The proteome of chicken skeletal muscle: Changes in soluble protein expression during growth in a layer strain

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The whole animal, and the pectoralis muscle in particular, grows at a greatly enhanced rate in chickens selected for meat production (broilers) when compared to those selected for egg production (layers). As part of an ongoing study to analyse muscle protein dynamics under conditions of rapid growth, we have embarked upon a preliminary characterisation of the proteome of layer chicken pectoralis muscle, at specified time-points from 1 to 27 days after hatching. Soluble extracts of muscle homogenates were separated by two-dimensional (2-D) gel electrophoresis and selected spots were analysed by in-gel tryptic digestion and matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry. Of 90 spots, 51 gave mass spectra that matched to existing chicken proteins present in on-line databases, 12 matched equivalent proteins from non-avian species and 11 yielded good quality spectra but were unable to be matched against existing databases. For many of these proteins, growth over 27 days elicited dramatic changes in relative expression levels. Chicken skeletal muscle offers an excellent system for developmental proteomics.

Keywords: Chicken / *Gallus gallus* / Matrix assisted laser desorption/ionization-time of flight / Pectoralis muscle / Skeletal muscle / Two-dimensional gel electrophoresis

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1 Introduction

Selection of chickens (*Gallus gallus*) for meat production (broilers) has led to the generation of inbred strains that show accelerated growth, and in particular, an enhanced growth of the pectoralis (breast) muscle [1–4]. By contrast, chickens selected for egg production (layers) grow much more slowly, with no disproportionate accretion of pectoralis muscle protein. The production of poultry meat increased worldwide by 8.3% *per annum* between 1987 and 1997 and is today a major growth industry. In 2002, 54 million metric tonnes of “ready to cook” broiler meat was produced worldwide with an increase predicted for 2003 (www.poultryegg.org). In the UK alone, over 10 million eggs were produced for commercial use in 2002 (www.defra.gov.uk). As the demand for quality poultry products has risen, the drive to produce fast growing, lean birds has increased [5]. However, it is important that such growth is tempered with maintaining the physical well-being of the birds. A number of disorders have been associated with accelerated muscle growth, including ascites and pulmonary hypertension syndrome [4, 6].

The pectoralis muscle of the chicken comprises almost exclusively white “fast twitch” fibres and thus provides a relatively simple tissue system to assess protein expression and dynamics, and in particular, to assess the influence of the genetic differences between the broiler and layer strains. As such, a comparison of muscle growth between the two strains provides an excellent tool for analysis of the processes of muscle accretion [7]. Protein accumulation reflects the balance between two opposing processes of protein synthesis and protein degradation, both intracellular processes. Our long-term goal is to assess the relative rates of synthesis and degradation

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Abbreviation: 2-D GE, two-dimensional gel electrophoresis

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during accretion of individual proteins in pectoralis muscle in a similar manner to the analysis carried out in yeast [8]. However, before one can embark on those studies, it is necessary to define the protein complement of chicken skeletal muscle, as resolved by two-dimensional gel electrophoresis. In order to define the post-hatching and developmental changes in protein complement in pectoralis muscle, we have assessed the soluble protein patterns between 1 and 27 day old chickens.

2 Materials and methods

2.1 Experimental animals

Male layer chickens (HISex Brown) were obtained at 1 d of age and maintained at 23 ± 2°C, under 12 h light, 12 h dark cycles, on a diet comprising 26% protein (Farmway, Durham, UK). Animals were weighed every third day and a randomly selected subset were killed by CO_2 asphyxiation at 1 d, 5 d, 9 d, 13 d and 27 d.

2.2 Sample preparation

Pectoralis muscles were dissected immediately, weighed and stored at −80°C until required. The muscle was homogenised (Ystral homogeniser; SIC, Eastleigh, Hampshire, UK) in 10 volumes of phosphate buffer (20 mM, pH 7.0) containing protease inhibitors (Complete Protease Inhibitors, Roche, Lewes, UK). The homogenate was centrifuged at 4°C, 15 000 × *g* for 45 min and the supernatant was retained for analysis. The protein content of each sample was assayed using the Coomassie Plus Protein Assay (Perbio Science UK, Tattenhall, UK).

2.3 Two-dimensional gel electrophoresis (2-D GE)

Prior to loading onto immobilised pH-gradient (IPG) strips (pH 3–10L, 13 cm; Amersham, Buckinghamshire, UK) each sample (300 μg protein) was incubated in buffer containing CHAPS (4% w/v), 7 M urea, 2 M thiourea, 20 mM dithiothreitol and ampholytes (0.5% v/v) for 1 h. The first-dimensional separation was on an IPGPhor unit (Amersham). In-gel rehydration (150 Vh at 30 V, 300 Vh at 60 V, 20°C) was followed by isoelectric focusing (500 Vh at 500 V, 1000 Vh at 1000 V and 48 000 Vh at 8000 V). The focused IPG strips were equilibrated in 50 mM Tris-HCl, pH 8.8, containing 6 M urea, 30% v/v glycerol, 2% w/v SDS, and a trace of bromophenol blue. Dithiothreitol (10 mg/mL) was present as a reducing agent for the initial equilibration. A second equilibration step was carried out with iodoacetamide (25 mg/mL) present in place of dithiothreitol. The proteins were electrophoresed through a linear 12.5% acrylamide gel and the gels were stained using Coomassie blue (Bio-Safe™; Bio-Rad, Hercules, CA, USA). Gels were visualised using PDQuest software (Bio-Rad). Three birds were analysed at each time point. The expression levels of key proteins were calculated per gram of pectoralis muscle. This was achieved by determining the spot volume corresponding to each protein as a fraction of the total integrated spot density on the gel. From this, as the total amount of protein loaded was known, the absolute amount of each protein could be calculated. This was subsequently corrected to account for mass of tissue per bird. Gel plugs containing protein spots of interest were excised and the proteins were subjected to in-gel tryptic digestion and peptide extraction using a MassPrep™ digestion robot (Micromass, Manchester, UK).

2.4 MALDI-TOF-MS

Peptides were analysed using a MALDI-TOF mass spectrometer (M@LDI™, Micromass) over the range of 1000–3500 thomsons. Proteins were identified from their peptide mass fingerprint by manual searching using a locally implemented MASCOT server. The initial search parameters allowed a single trypsin missed cleavage, carbamidomethyl modification of cysteine residues, oxidation of methionine and an *m/z* error of ± 250 ppm. The taxonomic search space was restricted to Chordata.

3 Results and discussion

The primary aim of this work was to define the first proteome map of chicken skeletal muscle and to characterise developmental changes in the soluble protein profile during the first month post-hatching. Chickens increase dramatically in size over the first month post-hatching. This growth is reflected in the increase in mass of the pectoralis muscle (Fig. 1). The proteins studied in this analysis correspond only to the soluble fraction of the tissue upon homogenisation. The extraction and solubilization process was designed to resolve the soluble proteins from the contractile apparatus, to diminish the impact of the large quantities of actin and myosin that otherwise dominate and distort the gel image.

Two-dimensional SDS-PAGE gels were generated in triplicate at five time-points over a 27 d period post-hatching (Fig. 2). Profiles obtained were remarkably consistent. Several features are immediately evident. First, it is striking that relatively few proteins comprise most of the mass of soluble protein, particularly in muscle samples from older birds. At day 1, around 90% of the protein is accounted for by 60 spots. By day 27, only 35 spots accounted for the same percentage of the total soluble

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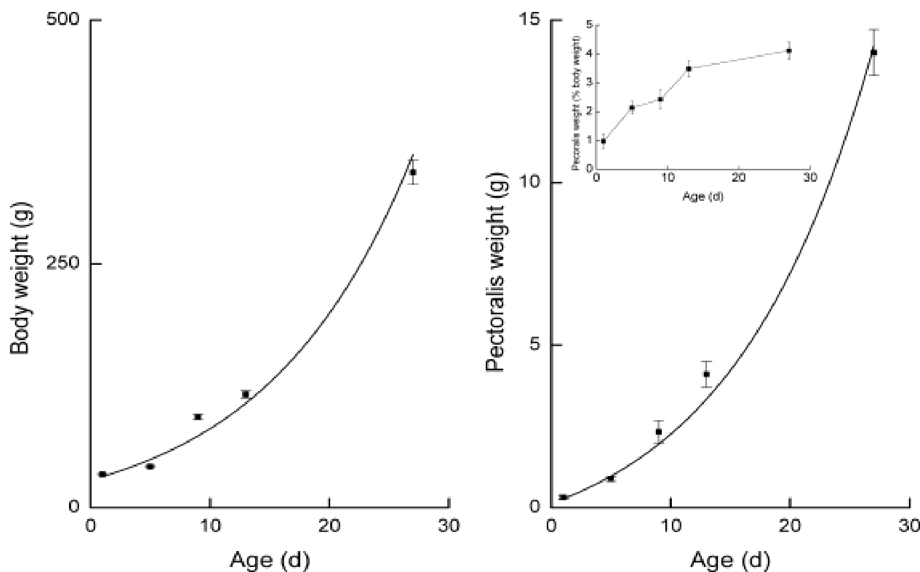

Figure 1. Bird growth and increase in muscle mass post-hatching. Male layer chicks were obtained immediately post-hatching and grown for 27 days. At each time point, total body weight and pectoralis muscle weight were recorded. The inset graph records the pectoralis weight as a percentage of body weight.

Figure 2. Growth related changes in soluble protein expression pattern in chicken pectoralis. Pectoralis muscle was obtained from 1, 5, 9, 13, and 27 day old male layer chicks of age and soluble proteins (300 μg total protein) from a 10% w/v homogenate of chicken pectoralis muscle were resolved on full range 2-D GE. The gels were stained with colloidal Coomassie blue.

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protein complement. Indeed, with the possible exception of plasma, skeletal muscle shows the greatest degree of specialisation and consequent dynamic range in protein expression. The predominance of a few proteins is particularly striking in view of the fact that the otherwise predominant myofibrillar proteins were not present in the sample analysed, which consisted only of the protein fraction soluble in low-ionic-strength buffers. Any future display and analysis of the many less abundant proteins will require prefractionation methods to simplify the preparation and diminish the effect of the dominant constituents.

It is also evident that the pattern of protein expression undergoes a substantial change over the 27 d time period. At 1 d, some 90 protein spots are visible, covering the entire gel area (p*I* 3–10, M_r 6–200 kDa). The majority of the proteins are between 30 and 60 kDa and cover the whole span of p*I* values separable by these gels. By the time the birds have reached five days of age, the pattern of proteins has changed substantially, consistent with a major post-hatching developmental shift. This apparent "reprogramming" of the tissue is even more pronounced in the samples derived from older birds. By 9 d, the gel is dominated by the proteins in the top-right quadrant, relating to proteins of high (30 kDa and above) molecular mass and isoelectric points between 6.5 and 9. At 27 d, the pattern is similar to that at 9 d, but even more pronounced.

Individual protein spots were excised from the gels, subjected to automated in-gel digestion and analysed by MALDI-TOF-MS (Fig. 3, Table 1). A total of 53 individual proteins were recognised on the basis of the tryptic mass profiles. In many cases, the same profiles were observed for multiple gel spots. (This is denoted by alphabetical subscripting; *e.g.*, 3a, 3b and 3c each related to separate spots on the same gel that correspond to creatine kinase. Further, the spots have been labelled so as to maintain consistency between the gel images). The majority (38/53) of the proteins were matched to sequences derived from the chicken, either as experimentally determined protein sequences or cDNA sequences. At this time, the full genome sequence of *Gallus gallus* is not available [9] and a subset of protein identities were established by cross-species matching. A number of the matches were to proteins from human or mouse, although rabbit, pig, trout and frog were also represented. The chicken genome sequence is predicted to be available by March 2004 [9, 10]. This should enhance identification of proteins in this type of study. Previous work from our group [11] has confirmed the identities of some of the major chicken muscle proteins by MS/MS sequencing. One entry (spot 24) matched to a 166 amino acid sequence inferred from cDNA that relates to a 18 kDa fragment of fructose-1,6-bisphosphatase

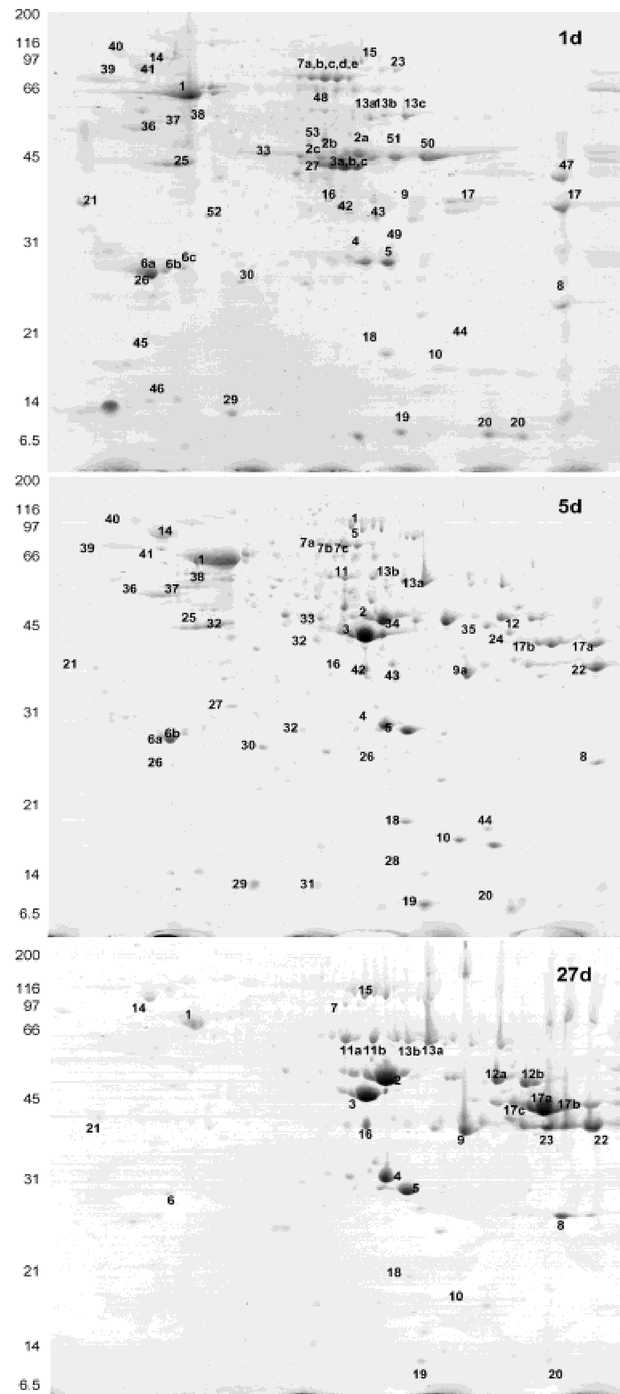

Figure 3. Identification of soluble protein expression from chicken pectoralis muscle during muscle growth. Because the protein pattern changes so dramatically over time, 2-D GE from three ages (1, 5, and 27 d) are included. On each gel, spots excised for protein identification by MALDI-TOF-MS of the tryptic hydrosylates are identified by a number, whilst subscripted letters identify clearly resolved protein spots that yielded an identical identification. The numbering scheme is consistent across all gels, and the identifications are listed in Table 1.

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Table 1. Protein identifications from chicken skeletal muscle

Spot No.	Identification	Mowse score[a)]	% Coverage	Peptides matched	Mass[b)]	p*I*[c)]	Species	Acc. No.
1	Serum albumin	309 (64)	57	36	71.8 (70)	5.51 (5.5)	Chicken	P19121
2	β-Enolase[e)]	92 (67)	46	19	47 (47)	6.8 (7–7.5)	Chicken	P07322
3	Creatine kinase	195 (58)	42	17	43 (45)	6.5 (7–7.5)	Chicken	P00565
4	Phosphoglycerate mutase	96 (58)	39	9	28.8 (29)	7.25 (7.5)	Human	P18669
5	Triosephosphate isomerase	242 (58)	73	12	26.7 (27)	6.8 (7.8)	Chicken	P00940
6	Apolipoprotein A1	246 (58)	55	19	31 (27)	5.6 (5.5)	Chicken	P08250
7	Ovotransferrin	329 (64)	46	31	79.5 (80)	6.85 (7–7.5)	Chicken	P02789
8	Adenylate kinase isozyme 1	114 (58)	35	7	22 (25)	8.68 (9.5)	Chicken	P05081
9	L-Lactate dehydrogenase A-chain	169 (58)	41	15	36.6 (37)	7.74 (8)	Chicken	P00340
10	Nucleoside diphosphate kinase[d)e)]	127 (67)	52	8	17.5 (18)	7.72 (8.5)	Chicken	O57535
11	Phosphoglucomutase	114 (58)	27	16	62 (60)	6.62 (7–7.5)	Rabbit	P00949
12	Phosphoglycerate kinase	166 (58)	43	10	45 (46)	8.31 (8.5)	Chicken	P51903
13	Pyruvate kinase[d)e)]	150 (67)	37	22	58 (55)	7.28 (7.5)	Chicken	P00548
14	Heat shock protein 90-α	182 (58)	26	23	84 (96)	5.01 (5)	Chicken	P11501
15	Elongation factor 2	113 (58)	20	15	96.2 (100)	6.42 (6.5)	Chicken	Q90705
16	Glycerol-3-phosphate dehydrogenase	100 (58)	43	10	38 (38)	6.83 (7)	Mouse	P13707
17	Glyceraldehyde 3-phosphate dehydrogenase	137 (58)	34	15	36 (37)	8.71 (9)	Chicken	P00356
18	Cofilin	64 (58)	46	6	18.8 (19)	7.66 (7.5)	Chicken	P21566
19	Hemoglobin α-D chain	123 (58)	65	9	15.7 (14)	7.08 (8.5)	Chicken	P02001
20	Hemoglobin α-A chain	95 (58)	71	8	15.4 (14)	8.56 (9.5)	Chicken	P01994
21	Apolipoprotein AIV	262 (67)	63	20	40.8 (40)	4.8 (4.5)	Chicken	O93601
22	c-Jun *N*-terminal kinase 2[d)e)]	68 (67)	39	16	44.5 (44)	5.97 (9)	Chicken	P79996
23	Glycogen phosphorylase	59 (58)	18	12	98.5 (97)	6.08 (7)	Rabbit	P00489
24	Fructose-1,6-bisphosphatase[d)e)]	74 (67)	31	9	Fragment	Fragment	Chicken	Q9I8D3
25	α-Actin	106 (58)	28	16	42 (45)	5.23 (5)	Human	P02568
26	Myosin light chain I[e)]	71 (67)	48	8	21 (24)	4.98 (5)	Mouse	P05977
26	Tropomyosin α-chain	120 (58)	38	16	32.8 (25)	4.7 (7.5)	Chicken	P02559
27	Casein kinase 1 α-isoform	58 (58)	25	8	38 (33)	9.59 (5.5)	Chicken	P70065
28	Myoglobin	115 (58)	66	8	16.9 (17)	6.83 (7.5)	Pig	P02189
29	Fatty acid-binding protein	61 (58)	54	4	7.97 (10)	5.17 (6)	Chicken	P80565
30	Actin polymerisation inhibitor	87 (58)	54	9	22 (24)	5.77 (6)	Chicken	Q00649
31	Protein kinase C inhibitor[e)]	49 (67)	22	4	13.8 (14)	6.28 (6.5)	Chicken	Q9I882
32	Aldolase C	91 (58)	31	5	39 (30)	5.8 (7)	Chicken	P53449
32	β-Actin	63 (58)	22	8	42 (45)	5.3 (5.5)	Chicken	P53478
33	α-Enolase	69 (64)	33	97	47.5 (48)	6.16 (6.5)	Chicken	P51913
34	T-complex protein	59 (58)	15	97	60 (48)	5.45 (7.5)	Human	P48643
35	Citrate synthase	60 (58)	16	229	47.5 (46)	8.31 (8.5)	Chicken	P23007
36	Tubulin β-7 chain	208 (58)	48	2222	50 (47)	4.78 (5)	Chicken	P09244
37	Tubulin α-chain	148 (58)	46	1022	46 (48)	4.96 (5)	Chicken	P02552
38	Vimentin	65 (58)	21	710	53.5 (55)	5.08 (5)	Trout	P48674
39	PIT 54[e)]	84 (67)	20	87	53 (66)	4.61 (4.5)	Chicken	Q98TD1
40	Endoplasmin	113 (58)	21	248	92 (100)	4.83 (4.5)	Chicken	P08110
41	Immunoglobulin heavy binding protein	165 (58)	40	1224	72 (72)	5.12 (5)	Chicken	Q90593
42	L-Lactate dehydrogenase B-chain	101 (58)	34	1412	36.6 (35)	7.23 (7.25)	Chicken	P00337
43	L-Lactate dehydrogenase M-chain	72 (58)	29	514	36.7 (35)	7.77 (7.75)	Mouse	P06151

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Table 1. Continued

Spot No.	Identification	Mowse score[a)]	% Coverage	Peptides matched	Mass[b)]	p*I*[c)]	Species	Acc. No.
44	Destrin	60 (58)	32	105	18.9 (20)	7.52 (8.5)	Chicken	P18359
45	Myosin regulatory light chain 2	117 (58)	50	510	19 (19)	4.82 (5)	Mouse	P97457
46	Transthyretin	59 (58)	65	105	16.4 (16)	5.11 (5)	Chicken	P27731
47	Fructose bisphosphate aldolase	68 (58)	37	710	40 (46)	8.4 (9.5)	Mouse	P05064
48	Vitamin D binding protein[e)]	93 (67)	20	157	55 (55)	6.47 (7)	Chicken	Q9W6F5
49	Carbonic anhydrase	172 (58)	70	1015	29.6 (31)	7.86 (8)	Mouse	Q9ERN8
50	Aldehyde dehydrogenase	72 (58)	21	810	56 (48)	7.49 (8)	Chicken	P27463
51	Guanine nucleotide binding protein	60 (58)	33	98	42 (48)	5.63 (7.5)	Xenopus	O73819
52	Annexin V	110 (58)	33	149	36.2 (35)	5.6 (5.5)	Chicken	P17153
53	Adenosyl homocysteinase	90 (58)	27	14	48 (50)	6.04 (6.5)	Xenopus	P51893

a) Mowse baseline significance score in brackets
b) Mass predicted from gel in brackets
c) p*I* predicted from gel in brackets
d) Full protein sequence from chick not available
e) Identified using MSDB database (all other identifications made using the Swiss-Prot database)

(accession No. Q9I8D3). The full amino acid sequence of this protein is not yet available. A BLAST (http://www.ncbi.nlm.nih.gov/blast/) search of the fragment sequence displayed high score matches to homologous fructose-1,6-bisphosphatase proteins from rabbit, human and rat with mass and p*I* similar to that predicted from the gel analysis (37 kDa, p*I* 6.8).

Even on cursory inspection of the gels, it is obvious that substantial changes are occurring in the pectoralis muscle of the birds during the early stages of growth. The young birds (1 d) display relatively low quantities of the glycolytic enzymes such as enolase, triosephosphate isomerase and phosphoglucomutase. Also present are haemoglobin, heat shock proteins, and proteins involved in the synthesis and modification of new proteins such as elongation factor 2 [12]. Of the contractile proteins, actin is the most prominent in the soluble fraction of the youngest birds. Fragments of myosin have been identified in a number of spots on the gels but never as a major constituent. Indeed, myosin does not readily transfer from the first to second dimension in 2-D GE (unpublished data). In addition a number of proteins involved in the construction of the actomyosin complex are observed in the youngest samples. These include T-complex protein 1, a molecular chaperone which assists the folding of actin and tubulin and destrin and cofilin, which are both actin binding proteins that control (de)polymerisation of actin *in vivo*. At 9 d, the tissue has become markedly more specialised and the soluble fraction is dominated by glycolytic enzymes (Figs. 2 and 3). The major soluble protein complement is dominated by β-enolase, glyceraldehyde 3-phosphate dehydrogenase, lactate dehydrogenase, triosephosphate isomerase, phosphoglycerate mutase, creatine kinase, pyruvate kinase and phosphoglycerate kinase. Proteins such as actin and tubulin are no longer apparent in the soluble fraction.

In the predominantly fast twitch pectoralis muscle, glycolysis is one of the main pathways through which the bird derives energy for muscle contraction and to fuel energy demands for growth. The growth curve for layer chicks has been well documented by various studies [13, 14]. After a relatively slow initial phase, the birds rapidly increase body weight. In this study, the birds at 1 d had an average body mass of 34 g. This doubled at 1 week of age. However, by 27 d, the mass of the bird has undergone a 10-fold increase to 344 g. In addition, the pectoralis muscle increases approximately 44-fold from 315 mg at 1 d to an average of 14 g at 27 d (Fig. 1). To maintain this mass of tissue, the birds require substantial energy and it is not surprising that as the birds age the glycolytic enzymes dominate the soluble protein fraction. However, muscle development is also associated with a marked increase in contractile capabilities, and there would be expected to be a strong link between the demands of the contractile apparatus and the energy producing machinery. The most striking increase was observed for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). GAPDH (spot 17) catalyses the NAD(H) dependent conversion of D-glyceraldehyde 3-phosphate to 3-phospho-D-glyceroyl phosphate. At 1 d the bird expresses only 0.33 ± 0.16 mg/g GAPDH rising to over 10 ± 2.9 mg/g at 27 d (Fig. 4). This change is clearly apparent on the gels (Figs. 2 and 3).

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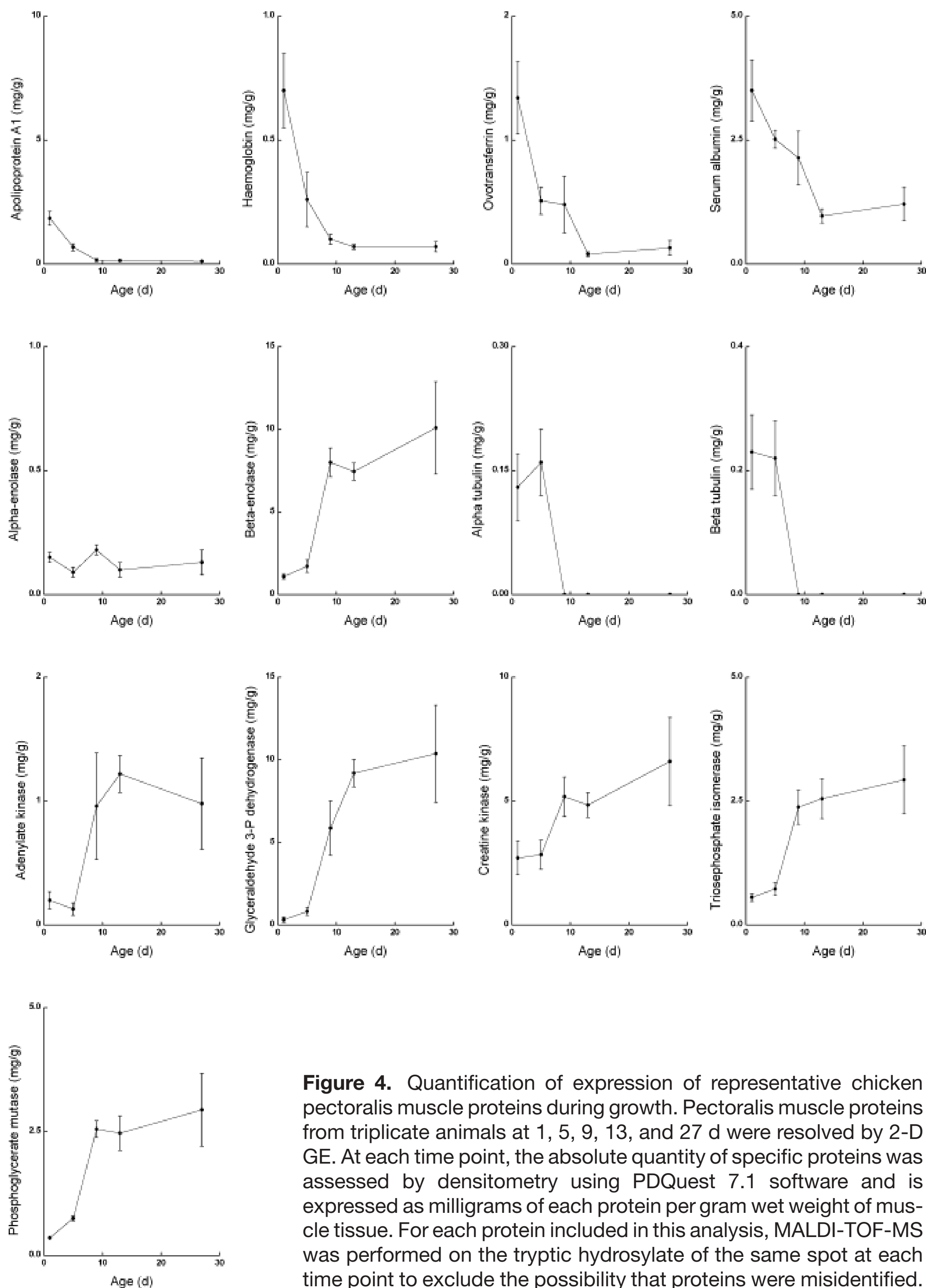

Figure 4. Quantification of expression of representative chicken pectoralis muscle proteins during growth. Pectoralis muscle proteins from triplicate animals at 1, 5, 9, 13, and 27 d were resolved by 2-D GE. At each time point, the absolute quantity of specific proteins was assessed by densitometry using PDQuest 7.1 software and is expressed as milligrams of each protein per gram wet weight of muscle tissue. For each protein included in this analysis, MALDI-TOF-MS was performed on the tryptic hydrosylate of the same spot at each time point to exclude the possibility that proteins were misidentified.

Two spots gave spectra corresponding to enolase. Enolase is also a key glycolytic enzyme [15–17]. Two enolase isozymes (α and β) are present in muscle while a third enolase isozyme, γ-enolase, which has been identified in chicken, is brain-specific [17]. The sequence similarity between the chick α- and β-enolase is 83% at the protein level [16], but the peptide mass fingerprints were of sufficiently high quality and sufficiently distinct to confirm that the two spots contained the different enolase isozymes (Fig. 5). Despite the high sequence similarity, it was

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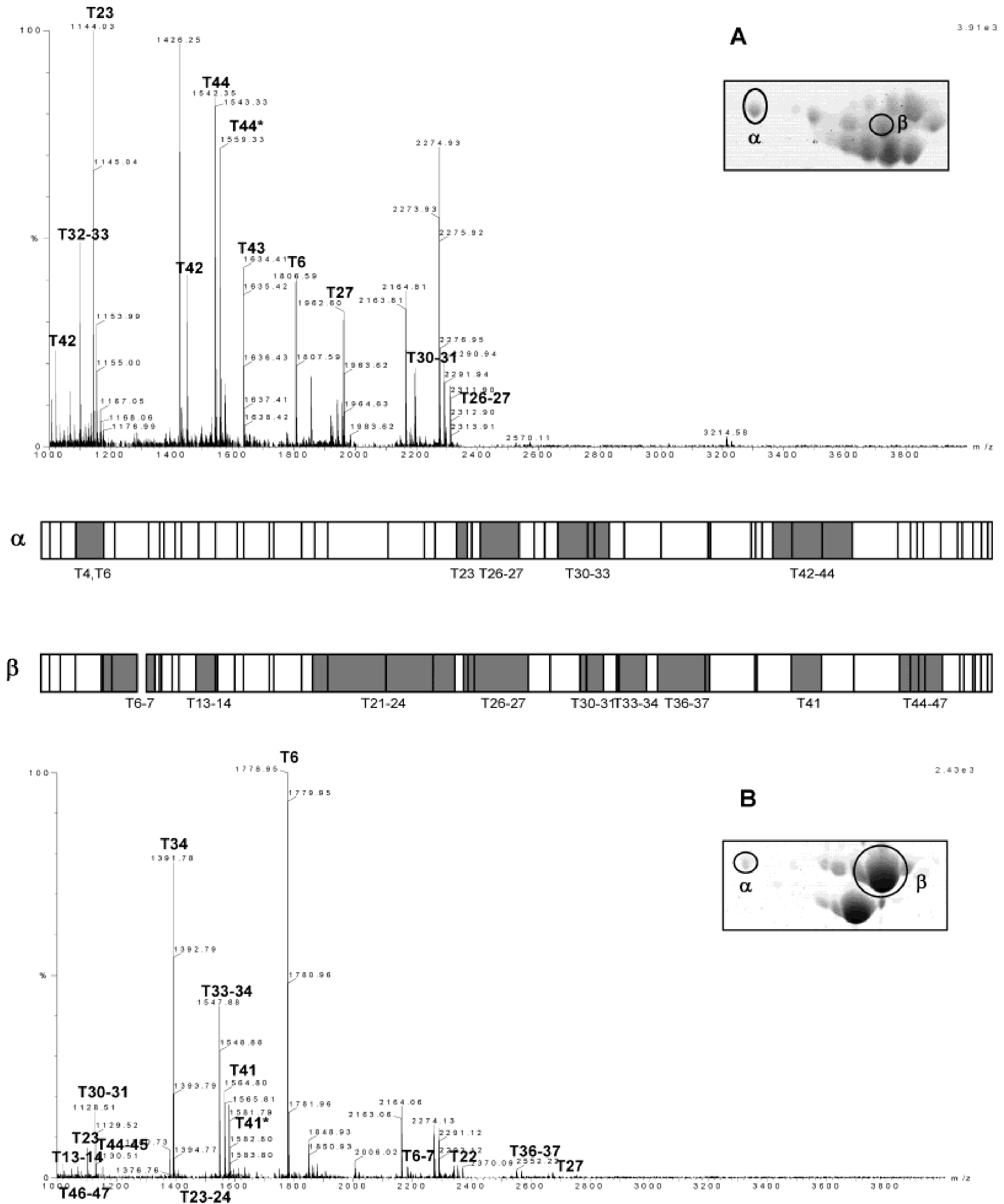

Figure 5. Discrimination between α- and β-enolase. MALDI-TOF mass spectra of the tryptic hydrosylate from two spots, separated predominantly in the charge dimension matched to chick enolase. Detailed examination of the spectra allowed assignments of peptides that permitted clear discrimination of the α- and β-isoforms. (A) MALDI-TOF spectrum obtained for the spot identified as α-enolase; (B) MALDI-TOF spectrum of the spot identified as β-enolase. In the middle region, peptide coverage maps for the α- and β-isoforms are used to highlight peptides identified from each isoform (shaded).

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observed that there were no common tryptic peptides between the two isoforms.

Previous studies have indicated that the α-isoform predominates in immature tissue whereas the β-enzyme is predominant in mature muscle [16]. Analysis of translation products *in vitro* from four-day old chicken muscle samples was consistent with translation of both α and β-enolase mRNA [16]. Further, in 13 d embryo muscle tissue the α-enolase mRNA is predominant, but decreases during subsequent development. The β-enolase precursor mRNA, in contrast, is almost undetectable during the embryonic phase and increases greatly post-hatching [15]. A similar expression pattern has been noticed in mouse somites [18]. The analysis of transcript and isoenzyme patterns has provided strong evidence for the switch in isozyme form from α to β during development.

On 2-D GE, the spot corresponding to the more acidic α-isozyme diminished by 9 d whereas the β-isozyme had become prevalent (Fig. 6). However, because a fixed amount of protein was loaded onto each gel, any enhanced expression of some proteins would lead to an apparent diminution in others. Therefore, we used scanning densitometry to estimate absolute levels of the enolase isoforms in pectoralis muscle. After this correction, it was clear that although the α-isoform decreases in relation to the total soluble protein complement, the concentration of the protein in the tissue was virtually unchanged. In turn, this means that as tissue mass increased with age the α-enolase pool in the tissue was expanded from 0.05 ± 0.01 mg to 1.82 ± 0.5 mg (mean ± SEM, n = 3) over the growth period. Although the level of mRNA in the tissue is known to decline [15], synthesis must have continued as the birds aged. By contrast, the β-isoform is present at relatively low levels during early development. Around 9 d, production of this enzyme is enhanced. Thereafter, the tissue concentration of this enzyme reached a plateau, comprising one of the most abundant soluble proteins. The relative ratio of the enolase isoforms changes from 1:7 (α:β) at 1 d to 1:93 at 27 d. These data indicate that although the α.isoform is still present in 27 d chicken muscle, the β-isoform is predominant.

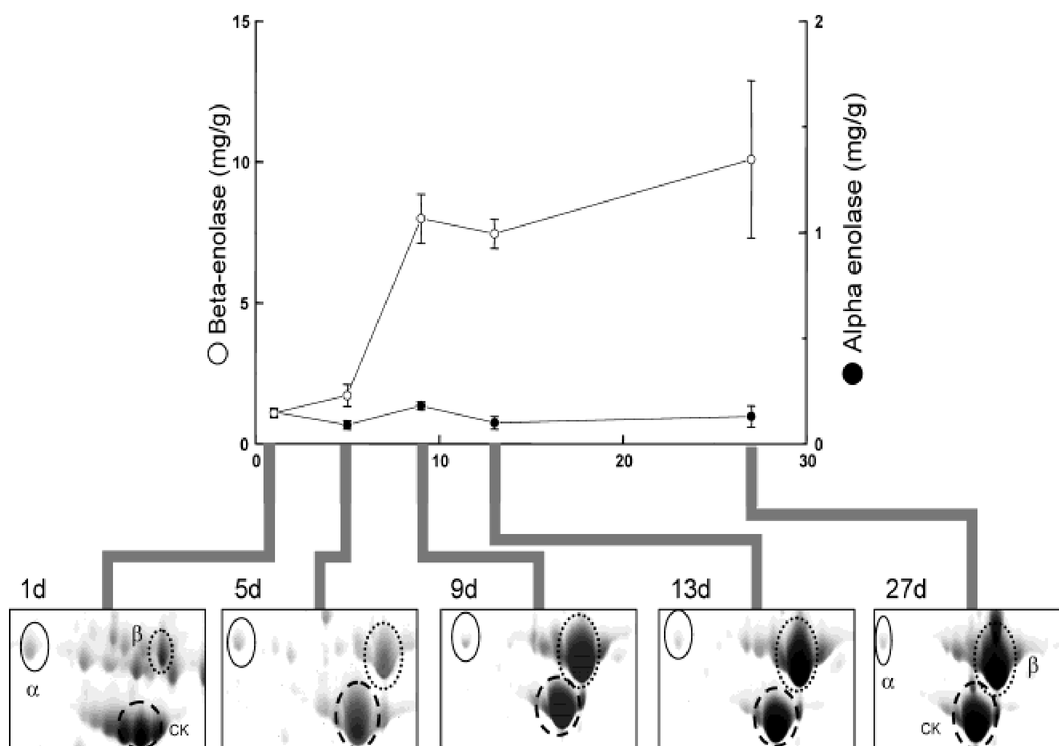

Figure 6. Post-hatching developmental switch in enolase expression. Spots identified as α- and β-enolase were used to quantify the expression of these proteins throughout 27 days of growth post-hatching. Data are presented as absolute levels of protein (mg protein per g wet weight of tissue; mean ± SEM, n = 3) for five time points. A representative subsection of the gel at each time point is also included. α-Enolase is circled by a solid line, β-enolase by a dotted line, and creatine kinase by a dashed line.

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A complex change in expression was also observed for creatine kinase (CK). CK is present in muscle immediately post-hatching (Fig. 3, spot 3), and at 1 d is present at 2.7 ± 0.7 mg/g, rising to 6.6 ± 1.8 mg/g at 27 d. At 1 d, MALDI-TOF spectra characteristic of the tryptic hydrosylate of CK are obtained from three spots of near-equal intensity, differing in charge but not differing significantly in mass. As the birds age, whilst all CK spots remain visible, the expression pattern becomes more asymmetric, such that after 5 d, one form of the enzyme predominates. At 27 d, this spot accounts for virtually all of the CK skeletal muscle.

The MALDI-TOF spectra give no indication of the nature of the difference between these CK variants. There are four isoforms of the enzyme and the form expressed almost exclusively in differentiated skeletal muscle is M-CK [19, 20]. An isozyme switch from brain-type (B-CK) to muscle-specific CK takes place during myotube formation [21]. However, all spectra yield database matches that confirm that they are M-CK. CK is reported to be a phosphoprotein *in vivo*. Rabbit M-CK has been shown to autophosphorylate *in vitro* while the human and mouse forms are phosphorylated by protein kinases [21]. It has been shown using ^{32}P-labelled cultures of chicken myogenic skeletal muscle cells that chicken M-CK is subjected to (de)phosphorylation *in vivo* [21]. Further, M-CK is ambiquitous, being located in both the soluble and myofibrillar components of skeletal muscle; approximately 5–10% of M-CK is specifically bound to the myofibrillar M-band [22–24]. It is possible that the development of the contractile apparatus could, by virtue of selective binding of different variants, lead to selective depletion of some proteins in the more mature tissue.

Triosephosphate isomerase (TPI) is an essential "housekeeping" enzyme in all living cells [25], catalysing the interconversion of D-glyceraldehyde 3-phosphate and glycerone phosphate. It is well characterised and has been used as a model for ageing-dependent accumulation of protein damage [26]. Various ageing-dependent isoforms of TPI have been identified [25–27]. These result from deamidation of specific amino acid residues and oxidation of a conserved cysteine residue. Also, as it exists *in vivo* in a dimeric form and can link to form heterodimers, 3 isozymes can be expressed [28]. TPI is present in substantial amounts in the soluble muscle extract from day 1 post hatching. The adjacent spot (spot 5) corresponds to phosphoglycerate mutase (PGM), responsible for the interconversion of 2-phosphoglycerate and 3-phosphoglycerate [29–31]. At 1 d TPI and PGM are present at 0.6 ± 0.08 and 0.4 ± 0.01 mg/g, respectively. This rises to around 3 mg/g for each enzyme representing an order of magnitude increase.

A number of proteins in the soluble fraction from the youngest birds declined substantially with time. The majority of these are serum constituents and include ovotransferrin, an iron-transporting protein, haemoglobin, albumin, and apolipoproteins A-I and A-IV. Apolipoprotein A-I is involved in cholesterol transfer from tissues to liver. It acts as a cofactor for lecithin cholesterol acyltransferase. Apolipoprotein AIV has been linked to lipid absorption and the modulation of body weight in humans [32, 33] but in chicken, a unique role has been postulated in the formation and secretion of as yet unidentified triglyceride-rich lipoproteins [34–36]. As the birds develop from the late-embryonic phase to the hatching chick, large amounts of cholesteryl esters accumulate in the liver [37, 38], derived from the uptake of cholesterol-rich lipoproteins from the yolk sac membrane coupled with the inefficiency of the developing liver [39–41]. This increase in lipoproteins is the stimulus for the production of the apolipoproteins. Unlike mammalian apolipoproteins which are expressed predominantly in the liver and intestine, avian apolipoproteins are expressed in numerous other tissues including skeletal muscle. Elevated synthesis of apolipoprotein AI in the skeletal muscle of newborn chick led to the hypothesis that the protein acts as a local lipid transporter in early post-hatching development [42]. All of these proteins were present in 1 d muscle as 5% of the soluble protein content or above. However, as the birds age, the amount of these proteins with respect to the total soluble fraction is greatly diminished (Fig. 4). As these proteins were only present at relatively low percentage of the soluble fraction initially, by 27 d their contribution to the total protein pool is minimal.

Immediately post-hatching, birds express a number of proteins in the soluble fraction that are normally associated with the myofibrillar structure. These include actin, myosin light chain, cofilin, destrin, actin-depolymerising factor and tubulin. After 9 d, these proteins are absent from 2-D gels and are presumed to have been incorporated into the maturing myofibrillar structure. Up to 5 d of age, the myofibril compartment contributes a maximum of 30% of the protein complement [43] but by 9 d this has increased to almost 70% and at 27d, the myofibrils account for almost 80% of skeletal muscle protein. Tubulins are dimeric proteins of microtubules [44] composed of two nonidentical acidic monomers of about 50 kDa, denoted α- and β-tubulin. The dimer provides an interaction domain for microtubule-associated proteins and is associated with motor proteins such as kinesin and dynein [45, 46]. In most eukaryotes, tubulins undergo several types of conserved post-translational modification, such as detyrosination, acetylation, phosphorylation, palmitoylation, polyglutamylation, and polyglycylation [47]. Detyrosination, polyglycylation, and glutamylation influ-

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ence the binding of motor proteins to the microtubule surface [47, 48]. These modifications are all located at the carboxyl terminus of the protein, a segment of the polypeptide chain critical for the processive movement of single-headed kinesin along the microtubule. In gels from chicken skeletal muscle, the two tubulin monomers are located in the upper left quadrant of the gels derived from animals at 1 and 5 days of age (Fig. 3, spots 36 and 37), as would be predicted from their calculated molecular mass and p*I*. For α-tubulin (α-1) the calculated molecular mass is 46 kDa and p*I* is 4.96, for β-tubulin (β-7), these are 50 kDa and 4.78, respectively. The proteins both appear on the 2-D gels as "charge trains" indicative of a range of post-translational modifications. The same effect has previously been observed on isoelectric focusing [49] where between 27 and 30 charge variants of rat brain tubulin were identified. The tubulins are only observed in the soluble protein fraction in 1 d and 5 d birds ($\sim$0.4 $\pm$ 0.05 mg/g at 1 d falling to under 0.2 $\pm$ 0.06 mg/g at 5 d). By 9 d, there is no evidence of tubulin in the soluble protein fraction (Figs. 3 and 4). Although it is possible to differentiate between α and β-tubulin using MALDI-TOF-MS of the tryptic hydrosylates, we have not been able to define the individual components of each charge train.

4 Concluding remarks

The complex changes in protein expression patterns, of which a representative set are discussed here, serve to highlight the dramatic changes that occur in this experimental model. The transition seen in protein expression as the birds age has not been documented previously and indeed, the scale of reprogramming observed was unexpected. This work has taken the first step in understanding post-hatching development on a proteome-wide scale, and has indicated the complexity in such an analysis. Isoenzyme shifts, association with structural elements and post-translational modifications all characterise growth in this muscle system. A key challenge in such studies lies in defining the proteome changes in terms of shifts in the rate of synthesis or degradation of each protein. Although estimates have been made of the rate of total protein turnover in these tissues, there has been no study targeted to individual proteins. It is pleasing that so many of the major proteins in muscle were positively identified, and that another significant proportion of the proteins could be convincingly matched to proteins from other organisms. The next challenge lies in the use of stable isotope labelled amino acids to define, on a protein-by-protein basis, the dynamics of the individual components of the proteome.

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