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Differential Protein Expression During Tail Regeneration of Anolis carolinensis

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Abstract – Some animals have the ability to regenerate lost limbs. Lizards are unique in that they can autotomize their tails and regrow them. In this study, changes in protein expression occurring within the regenerating tail of *Anolis carolinensis* (Green anole) 72 h post amputation were examined. Proteins were separated using 2-dimension gel electrophoresis followed by detection of differential expression using SameSpots software. Six different protein spots of interest (t-Test, $p < 0.05$) were excised and MS/MS performed for protein identification. Within those spots, proteins involved in immunity, energetics, and protein folding and degradation were identified. The proteins that were up-regulated were transferrin, nucleotide-binding domain of the sugar kinase superfamily, and CH1 domain in immunoglobulin. The down-regulated proteins include T-complex protein 1, phosphoglucomutase 1, AAA, creatine kinase, ESP15 homology domain, and PINT motif.

Introduction

Many organisms have strategies to maintain their forms and cope with physical damage. In order to recover from injury, the damaged tissue must be replenished with new cells. Although regeneration of differentiated cells occurs in all animals, regeneration of lost body parts is not a universal trait. Some invertebrates can regrow almost any parts of their bodies, such as sea stars (Mladenov et al. 1989), planarians (Reddien and Sánchez Alvarado 2004), and hydras (Shimizu

et al. 2002). Among the vertebrates, newts have an exceptional regeneration capacity; they can regrow lens (Reyer 1954), jaws (Oberpriller and Oberpriller 1974), limbs (Ghosh et al. 1994) and even their hearts (Brockes 1997). The regenerative ability of other vertebrates is more limited; zebra fish can regrow their fins (Akimenko et al. 1995), and most of the lizard species are able to autotomize their tails when threatened by predators and regrow them rapidly (Fitch 2003, Meyer et al. 2002).

Studies in lizard tail regeneration have been performed focusing on morphological (Cox 1969) and physiological changes (Meyer et al. 2002). Advances in knowledge in signaling pathways have broadened the scope on various mechanisms of control for regeneration of limb (Mercader et al. 2005), and their implications in regenerative medicine (Stoick-Cooper et al. 2007). In this study, we are interested in identifying the proteins that are differentially regulated during tail regeneration process of *Anolis carolinensis* (Green anole). Our study will contribute to a better understanding of the limb regeneration process.

Materials and Methods

Housing conditions

Male anoles purchased from BackwaterReptiles.com were housed in 38 L terrarium. Anoles were fed, twice weekly, a diet of vita-crickets (BackwaterReptiles.com). Incandescent lamps (60W) were used to maintain proper rearing temperatures.

Sample Collection

Two weeks habituation was allowed prior to tissue collection. Tails were excised 2 cm posterior to the hip. One-half centimeter tissue samples were collected from the excised tissue, frozen in liquid nitrogen, and stored at -80˚C. After 72 h, 0.5 cm samples were collected from the regenerating tissue for comparison and stored at -80˚C.

Protein extraction

Total protein extraction was performed in a similar manner to Thornton (2010) and Hajduch et al. (2005). Aliquots of tissue (0.20 g) were ground in liquid nitrogen with a mortar and pestle as described by Leutwiler (1984). Tissue extraction medium (0.1 M Trizma base, 10 mM EDTA, 0.9 M sucrose, 0.4% B-mercaptoethanol, pH 8.8) and tris-saturated phenol (pH 8.8) were added to the samples followed by brief sonication (Amplification set at 25 for 15 seconds; Q700, QSonica, LLC, Newtown, CT) and incubated at -20˚C for 30 minutes on a rocker. Phenol and aqueous phases were separated by centrifugation at 5000 g for 10 min at 4˚C. The top phenol phase (1 ml) was removed and proteins were precipitated by adding 5 mL of cold 0.1 M ammonium acetate in 100% methanol. The samples were incubated overnight at -20˚C (Hajduch et al. 2005).

Protein purification

Samples were centrifuged for 10 min at 5,000 g and the supernatant decanted. The protein was washed once using 0.1 M ammonium acetate in 100% methanol, two times in 80% (v/v) acetone, and once in 70% (v/v) ethanol. Proteins were incubated in each solution at -20˚C for 15 min and centrifuged. In each of the washes, the supernatant was decanted and the proteins were resuspended in the next solution. After washing, samples were resuspended in a minimal volume of isoelectric focusing (IEF) buffer (8 M urea, 2 M thiourea, 2% CHAPS, 2% Triton X-100, and 50 mM DTT) needed to dissolve all protein (Hajduch et al. 2005). Samples were quantified with the BioRad Protein Assay 425584 (Hercules, CA) against a standard curve of bovine serum albumin using a 96-well plate reader (Multiskan Go, Thermo Scientific, West Palm Beach, FL). Samples were stored at -80˚C.

Isoelectric focusing

Protein samples were focused using immobilized pH gradient (pH range: 5-8) 11 cm strips. The strips were actively rehydrated at 50V using 185μL IEF buffer (with DL-dithiothreitol and pH 3–10 ampholytes) along with 450μg of protein. IEF was performed in a BioRad Protean IEF cell (Hercules, CA) for a total of 45,000 Vh. Samples were held at 500V until they were stored at -80˚C. Equilibration was performed for 20 minutes in an equilibration buffer with 10 mg of DL-dithiothreitol/mL, and then in equilibration buffer with 25 mg of iodoacetamide/mL for another 20 minutes (Hajduch et al. 2005).

Second-dimensional electrophoresis

Immobilized pH gradient strips were rinsed in SDS running buffer (25 mM Tris, 0.192 M glycine, and 20% [w/v] SDS) and applied to a 16 cm, 14% (w/v) acrylamide gel with a 5 mm 10% (w/v) acrylamide stacker. Strips were then overlaid with 0.75% (w/v) low-melting-point agarose in SDS-PAGE running buffer with bromophenol blue added. The second dimension was briefly run at 110 V driving the sample through the stacker and then at 330 V (Hajduch et al. 2005).

Data analysis

Normalized protein differences were analyzed using SameSpots software from Nonlinear Dynamics Ltd. (Newcastle, United Kingdom). Spots of interest (spots with >±1.1 average fold-difference in normalized protein volume, and $p<0.05$) were excised from the gels and sent to the University of Nebraska Mass Spectrometry Core Facility (Lincoln, NE) for protein identification using tandem mass spectrometry (MS/MS) with a Waters Q-TOF Ultima mass spectrometer (Micromass/Waters, Milford, MA). Sequences were searched against Matrix Science database 20100701 (www.matrixscience.com) for protein mass fingerprints of possible homologues (147241 sequences in the database) with the significant threshold set at $P < 0.05$. Significant

differences between pre- and post-amputation samples were identified with *T*-tests calculated with SameSpots software Version 4.5.4325.3262. Molecular weight search scoring (MOWSE) used an algorithm in Pappin et al. (1993) to determine the "rank" of the peptide compared to all matches in the database. MOWSE was also used in conjunction with the percent coverage and individual ion scores (not shown) for each amino acid in the peptide to verify homology or the identity of the protein.

Results and Discussion

There were eleven protein spots in our gels that showed significant alterations in expression. Many spots had more than one protein, and some proteins were found in multiple spots. Therefore, these changes cannot be attributed to differential expression of one particular protein per spot. Ion masses derived from mass spectra were submitted to the MASCOT online database for identification. Within these spots, only proteins with over 20% coverage were analyzed (Table 1).

Proteins that were significantly down-regulated during tail regeneration are shown in Figure 1A. They include t-complex protein-1 (#393), phosphoglucomutase-1 (#393), AAA-family (#411), creatine kinase (#485), ESP15 homology domain (#485), and PINT motif (Proteasome subunits, Int-6, Nip-1, and TRIP-15; #614).

T-complex protein-1 directs protein folding, especially actin and tubulin (Sternlicht et al. 1993). Eps 15 homology domain plays a vital role in endocytosis (Carbone et al. 1997). Phosphoglucomutase-1 catalyzes conversion of glucose-1-phosphate to glucose-6-phosphate (Najjar 1948). Creatine kinase catalyzes conversion between phosphocreatine and creatine, both of which are involved in cellular energetics (Wallimann et al. 1992). AAA family (ATPases associated with a variety of cellular activities) is involved in various cellular activities, such as

controlling gene expression and proteolysis (Leonhard et al. 1996). PINT motif is a subunit within a proteasome, which selectively degrades proteins within eukaryotic cells (Aravind and Ponting 1998).

Proteins that were significantly up-regulated are shown in Figure 1B including transferrin (#307), nucleotide binding domain of the sugar kinase/HSP 70/actin superfamily (#310), and CH-1 domain of immunoglobulin (#310). Transferrin is an iron-binding protein in plasma, regulating the level of iron in the body (Aisen et al. 1966). Nucleotide binding domain of sugar kinase/HSP70/actin superfamily acts as an ATPase and binds to ATP. The hydrolysis of ATP is important in HSP70 cycle (Golas et al. 2015). CH1 domain is the first constant domain of the immunoglobulin heavy chain, which is involved in a body's immunity against bacteria or disease (Macpherson et al. 1996).

It is difficult to correlate the function of some of these proteins with the processes of the limb regeneration. Seventy two hours after the initial tail amputation, altered expression of proteins involved in the immune system, cellular energetics, and protein folding and degradation was detected in this study.

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Table 1. MALDI-TOF MS identification of differentially expressed proteins from *A. carolinensis* 72 h post tail amputation; $\alpha = 0.05$, $n = 3$.

Spot #	Calculated/ Observed pI*	Nominal/ Observed mass (kD)	Protein I.D. (accession #)	Coverage (%)	MOWSE score	Average fold change	T-test (P)	Function
307	5.7/6.5	76.455/75	cd13617	29	560	1.1	0.016	Transferrin (iron-binding blood plasma glycoprotein)
310	5.23/5.3	42.024/73	cd00012	30	347	1.4	0.034	Nucleotide-Binding Domain of the sugar kinase superfamily
310	5.98/5.3	57.643/73	cd04985	21	269	1.4	0.034	CH1 domain in immunoglobulin
393	5.60/6.3	60.439/60	cd03335	31	545	-1.5	0.016	T-complex protein 1 (involved in productive folding of protein)
393	6.01/6.3	61.717/60	cd03085	21	358	-1.5	-0.016	Phosphoglucomutase 1 (sucrose catabolism)
411	5.87/6.6	49.196/55	cd00009	63	938	-2.0	0.023	ATPases Associated with a wide variety of cellular Activities.
485	6.47/6.5	43.842/46	cd00716	32	795	-1.3	0.017	Creatine kinase
485	6.18/6.5	52.928/46	cd09913	27	795	-1.3	0.017	Eps15 homology domain (endocytic events)
614	5.37/5.8	42724/36	pfam01399	43	427	-1.1	0.015	PINT motif (Proteasome, Int-6, Nip-1 and TRIP-15)

* - Isoelectric point.

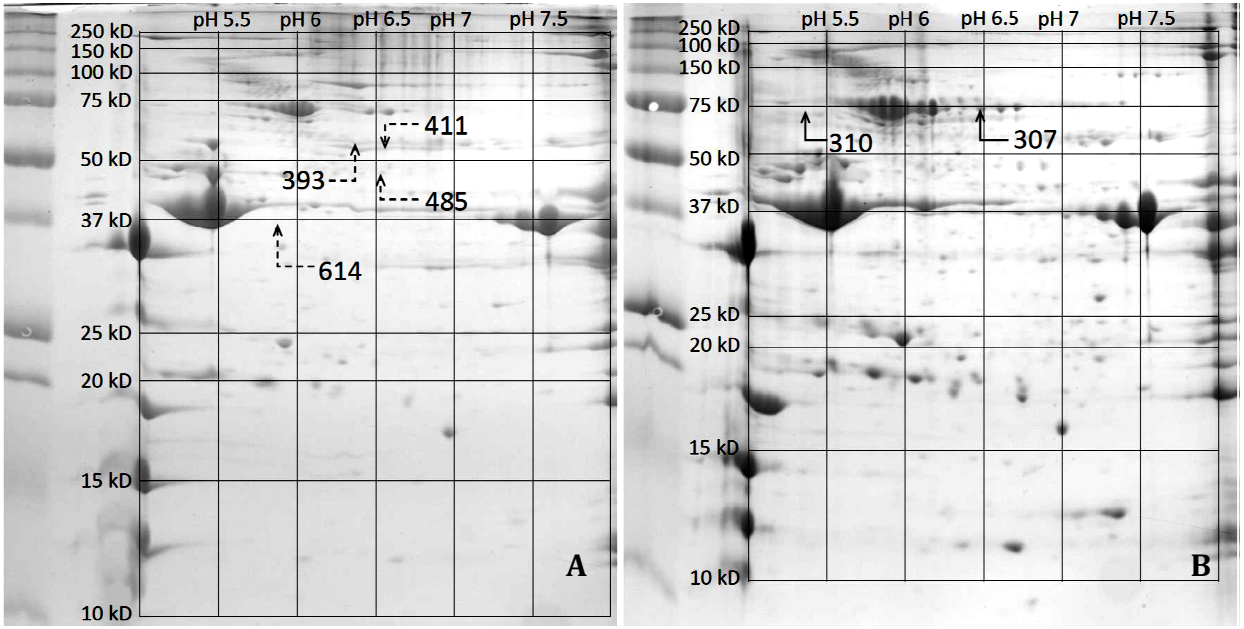

Figure 1. Representative 2-D gels of male *Anolis carolinensis* stained in coomassie blue; (A) 0 h, N = 3 (B) 72 h, N = 3. Molecular weight standards are on the left side of the gel, and isoelectric focusing point at the top. The selected spots were up- or down-regulated by 1.1-fold or greater ($p < 0.05$) between 0 h and 72 h gels and had over 20% coverage (Table 1). Solid arrows indicate up-regulated proteins, and dashed arrows down-regulated proteins.