ 1 min, followed by an extension of 72 $^{\circ}\text{C}$ 10 min. A cDNA product (689 bp) was cloned into a plasmid (pCR2.1, Invitrogen) cloning vector and sequence analysis confirmed it was the cathepsin D cDNA. This cDNA fragment was excised from the

plasmid using *EcoRI* restriction enzyme and used as a probe for northern blot analysis.

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, Amersham Pharmacia Biotech) by capillary blotting using 20 \times SSC. The RNA was then fixed to the membrane by baking at 80 °C for 2 h. The membranes were prehybridised at 65 °C in rapid-hyb buffer (Amersham Pharmacia Biotech) for 2 h. The filters were hybridised overnight with cathepsin D cDNA. For probe labeling cDNA was gel purified from digested plasmid and random primed with ^{32}P dCTP using labelling beads (Amersham Pharmacia Biotech) to a specific activity of $\sim 1 \times 10^8$ cpm μg^{-1} . Following hybridisation the filters were washed to a stringency of 0.2 \times SSC, 0.1% SDS at 65 °C and then exposed to X-ray film for autoradiography.

The probe was removed from the membrane after autoradiography by incubation with 0.1% SDS 95 °C for 15 min. This wash was repeated twice to ensure complete removal of the probe. Confirmation of loading was carried out by reprobing the membranes with a rainbow trout β actin cDNA clone, hybridization and washing conditions for this were the same as for the cathepsin D probe. Densitometric analysis of northern blots was performed using Gelworks 1 D Advanced program (Non-Linear Dynamics).

Results

Generation of the 2D reference map for trout liver proteins

All fish survived the 2 weeks starvation protocol described in Materials and methods. The fed fish group had mean growth rate 1.6% \pm 0.08/day (mean \pm SEM $n = 7$) with the starved fish losing weight as indicated by negative growth rate -0.34% \pm 0.02/day (mean \pm SEM $n = 3$). Liver protein extracts were subjected to 2D electrophoresis as described in Materials and methods. Liver protein extracts from three fish from the starved group (S1–S3) and three fish from the fed group (F1–F3) were compared by this approach.

The number of spots detected on the gels varied between the six gels from 717 to 800. After editing the number of protein spots maintained for analysis

Table 1. Twenty four protein spots that were shown to be of different abundance in fed or starved trout. The measured charge (pI), molecular weight (kDa) and the direction of change indicated (F indicates increased in fed fish, S indicates increase in starved fish) are shown with the result of a paired *t*-test on 3 samples from fed and starved animals

Spot	pI	kDa	Change	<i>t</i> -test
103	6.2	70.8	F	0.036
194	6.9	53.0	F	0.055
207	6.5	51.0	S	0.003
212	5.4	50.9	S	0.053
220	6.1	49.5	F	0.088
231	6.5	47.9	S	0.118
243	6.2	47.3	S	0.030
260	5.3	44.6	S	0.044
317	5.2	38.6	S	0.004
353	6.6	36.4	S	0.007
382	6.5	34.1	F	0.126
384	5.8	34.2	S	0.014
416	6.3	32.2	S	0.024
441	6.9	31.0	F	<0.001
467	6.9	29.4	S	0.133
471	4.9	29.4	F	0.186
473	6.4	29.0	F	0.058
487	5.4	28.1	F	0.116
489	5.9	27.8	S	0.041
490	6.0	28.0	S	0.003
517	4.5	24.8	S	0.036
524	6.9	24.4	S	0.132
528	6.4	23.3	S	0.044
537	6.2	20.9	S	0.024

was 780. The detected proteins had molecular masses of between 97,400 and 12,400 and pI's between 4 and 7. The abundance of the individual protein spots, detected on the gels, ranged between 0.0016 and 5.3% of the total detected protein on the gel.

The protein profile (Figure 1) from fish F1 was chosen for production of a reference gel for the current study. Following the matching of the other 5 gel profiles (2 fed and 3 starved) to the reference profile, 780 proteins were assigned to the reference protein map. All proteins assigned to the reference profile were detected for at least 2 out of 3 of the gels for either fish group.

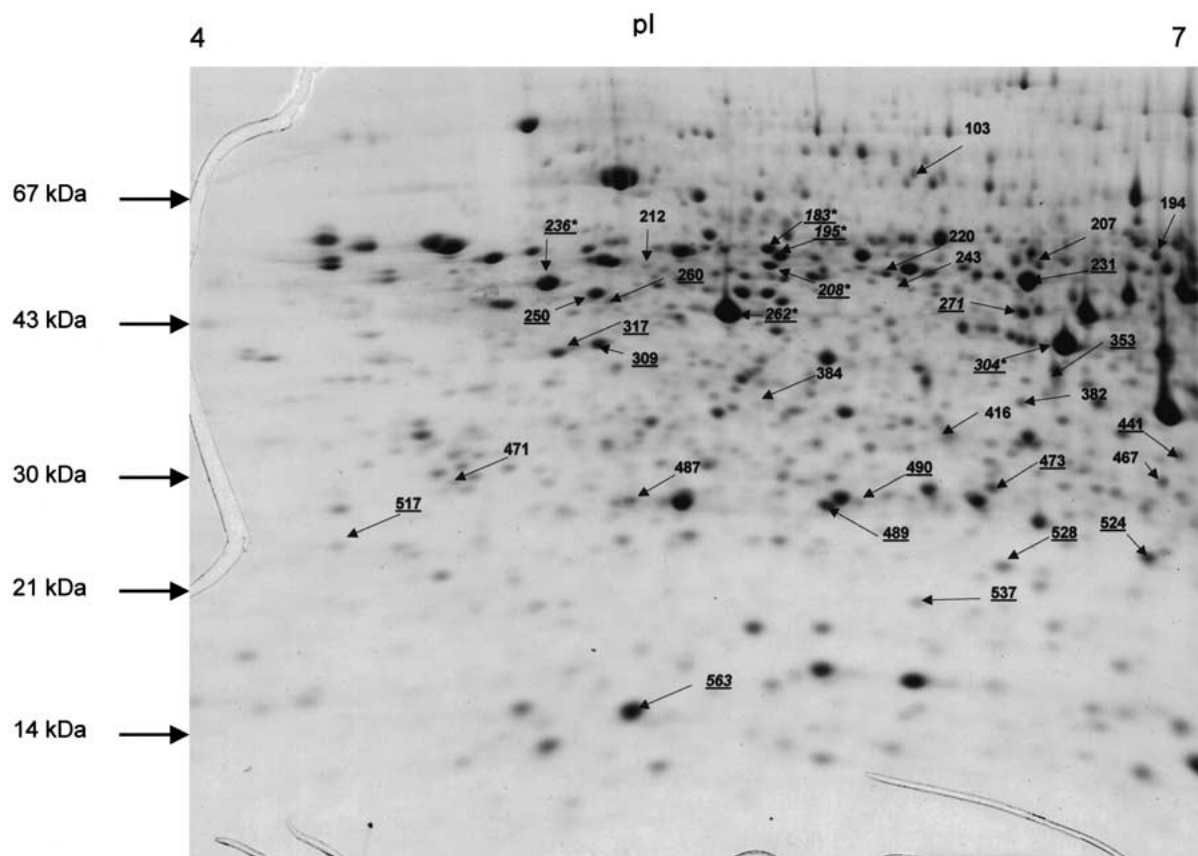


Figure 1. Representative gel of rainbow trout liver proteins and analysed by 2DE. A total liver protein extract was prepared from fish F1 and analysed by 2DE as described in Methods. The proteins were located by staining with colloidal Coomassie blue G250. Protein spots showing significant changes in their abundance as a result of starvation are indicated by the arrows. Spot numbers that are underlined were analysed further by peptide mass mapping. Protein spots that were used only for mass mapping and not of different abundance have an asterisk (*) after the number.

Detection of differentially expressed proteins

Proteins were considered to be differentially expressed between the two groups of fish based on the following criteria: (1) there was ≥ 2 -fold difference in the spot abundance between starved and fed fish, (2) the change was consistent for the three replicate analyses for each group of fish. Twenty-four proteins fulfilled these criteria and are shown in Figure 1 on a representative profile of rainbow trout liver proteins derived from fish F1. Table 1 shows the quantitative changes observed from these 24 proteins as a result of the withdrawal of food. Figure 2 shows the mean normalised volume value of the proteins from the fed and starved groups of fish. The greatest changes in abundance were observed for spots 317 and 243, which increased 6.5- and 7-fold in abundance respectively in starved compared to fed fish. The consistent increase in abun-

dance of spot 317 is shown as enlarged sections of gels in Figure 3.

Identification of protein spots by peptide mass fingerprinting and mass spectrometry

Protein spots were excised from the gels run specifically for spot excision. Twelve out of the 22 proteins analysed were found to be differentially expressed between the two groups of fish (Figure 1, Table 1); the remaining differentially expressed proteins were not excised because of low level of protein or they were too close to neighbouring proteins to be confident of no contamination. The ten other protein spots were selected for analysis as they were present as highly abundant spots (marked on Figure 1). The extra protein spots were to help determine how effective peptide mass mapping is for identification of trout proteins. A selection of which proteins that could be removed

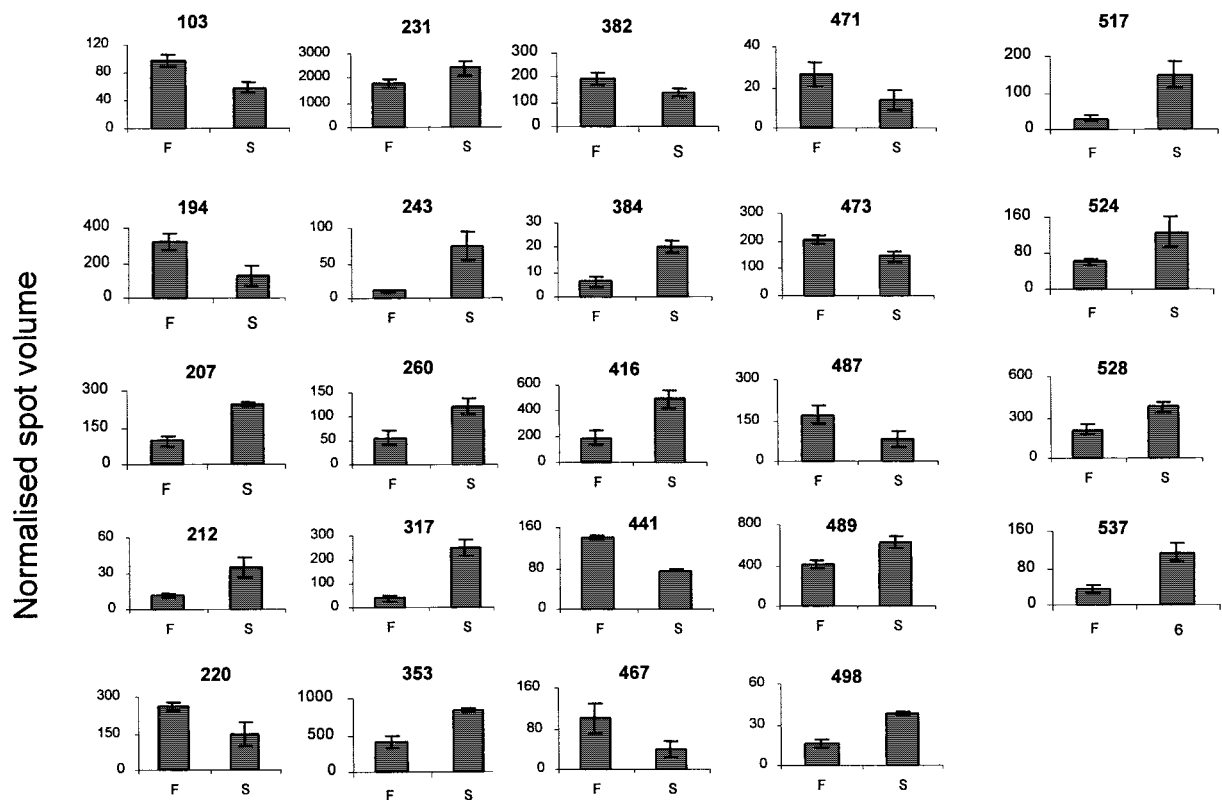


Figure 2. Graphs of differences in abundance. Means of spot volume and S.E.M. of fed (F) and starved (S) liver proteins for the spots marked by arrows in figure 1. Spots analysed here were present on all gels in both fed and starved groups.

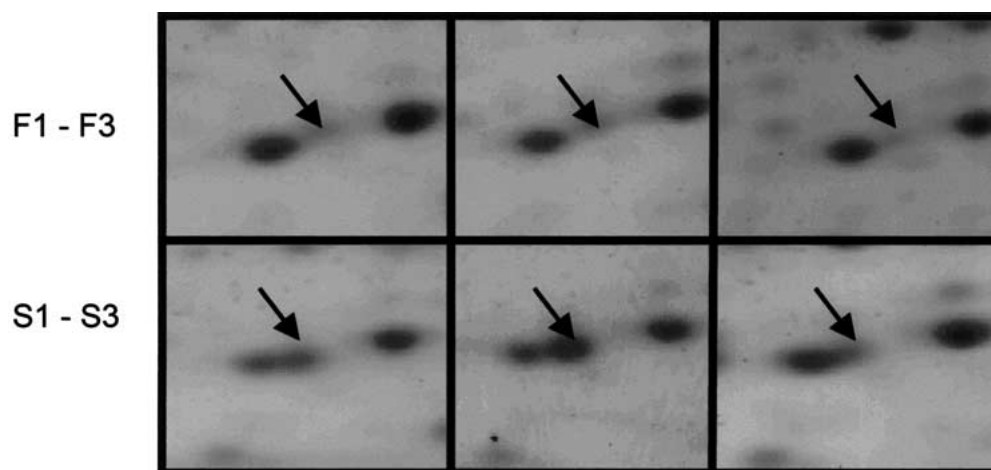


Figure 3. Changes in synthesis of protein 317 due to starvation. Total liver protein extracts were prepared from fed (F1-F3) and starved (S1-S3) fish and analysed by 2DE as described in Methods. Each panel shows enlarged regions of the gels for spot 317 (Figure 1). The position of spot 317 is indicated by the arrow.

from the gels was based on the criteria that a single spot could be cut without contamination from neighbouring spots. Nineteen of the 22 spots processed yielded tryptic peptides that were suitable for data base searching. The remaining 3 protein spots (524, 528, 537) failed to produce tryptic peptides possible due to their low abundance in the gels.

The peptide fragment masses were used to search the GenBank database for protein homologies using the Mascot search program (Perkins et al. 1999). Protein homologies were obtained for 13 spots. Five of the proteins were identified from protein or cDNA sequences already described in teleost species, four from rainbow trout (spots 183, 250, 262, 317) and one from a cyprinid fish (236). The remaining protein homologies were distributed between lower vertebrates (spots 231, 304 and 473), invertebrates (spots 260 and 271) and mammal sequences (spots 195, 489 and 517). Table 2 summarises the data of the MASCOT search for all the protein characterised by this approach in the current study. A representative trypsin digest fingerprint is shown for protein spot 317 (Figure 4a) together with the predicted peptide fragments and the coverage (25%) of the identified protein (Figure 4b). This protein was identified as rainbow trout cathepsin D (Brooks et al. 1997) and was used for further experiments to determine gene expression.

Northern blot analysis

Northern blot analysis on RNA isolated from different tissues of trout showed that the cathepsin D probe hybridised a single transcript (1.8 Kb). The highest level of expression was seen for RNA extracted from kidney and muscle, with lower levels in liver and spleen (Figure 5a). To ascertain if there was a corresponding change in mRNA level as seen for protein (Figure 3), RNA from the livers of fed and starved fish was extracted and used for northern blot analysis. This showed there was an increase in the cathepsin D transcript under the starvation conditions (Figure 5b). The blot was reprobbed with rainbow trout cytoplasmic β actin cDNA probe corresponding to spot 262 (which was shown to have no difference in abundance in all six gels analysed) as a control to normalise RNA between lanes, no difference in expression of β actin mRNA was observed. The densitometric analysis showed there was a significant increase in the expression of cathepsin D mRNA as compared to β actin mRNA ($p > 0.05$). The results of this analysis are shown in Figure 6.

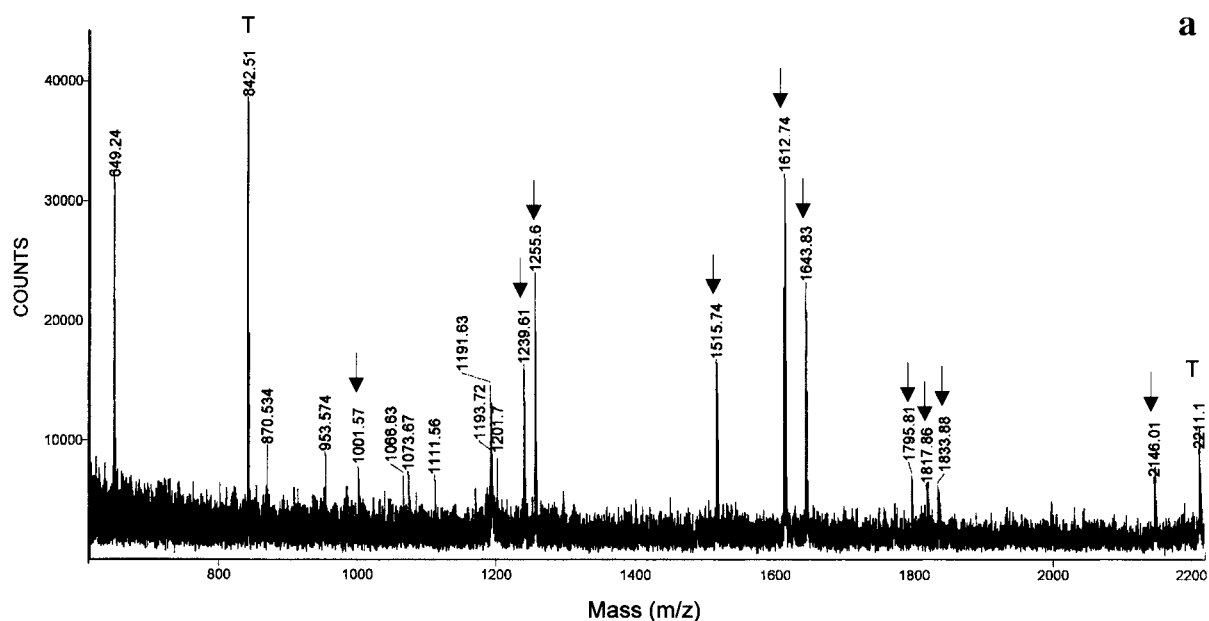
Discussion

Proteomics, the global analysis of protein synthesis, observes the end product of gene expression, the protein. Changes in protein abundance can be brought about by alterations in the rate of transcription and half life of individual mRNA species as well post-translational events. Other approaches for analysing differential gene expression, for example reverse transcription PCR differential display (Liang and Pardee 1992), subtractive cloning (Diatchenko et al. 1996) and mRNA profiling using micro arrays (Lockhart and Winzler 2000; Gracey et al. 2001) assay only mRNA expression, which are not always reflected in the levels of protein synthesis. Consequently, proteomics has the potential to provide a more comprehensive view of gene expression than nucleic acid based technologies. Two-dimensional protein gels, combined with peptide mass mapping by MALDI-TOF for protein identification, are widely used for determining differential protein synthesis (i.e., protein profiling) in biological systems. In the current study, we describe the use of protein profiling in a non model organism rainbow trout to demonstrate, for the first time in teleosts, that proteomics has potential to help us study cellular mechanisms involved in protein degradation.

Gel images

Under the analytical conditions employed in the current study we were able to measure the abundance of over 700 protein spots in the fed and starved fish. Studies in mouse liver identified 1,300 protein spots of which 800 were chosen for analysis (Fountoulakis et al. 2000). Previous studies on analysis of fish proteins using 2DE examined gill tissue in *Gillichthys mirabilis* (Kultz and Somero 1996) and in whole embryo during development of *Oncorhynchus* species (Kanaya et al. 2000). Neither of these studies integrated protein identification using peptide map fingerprinting. N terminal sequencing was used (Kanaya et al. 2000) to identify the protein vitellogenin which was present as multiple spots in the protein profile of embryonic rainbow trout.

We have identified protein spots that are present at different levels as a result of starvation. Twenty-four spots were found to show consistent differences in their abundance between fed and starved trout, this represents 3.6% of the protein spots analysed in the study. Eight of the proteins showed an increased abundance in the fed fish while and 16 proteins had an



Observed	Mr(expt)	Mr(calc)	Start	End	Miss	Peptide
1001.57	1000.56	1000.57	173	182	0	QPGVAFIAAK
1239.61	1238.60	1238.6	183	193	0	FDGILGMAYPR
1255.60	1254.59	1254.61	183	193	0	FDGILGMAYPR 1 Oxidation (M)
1515.74	1514.73	1514.75	212	223	0	VEQNVFSFYLNLR
1612.74	1611.73	1611.72	242	254	0	YYSQDFQYLDVSR
1643.83	1642.82	1642.85	211	223	1	KVEQNVFSFYLNLR
1795.81	1794.80	1794.86	224	241	0	NPDSEPGGELLGGTDPK
1817.86	1816.85	1816.90	194	210	0	ISVDGVAPPPDNIMSQK
1833.88	1832.87	1832.90	194	210	0	ISVDGVAPPPDNIMSQK 1 Oxidation (M)
2146.01	2145.00	2145.07	276	297	0	GGCEAIVDTGTSLLITGPAAEVK

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1 MKVLYLCLFAALALASDALVRIPLRKFRSIRRTLTDSGRAAEELLAGQEH
51 TKYNNLGFPPSSNGPTPETLKNFMDAQYYGEIGLGTPTVQTFVFDTGSS
101 NLWVPSVHCSFTDIACLLHHKYNGAKSSTYVKNGTAFAIQYGGSLSGYL
151 SQDTCITIGLSIEDQGFGEAIKQPGVAFIAAKFDGILGMAYPRI SVDGVA
201 PFFDNIMSQKKVEQNVFSFYLNLRNPDSEPGGELLGGTDPKYYSGDFQYL
251 DVSRQAYWQIHMDGMGVGSQLSLCKGGCEAIVDTGTSLLITGPAAEVKALQ
301 RAIGATPLIQGEYMVNCDKIPTMPVITFNLGGQSYSLTAEQYVLKESQAG
351 KTICLSGFMLDIPAPAGPLWILGDVFIGQYTVFDRDNNRVGFAKSK

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Figure 4. (a) MALDI-TOF spectrum of an in-gel tryptic digest of protein spot 317. The peptide mass spectrum was calibrated using two trypsin autolysis peaks at 842.51 and 2211.10 Da (marked by T). Other arrow heads indicated peptides identified as those derived from rainbow trout cathepsin D. (4b) The peptide fragments identified during the mascot search and the coverage of the rainbow trout cathepsin D sequence is shown with peptide fragments highlighted in black.

increased abundance in the starved fish. Proteins were excised from gels for identification using peptide mass fingerprinting. In parallel to differentially expressed protein, several major protein spots were also selected to help ascertain the usefulness of this approach, at present there is not published data on the effectiveness of protein identification in fish using peptide mass fingerprinting. Three of the protein spots excised failed to produce a tryptic peptide profile. These proteins were

of low abundance and it is probable there was insufficient protein present for analysis. In parallel, proteins of low molecular weight may not produce sufficient peptide fragments for successful searching of the data base, a size range of proteins from 12 to 66 kDa has been shown to produce most reliable peptide masses for analysis (Lahm and Langen 2000).

Table 2. Results from peptide mass fingerprinting of protein spots excised from the 2D gels. The mascot score refers to the probability of identification, where P is the absolute probability ($-10 \times \log_{10}(P)$)

Spot number	PI	Mol wt.	Homology	Species	Accession number	Mascot score
183	5.7	54.5	Simple Keratin type II	<i>Oncorhynchus mykiss</i>	X92522	88
195	5.8	52.9	Golgin-245	<i>Homo sapiens</i>	U31906	69
208	5.7	51.0	No Homology			
231 ^S	6.5	47.9	Enolase (2-Phosphoglycerate dehydrogenase)	<i>Xenopus leavis</i>	P08734	82
236	5.1	47.6	ATP synthase beta subunit	<i>Cyprinus carpio</i>	AB023582	133
250	5.3	45.8	S6 ribosomal protein	<i>Oncorhynchus mykiss</i>	AF009665	56
260	5.3	44.6	RNA polymerase I	<i>Drosophila melanogaster</i>	AE003589	76
262	5.6	42.9	Beta actin	<i>Oncorhynchus mykiss</i>	AF157514	135
271	6.5	42.8	Aromatic-L-amino-acid decarboxylase	<i>Caenorhabditis elegans</i>	P34751	74
304	6.6	38.8	Kinase -like protein	<i>Xenopus leavis</i>	I51617	67
309	5.3	38.9	No Homology			
317 ^S	5.2	38.6	Cathepsin D	<i>Oncorhynchus mykiss</i>	U90321	119
353 ^S	6.6	36.4	No Homology			
441 ^F	6.9	31.0	No Homology			
473 ^F	6.4	29.0	Hypoxanthine guanine phosphoribosyl transferase	<i>Gallus gall</i>	AJ132697	75
489 ^S	5.9	27.7	Serine/theonine kinase	<i>Mus musculus</i>	NP 033314	112
490 ^S	6.0	28.0	No Homology			
517 ^S	4.5	24.8	Cytochrome C oxidase	<i>Bos taurus</i>	P 00006	107
524 ^S	6.9	24.4	No Homology			
528 ^S	6.4	23.3	No Homology			
537 ^S	6.2	20.8	No Homology			
563	5.4	15.1	No Homology			

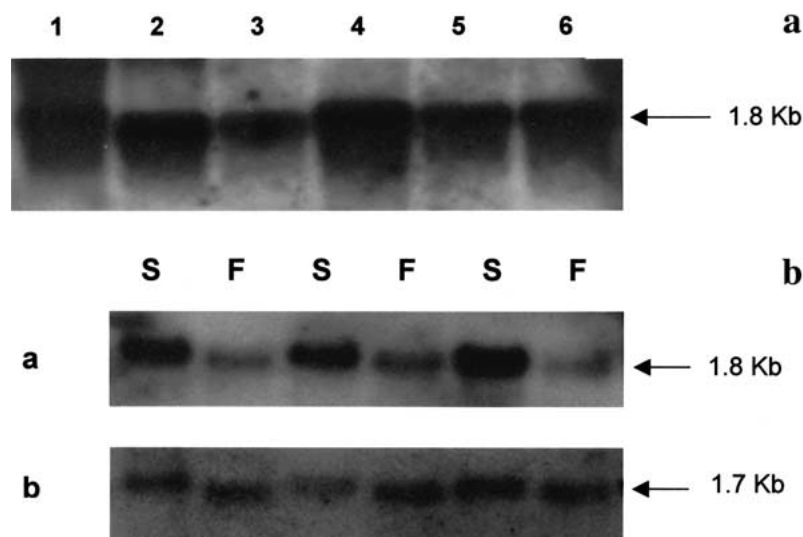


Figure 5. (a) Northern blot analysis of trout total RNA (10 μ g) isolated from different tissues hybridized with cathepsin D probe, lanes: 1, Spleen; 2, White muscle; 3, Liver; 4, Kidney; 5, Heart; 6, Gill. (b) Northern blot analysis of total RNA isolated from rainbow trout liver. RNA (10 μ g) probed with the rainbow trout cathepsin D cDNA probe (panel A). The probe was stripped from the membrane and hybridization using β actin cDNA probe is shown (panel B) to confirm equal loading between lanes.

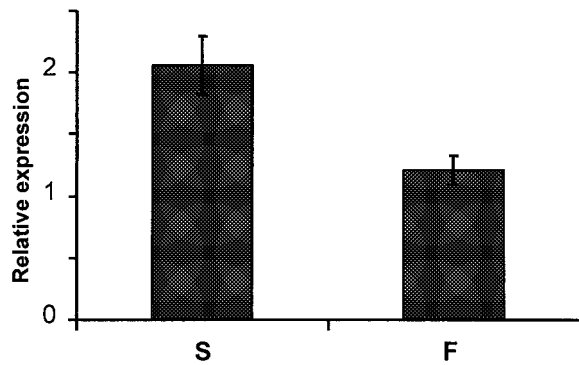


Figure 6. Graph of the mean (\pm S.E.M.) relative expression of cathepsin D mRNA from starved (S) and fed (F) rainbow trout liver RNA. The intensity of the cathepsin signal was normalised to β actin mRNA top calculate relative expression.

Fingerprinting

The post-electrophoretic method used for protein identification in the current study was peptide mass mapping. The tryptic peptide profiles generated by mass spectrometry through this method were used to search the program which provides a statistical probability for the protein identification. At the time of this study [20/11/00] the data were searched against all metazoan gene sequences, of which 13,737 were teleost sequences including 913 rainbow trout sequences. Protein identification using peptide mass fingerprinting relies on the presence in the database of either a closely related fish gene (or protein) sequence or a related protein which has a high level of amino acid sequence identity. Thirteen of the 24 (60%) rainbow trout proteins we processed for peptide mapping were found to have significant homologies with existing sequences in GenBank and were thus identified. Five out of the 13 protein identifications were made against teleost fish gene sequences in GenBank whereas the remaining proteins matched conserved proteins from other eukaryotes.

Five proteins that showed difference abundance levels in starved and fed fish were identified. One protein that was more abundant in fed compared to starved fish was identified as hypoxanthine guanine phosphoribosyl transferase (HGPRT), an enzyme involved in nucleotide biosynthesis. The mRNA encoding HGPRT increases as a result of growth factors (Gassmann et al. 1999), and the higher abundance of the HGPRT may simply reflect active growth of the fish. The remaining four proteins showed increased abundance in the starved fish. Two enzymes were identified which are involved in energy metabolism,

2-phosphoglycerate dehydrogenase (spot 231), and cytochrome c oxidase (spot 517). The reason for the increased abundance of these proteins may be due to the energy requirements for different cellular functions within the liver not used during normal feeding conditions. RNA polymerase I (spot 260) was present at a higher abundance in the livers of starved fish. The RNA content of the liver has been reported to be reduced following long term starvation of trout (70 days) (Peragon et al. 1999). Contradictory findings in brook trout (*Salvelinus fontinalis*) have shown higher concentrations of RNA in the livers of fasting compared to fed trout (McLaughlin et al. 1995).

Spot 317 was shown to be a cathepsin D from the cDNA sequence for rainbow trout ovarian cathepsin D (Brooks et al. 1997). The theoretical pI 4.92 and size 35.9 kDa for the mature cathepsin D protein, closely agrees with a calculated size of pI 5.2 and 38.6 kDa from our experimental gels. The principal route of protein degradation in liver is by the action of lysosomal enzymes. Lysosomes are membrane bound organelles containing 40–60 hydrolases. The free amino acids released from the degraded proteins are used either for further catabolism in the cytosol or reutilized in protein synthesis. A major group of proteases within the lysosomes are cathepsins, which are all aspartic peptidases classed in the pepsin family of proteases. Cathepsins D and L are endopeptidases whilst A and C are exopeptidases. The gene for cathepsin D has been cloned from various teleost fish including rainbow trout (Brooks et al. 1997), and seabream (*Sparus aurata*) (Carnevali et al. 1999). Both of these papers looked at the involvement of cathepsin D in the processing of the vitellogenin protein during early development. The level of cathepsin is also highest in the yolk sac of developing salmon embryos (Nemova and Sidorov 1985). Adult fish subjected to fasting for 30 and 60 days showed increased lysosome enzymes activities including cathepsin D (Krupnova et al. 1985). Cathepsin D activity increases in migrating salmon when the fish undergo prolonged fasting during their spawning migration (Mommensen et al. 1980). The data presented in our report are in agreement with increased cathepsin D during starvation, which may indicate a parallel increase in enzyme activity. Cathepsin D activity also increased in fish liver and gills following exposure to toxins (Kaivarainen et al. 1998). In mice cathepsin D activity increases after starvation of 2–3 days (Kovacs et al. 1989).

The level of cathepsin D mRNA was determined to compare gene expression and protein abundance. We

found that both the mRNA and protein levels increased in trout liver following two weeks starvation. β actin mRNA levels were shown to be unchanged between the starved and fed fish, the protein spot 262 identified as the β actin protein was also of similar abundance in fed and starved fish. A direct association between mRNA and protein abundance is not always found as demonstrated with for 23 proteins identified on 2-D gels for human liver (Anderson and Seilhamer 1997). A major factor influencing this may be that many highly abundant mRNAs encode for secreted proteins such as serum albumen which is not reflected in accumulations of these proteins in liver tissue (Kawamoto et al. 1996).

Biological significance of the findings

Amino acids play an important role in fish metabolism. For example, carnivorous fish such as salmonids require a high protein diet to achieve maximum growth rates and amino acids often replace carbohydrates as precursors of energy metabolism (Walton and Cowey 1982). During starvation energy requirements must be found from the body's reserves. In fish, catabolism releases amino acids for the synthesis of proteins and oxidation for energy production (Blasco et al. 1991). Many physiological adaptations are required during starvation and these are brought about by hormonal changes with the levels of insulin in the plasma falling (Moon 1998), whereas glucagon and glucocorticoids increase (Gutierrez et al. 1991). These hormonal alterations may be produced not only by starvation but also in response to seasonal changes and the fish reproductive cycle. These same modulators can also affect gene expression, in particular the pattern of gene expression for those genes encoding proteins involved in proteolysis as shown in this study.

In this report we demonstrated that protein profiling of trout liver tissue can be applied to study specific biological questions in the intact animals. The reference gel constructed as part of our work will form the basis of a liver protein map to which data will be added in future experiments. Peptide mass mapping was found to allow the identification of the majority of the proteins analysed even with an organism such as rainbow trout which has only been poorly characterised at the genomic level. As more gene sequences become available the capacity to reliably identify proteins resolved by 2DE will improve. The primary aim of this work was to increase our understanding of protein degradation in fish. We have identified a protein

that increased in abundance under starvation conditions as cathepsin D, a known lysosomal protease. Moreover, there was a parallel increase of both cathepsin D mRNA and mature protein under short-term starvation.

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