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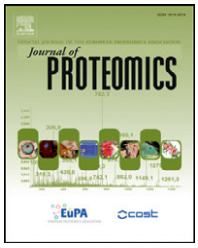

A proteomics strategy for determining the synthesis and degradation rates of individual proteins in fish☆

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ABSTRACT

In order to study the protein dynamics in the tissues of fish we have developed a proteomics-based strategy to determine the rates of synthesis and degradation of individual proteins. We have demonstrated the feasibility of this approach by measuring the turnover of multiple isoforms of parvalbumin (β1-7) in the skeletal muscle of common carp (*Cyprinus carpio*). A stable isotope-labelled amino acid ([$^2H_7$] L-leucine) was administered to the carp via the diet and its incorporation into the isoforms of parvalbumin in muscle over time was monitored by LC–MS analysis of signature peptides. The relative isotope abundance was calculated and used to deconvolute the data. The β7 parvalbumin isoform had a rate of synthesis that was greater than the rate of degradation. In contrast the rate of degradation of the β5 isoform exceeded its rate of synthesis, whilst the analysis revealed that the other parvalbumin β-isoforms (β1, β2, β3, β4 and β6) had a rate of synthesis that was equal to the rate of degradation. This work has addressed a number of technical challenges and represents the first study to use proteomic approaches to measure the turnover of individual proteins in fish. This article is part of a Special Issue entitled: Farm animal proteomics.

1. Introduction

Any change in the concentration of a specific protein reflects the net result of the opposing processes of protein synthesis and protein degradation. Even in a position of apparent steady-state, when the size of a protein pool remains constant, new proteins will be synthesised and older proteins will be degraded and recycled. Modulation of protein abundance mediated by post-transcriptional control of translation or protein post-translational modification, including degradation, is not formally or mechanistically linked to the abundance of mRNA. This discrepancy between mRNA and protein expression has been noted in a variety of biological systems [1–3].

An mRNA-focused perspective inevitably emphasises protein synthesis as the main influence on protein content in the cell, but ignores the equally critical contribution made by regulated or unregulated intracellular protein degradation. When a protein increases in intracellular concentration, this can be achieved by enhanced synthesis or, equally effective, diminished degradation. Whilst the outcome of these processes is the same (a change in the intracellular concentration of the protein), the mechanism and regulation of the two opposing

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processes is very different. To fully understand protein expression, it is important to give due cognizance to the flux of individual proteins as well as their concentration.

Conventional proteomic approaches provide a 'snap-shot' of the proteome and may mask physiologically meaningful differences in the synthesis and/or degradation rates of a given protein [4]. Bulk protein turnover in animals has previously been measured through the use of radioactive amino acids and stable isotopes [5–7], however these approaches do not allow disaggregation of the data into individual protein species. More recently novel methods using stable isotope labelling and mass spectrometry have been developed to calculate turnover rates of individual proteins in intact animal species [8–10]. We have now extended these proteomic strategies to determine the rates of synthesis and degradation of individual proteins in the tissues of fish. We have previously identified seven distinct β-isoforms of parvalbumin (β1–7) in the skeletal muscle from common carp (*Cyprinus carpio*) and shown that they are present in varying abundance [11]. In this study we have demonstrated the feasibility of our experimental approach by measuring the turnover of each of these parvalbumin isoforms.

2. Materials and methods

2.1. Fish and diet production

Common carp (mean weight = 19 g/fish) were maintained in the University of Liverpool aquarium at 25 °C ± 0.5 °C (at pH 6.5–7.0) on a 16 h light:8 h dark photoperiod throughout the study. The fish were initially fed with carp pellets for 2 months. Following this period the fish were fed twice a day with a synthetic diet in which the proximate composition of L-leucine was set to a level similar to standard carp diet [12,13]. As the diet had to be palatable to the carp and provide sufficient nutrients to allow the fish to grow at a normal rate, a total substitution of free protein with crystalline amino acids was not possible. The diet was cold pressed without steam in a PTM 6 (Plymouth Tropical Marines, Plymouth, UK) extruder. Ingredients were ground and sieved to less than 1 mm^2 before being thoroughly mixed prior to the addition of the oil and water fractions. The diet was then extruded through a 2 mm die. The resultant pellets were dried in a temperature controlled cabinet at 45 °C until the total moisture is less than 10% and then stored at 4 °C until use. Both the diet ingredients and the diet itself were analysed for proximate composition to ensure accurate formulation and confirm subsequent composition (Supplementary Table 1).

After 4 weeks the carp were switched to an experimental 'heavy' diet, in which 50% of the L-leucine in the diet (that proportion added as crystalline amino acid) was replaced with [$^2H_7$]L-leucine (98% purity) (Cambridge Isotope Laboratories, Andover, MA, USA). The fish were fed twice a day until visually satiated. Total daily food consumption was monitored on each day of the study and feeding did not exceed 1% body weight on any 1 d. The dietary changeover point was taken as t = 0. Fish were maintained for up to 7 weeks on the heavy diet. The fish ($n = 4$) were sampled at t = 0, 1, 2, 3, 4, 5 and 7 weeks following the dietary changeover. Immediately after sacrifice, fish were weighed and then the axial white skeletal muscle from midway down the body of each fish (under the dorsal fin and above the lateral line) was swiftly dissected. Tissues were then stored at −80 °C until analysed. The feeding regimen was carried out under licence granted by the UK Animal (Scientific Procedures) Act, 1986. Fish were killed in accordance with UK Home Office Schedule One regulations.

2.2. Sample preparation

The muscle samples (approximately 300–400 mg wet weight of tissue) were mechanically homogenised in 2.5 mL of 20 mM sodium phosphate buffer (pH 7.4) containing Complete Protease Inhibitors (Roche, Lewes, UK). The homogenate was centrifuged at 15,000 × *g* at 4 °C for 45 min and the supernatant was removed. The remaining pellet was re-suspended in 1 mL of sodium phosphate buffer, re-homogenised, and centrifuged and the supernatant was combined with the supernatant set aside in the previous step. This combined supernatant was then divided into two aliquots. One aliquot was used for determination of total parvalbumin content by 1-D SDS-PAGE. The second aliquot was heated at 95 °C for 5 min and then centrifuged at 15,000 × *g* for 10 min. Parvalbumin is thermostable and boiling is a well established procedure to produce a fraction highly enriched in parvalbumin [14]. The resulting supernatant was used for parvalbumin isoform analysis by 2-DE. The protein concentration of both the unboiled and boiled supernatant fractions was determined using the Coomassie Plus Protein Assay (Pierce Biotechnology, Rockford, IL, USA).

2.3. 1-D SDS-PAGE

The proteins from the unboiled fraction were separated by 1-D SDS-PAGE using a Mini-Protean 3 system (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK). Muscle samples (approximately 15 μg) of the soluble fraction of muscle were electrophoresed at a constant potential of 200 V through a 15% w/v polyacrylamide resolving gel with a 4% w/v stacking gel. Samples were incubated at 95 °C for 5 min in a reducing buffer (125 mM Tris–HCl; 140 mM SDS; 20% v/v glycerol; 200 mM DTT and 30 mM bromophenol blue) prior to loading. Gels were stained with Colloidal Coomassie Blue (Bio-Rad).

2.4. 2-DE

Samples (approximately 300 μg) of parvalbumin isoform extracts from the boiled muscle supernatants were precipitated with five volumes of ice-cold acetone and held at −20 °C for 1 h. The resulting pellet containing the proteins of interest was solubilised in buffer (4% w/v CHAPS, 8 M urea, 20 mM DTT and 0.5% v/v carrier ampholytes), incubated at room temperature for 1 h and was then applied to IPG strips (pH 3.9–5.1, 18 cm, Bio-Rad). The IPG strips were loaded face-down into a Protean IEF system (Bio-Rad) and rehydrated (12 h at 30 V, 20 °C) followed by isoelectric focusing (1 h at 250 V, 2 h at 1000 V and 45,000 Vh at 10,000 V). The focused strips were equilibrated in reducing buffer containing 50 mM Tris–HCl pH 8.8, 6 M urea, 30% v/v glycerol, 2% SDS w/v, 65 mM DTT and a trace of bromophenol blue. A second equilibration step was then performed using alkylating