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Identification of proteins interacting with lactate dehydrogenase in claw muscle of the porcelain crab *Petrolisthes cinctipes*

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Abstract

Biochemical adaptation of enzymes involves conservation of activity, stability and affinity across a wide range of intracellular and environmental conditions. Enzyme adaptation by alteration of primary structure is well known, but the roles of protein-protein interactions in enzyme adaptation are less well understood. Interspecific differences in thermal stability of lactate dehydrogenase (LDH) in porcelain crabs (genus *Petrolisthes*) are related to intrinsic differences among LDH molecules and by interactions with other stabilizing proteins. Here, we identified proteins that interact with LDH in porcelain crab claw muscle tissue using co-immunoprecipitation, and showed LDH exists in high molecular weight complexes using size exclusion chromatography and Western blot analyses. Co-immunoprecipitated proteins were separated using 2D SDS PAGE and analyzed by LC/ESI using peptide MS/MS. Peptide MS/MS ions were compared to an EST database for *Petrolisthes cinctipes* to identify proteins. Identified proteins included cytoskeletal elements, glycolytic enzymes, a phosphagen kinase, and the respiratory protein hemocyanin. Our results support the hypothesis that LDH interacts with glycolytic enzymes in a metabolon structured by cytoskeletal elements that may also include the enzyme for transfer of the adenylate charge in glycolytically produced ATP. Those interactions may play specific roles in biochemical adaptation of glycolytic enzymes.

Keywords

lactate dehydrogenase; protein-protein interactions; stability; metabolon; glycolysis; porcelain crab

1. Introduction

The organization of metabolic pathways, such as glycolysis and the tricarboxylic acid cycle, into structurally-coherent units termed metabolons can increase cellular metabolic flux and efficiency (Robinson et al., 1986; Velot et al., 1997). Metabolons are ordered groups of

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enzymes within a metabolic pathway that interact to form highly arranged enzyme complexes including sequential ordering of enzymes organized around cytoskeletal elements (Goetz et al., 1999). The enzyme ordering can increase metabolic flux by optimizing channeling of pathway intermediates and reducing diffusion distances (Velot et al., 1997). Compartmentalization of enzymes within a metabolon increases efficiency by preventing escape of metabolites to a different metabolic pathway (Velot et al., 1997). Metabolon organization of enzymes may also be a means by which enzyme thermodynamic properties (e.g., K_m , K_{cat} , V_{max}) and structural stability are regulated in responses to environmental cues.

Enzymatic proteins are marginally stable with a net free energy of the native conformation on the order of a few weak bonds (Branden and Tooze, 1999; Hochachka and Somero, 2002). A wide array of environmental conditions (e.g., temperature, osmotic content, intracellular pH (pHi)) can destabilize a protein's native conformation, and cause local to complete protein unfolding (Becktel and Baase, 1987; Branden and Tooze, 1999). Organisms that experience rapid variation in these environmental conditions that impact the intracellular environment (e.g., fermentative glycolytic reduction in pHi during burst activity) ensure protein stability by both intrinsic and extrinsic stabilization mechanisms. Intrinsic stabilization of proteins by amino acid substitutions in critical regions of the enzyme (Coquelle et al., 2007) can affect tertiary or quaternary structural stability and alter thermodynamic properties (e.g., K_m , K_{cat} , V_{max} , E_a) (Fields et al., 2002). Organisms that possess multiple paralogs and/or orthologs for a protein may respond to environmental shifts by expression of a different gene variant (Hochachka and Somero, 2002). Extrinsic stabilization of proteins through modification of water structure by methylamines (e.g., trimethylamine oxide, TMAO) can counteract enzyme destabilization by environmental gradients in hydrostatic pressure (Fields et al., 2001; Samerotte et al., 2007; Yancey, 2001, 2005; Yancey et al., 2004) and temperature (Fields et al., 2001). In contrast to what is known about the role of sequence variation or external milieu in alteration of protein structural stability, relatively little is known about how organisms use protein-protein interactions to protect the native state of proteins during destabilizing environmental conditions. Protein-protein interactions have been shown to enhance stability in purified protein mixtures (Layton and Hellinga, 2011), and in porcelain crab claw muscle, the stability of L-lactate dehydrogenase (LDH, EC 1.1.1.27: NAD⁺:lactate oxidoreductase) is enhanced by specific, but unidentified, protein-protein interactions, rather than by solute effects or protein concentration *per se* (Stillman and Somero, 2001).

In this study we focused on identifying extrinsic protein stabilizers of LDH in the porcelain crab *Petrolisthes cinctipes* using a co-immunoprecipitation approach. We hypothesize that the extrinsic stabilizing proteins are other glycolytic enzymes anchored to cytoskeletal elements in a metabolon (Goetz et al., 1999). Regulation of glycolytic enzyme assembly can alter glucose metabolism (Campanella et al., 2008), potentially through modification of the structural stability of glycolytic enzymes by protein-protein interactions. Protein-protein interactions in the glycolytic metabolon may be an important element in crab claw muscle for both fermentative glycolytic flux regulation as well as stabilization of glycolytic enzymes during physiological hypoxia and acidosis that occurs during prolonged activity (e.g., meral spread displays, pinching, crushing). By identifying these stabilizing proteins of crab LDH we hope to better understand basic components of protein interactions involving LDH activity or stability that are important in cellular regulation of glycolysis in hypoxia tolerance of metastatic solid tumors (Mathupala et al., 1997; Wissemann et al., 1991; Zhong and De Marzo, 1999).

2. Materials and Methods

2.1. Specimen Collection

Porcelain crab, *Petrolisthes cinctipes*, specimens were collected from sites including Cape Arago, Oregon (42°21'N; 124°19'W); Monterey Bay, California (36°36'N; 121°53'W), and around the San Francisco Bay, California (37° 46' N; 122° 26' W). Specimens were frozen in liquid N₂ or dry ice, and stored at -80 °C.

2.2. Antibody production

Polyclonal antibodies against *Petrolisthes cinctipes* LDH were produced in rabbits (Cocalico Biological, Inc.). Oxamate substrate affinity chromatography was used to purify *P. cinctipes* LDH following Fields and Somero (1997). Purity was checked by silver stained SDS-PAGE, and there was only one band present at ~35 kDa, the monomeric size of LDH, even when the stain was overdeveloped. Purified native LDH was injected in order to select for epitopes on the surface of the native LDH molecule. IgGs were purified from the rabbit serum using ProteinA affinity resin, dialyzed, concentrated with Centricon-30 centrifugal concentrators, and stored at -20 °C in 50% glycerol.

2.3. Co-immunoprecipitation

Petrolisthes cinctipes claw muscle tissue (50–100 mg) was dissected from frozen specimens and homogenized in 500 µL ice-cold radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris, pH 7.4, 1% NP-40 (w/v), 150 mM NaCl, 5 mM EDTA, 0.5% deoxycholic acid (w/v), 0.1% SDS (w/v), and 0.5× protease inhibitor (Sigma, P8340)), in ground glass homogenizers. RIPA was used in attempts to only capture strongly interacting proteins, and to minimize non-specific protein sticking. All subsequent procedures were performed on ice or at 4 °C unless otherwise noted. The homogenate was centrifuged (9,300 g for 2 min) and the supernatant was transferred to a fresh tube. General Co-IP procedure follows (Phee et al., 2006). For the first pre-clearing, 4 µL of non-immune rabbit serum (Sigma, R9133) was added to each homogenate supernatant. Protein A-sepharose 4B resin (Sigma, P9424) was prepared for use by washing 50 µL resin four times in 50 µL RIPA. Each wash involved mixing the resin with fresh RIPA and collection of cleaned resin by centrifugation. All centrifugations were at 112 g for 30 s. Cleaned resin was re-suspended in a 50% (v/v) RIPA slurry (total volume ~100 µL). Resin slurry (20 µL) was added to each muscle tissue homogenate supernatant and incubated for 1 h on a rotamix rotator. Samples were centrifuged and each supernatant collected for the second pre-clearing, and pellets saved for analysis of non-specific binding. For the second pre-clearing, 20 µL of resin slurry was added to the supernatant and incubated on rotation rack for 1 hr before centrifugation. Supernatants were collected for the IP step and pellets were saved for analysis using one-dimensional (1D) and two dimensional (2D) gel electrophoresis. For the IP step, 4 µL of IgG anti-*Petrolisthes cinctipes* LDH were added to the sample and incubated with mixing for six hours in order for LDH and antibody interaction to occur. Resin slurry (30 µL) was added and sample was incubated with mixing for 1hr followed by centrifugation. The pellet, which should have bound to the antibody-LDH-interacting proteins, was washed four times with RIPA. Each wash was saved for SDS-PAGE analysis to verify that all non-specific interacting proteins had been removed, and the cleaned pellet used for 1D or 2D PAGE. As controls, we performed the above procedures with purified LDH instead of homogenate supernatants, and without primary antibody to determine that LDH and interacting proteins are not able to precipitate without the antibody as well as any other proteins.

2.4. 1-D gel electrophoresis

The cleaned proteinA-sepharose pellet was incubated at 95–100°C for 2 min in 30 µL SDS sample buffer (62.5 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 2.5% β-mercaptoethanol, 0.0025% bromophenol blue (Laemmli, 1970)). Proteins were separated by 12% SDS-PAGE (Deslauriers et al., 1990; Laemmli, 1970) using a mini-PROTEAN-3 apparatus (BIO-RAD). Prestained SDS-PAGE molecular weight standards (BIO-RAD 161-0318) were run on each gel. Gels were stained using Coomassie Blue, imaged, and dried.

2.5. 2-D gel electrophoresis

Cleaned proteinA Sepharose pellets were suspended in isoelectric focusing buffer (7 M urea, 2 M thiourea, 2% CHAPS, 2% NP-40, 15 mM DTE, 0.5% ampholytes pH 3–10 NL (Amersham Biosciences, Uppsala, Sweden) and 0.002% bromophenol blue). Proteins (300 µg) were loaded onto each 11 cm, pH3-10NL IPG strips by passive rehydration overnight at room temperature. Isoelectric focusing was performed for at least 50,000 Vh then stored at –80 °C. Strips were bathed in equilibration buffer (375 mM Tris-HCl, 6 M urea, 30% glycerol, 2% SDS) to which 65 mM DTT was added to reduce proteins, followed by 135 mM iodoacetamide to alkylate the proteins. The IPG strips were then loaded onto 11% acrylamide slab gels (11.5 cm × 7 cm × 1 mm) for second dimension electrophoresis. The gels were stained with colloidal Coomassie blue and imaged using an Epson 1680 scanner. Images were analyzed using Delta2D software (Decodon GmbH, Greifswald, Germany). The all-to-one warping strategy was used to match the images to one another using the precipitate as the master image. Gel pairs were aligned using automatic (manually approved) and manual match vectors to overlay images to determine which spots were specific products of the co-immunoprecipitation. Spots were excised from the gel using a Beecher tissue array puncher.

2.6. In Gel Digestion

Protein bands/spots of interest were cut from the gels and were washed three times in 200 µL of 100% acetonitrile for ten minutes at 37 °C in a shaking incubator. To each sample, 70 µL of 200 mM NH₄HCO₃ pH 7.8 in acetonitrile (4:6 v/v) was added, and samples were incubated for 30 min at 37 °C. The gel materials were then re-hydrated by adding 70 µL of 200 mM NH₄HCO₃ pH 7.8 for 30 min at 37 °C, and dehydrated by adding 70 µL of acetonitrile for 2 min at room temperature. Trypsin (10 ng/µL) was added to cover the gel pieces. The gel pieces were re-hydrated at 4 °C for 10 min in the trypsin solution, and 55 µL of 25 mM NH₄HCO₃ pH 7.8 was added to the samples for incubation overnight at 37 °C. The supernatant was removed and 20 µL of 0.3% Trifluoroacetic acid in 60% acetonitrile solution was added and incubated for 30 min at RT to elute the remaining peptide out of the gel material. The supernatant was concentrated to a volume of ~1 µL, then 9 µL of 0.1% TFA in HPLC-grade water was added before samples were loaded into the mass spectrometer for peptide mass fingerprint analyses.

2.7. Protein identification LC/ESI-MS/MS analysis

Digested gel eluted protein tryptic-peptides were analyzed by nano-liquid chromatography/electrospray ionization-tandem mass spectrometry (LC/ESI-MS/MS) using a LTQ ion trap mass spectrometer (Thermo Electron, San Jose, CA) with a nano-electrospray ionization source. Reverse phase nano-LC analysis was conducted using a dual Thermo Surveyor HPLC pump system. Mobile phases for the reverse phase nano-LC were 0.1% HCOOH in water (mobile phase A) and 0.1% HCOOH in acetonitrile (mobile phase B). A three-step, linear gradient was used for the nanoLC separation (5% to 30% B in the first 65 min, followed by 30% to 80% B in the next 10 min, and 80% B in the last 10 min). ESI-MS/MS data acquisition was set up to collect ion signals from the eluted peptides using a cyclical

series of three different scan modes (1 full scan, 4 zoom scans, and 4 MS/MS scans for top four abundant ions). Full scan mass range was set from $m/z = 400$ to 1800. MS/MS analysis exclusion rule for the same precursor ion was set to a value of 3 during a 30 s period.

Resulting MS/MS spectra were searched against an assembled set of 98K ESTs from *P. cinctipes* (Tagmount et al., 2009) using the Sequest program to identify sequences of peptides based on the correlation scoring (Xcorr) of MS/MS fragments with the calculated fragments of the matching peptide. Protein identifications were based on a minimum of 2 peptide hits with a Xcorr score greater than 1.8 for a singly charged peptide ion, 2.5 for a doubly charged peptide ion, or 3.5 for a triply charged peptide ion.

2.8. Size Exclusion Column Chromatography

To determine whether LDH was present in protein complexes of different size, we used gel filtration size exclusion chromatography. Claw muscle tissue (~1 g) was dissected from frozen specimens and homogenized in 5 mL ice-cold RIPA buffer (the same buffer used in co-IP), in ground glass homogenizers. All subsequent procedures were performed on ice or at 4°C unless otherwise noted. The homogenate was centrifuged (9,300 g, 35 min) and the supernatant was transferred to a fresh tube. Supernatant (~4 mL) was combined with 1 mL molecular weight marker solution (a mix of dyes with different colors and molecular masses: 0.1 mg/mL vitamin B-12 (1.4 kDa), 0.1 mg/mL cytochrome C (12 kDa), 0.1 mg/mL blue dextran (2,000 kDa) in 20 mM TrisHCl, 1 mM EDTA, pH 8 acidic iodide 0.2% (w/v) starch). Sample was applied to a 100 mL column (1cm wide × 40 cm tall) packed with sephacryl S-300 with a constant flow of RIPA buffer running through the column at a flow rate of between 75–150 µL/min. An initial void volume was collected followed by fractions of 300–600 µl once the blue dextran reached the bottom of the column. Fractions were stored on ice for enzymatic activity assays and for Western blot analyses.

2.9. LDH Activity of Size Exclusion Chromatography Fractions

Enzymatic activity assays monitoring the oxidation of NADH were performed using a 96 well plate reader (Tecan Infinite F200) at 25 °C at 340 nm. 5 µL from each eluted fraction was loaded into a well on the plate. 195 µL of assay cocktail (80 mM imidazole, 150 µM NADH, 5 mM pyruvate) was added and absorbance was recorded each minute for 60 min. Maximum slopes over 20 data points were calculated using Magellan6 software.

2.10. Western Blot Analysis of Size Exclusion Fractions

Fractions were run out on a 12% SDS-PAGE as above. Proteins were transferred from the SDS gel to 0.45 µm pore size nitrocellulose membrane using the Bio-Rad mini protean II blotting apparatus following the manufacturer's instructions. After transfer, the blots were blocked by shaking overnight at 4 °C in a solution of 6% instant non fat dry milk with less than 2% fat (Safeway Brand) in phosphate buffered saline plus Tween-20 (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, and 0.05% Tween-20). The blots were then incubated at room temperature 2 h in a solution of 3% milk/PBS containing 1:1000 to 1:5000 immunoserum containing anti-*P. cinctipes* LDH (see above). The blots were washed in multiple washes of PBS-Tween for a total of 1 h, and incubated for 1.5 h in 3% milk/PBS containing secondary antibody (Anti-Rabbit IgG-Peroxidase Sigma A0545-1mL) at 1:2000 dilution. The blots were finally washed for 30 min in PBS-Tween, the excess buffer was drained and membranes were incubated for 5 min in the ECL reagent (Pierce Product #32109), and exposed to Kodak X-OMAT film for 30 s.

3. Results

3.1. Electrophoretic Separation of Co-Immunoprecipitated Proteins

Separation of co-immunoprecipitated (co-IP) proteins on 1D SDS-PAGE yielded at least five protein bands (one ~190 kDa, three ~90 kDa, and one ~45 kDa) that were not present in samples resulting from co-IP of pure LDH or co-IP without primary antibody (Fig. 1C). Greater separation of proteins was achieved using 2D gel electrophoresis (Fig. 2). To identify which spots on the 2D gels were co-IP proteins, the muscle homogenate Co-IP sample gel was overlaid with a gel of pure LDH-IgG to form a composite image (Fig. 2C). Seventy-one circled spots represent Co-IP proteins of interest that were not shared with the antibody spots. These proteins have a wide range of molecular weights, spanning the entire height of the gel (10–275 kDa), and a wide range of isoelectric points (pIs) spanning the length of the gel (pH 3–10).

3.3. Identification of co-IP proteins

All of the protein bands excised from the 1D gel matched a sequence in the EST database, and were identified as actin, myosin, and the oxygen carrying protein hemocyanin (Table 1). Thirty-two of the 71 protein spots excised from the 2D gel yielded adequate sequence data to achieve a match with a sequence from the EST database, based on total XCorr scores > 20 from at least two peptides matching the translated sequence (Table 2). These successfully identified proteins were fructose-bisphosphate aldolase C, phosphoglycerate mutase, enolase, arginine kinase, hemocyanin, actin, tropomyosin, and four and a half LIM domain protein (Table 2). Spot 19 was successfully sequenced and matched a *P. cinctipes* EST, but that EST had no homology to any known proteins from the major protein and DNA databases (e.g., GenBank, InterProScan). Half of the spots characterized were hemocyanin (16/32, Table 2), corresponding to a smear of basic proteins at about 75 kDa molecular mass (Fig 2C).

3.4. Chromatography Based Analysis of LDH-containing Complex Size

Size exclusion chromatography indicated LDH enzymatic activity in large protein size classes. LDH activity was observed after 7 or 8 fractions, gradually increased to approximately fraction 50–55, then rapidly decreased (Fig. 3). Western blots were performed on every fifth eluted fraction from the chromatography indicated that LDH was present in two to three separate size classes in each fraction (Fig. 4). Though all size classes were observed in each fraction, larger sized protein complexes containing LDH were observed in earlier fractions, and smaller sized protein complexes were observed in later fractions (Fig. 4).

4. Discussion

Our results indicate that lactate dehydrogenase in crab claw muscle is a member of a complex of glycolytic and energy transfer enzymes organized on cytoskeletal elements. Hemocyanin is commonly observed in co-immunoprecipitation of proteins from crab muscle tissue (Don Mykles, pers. comm.), reflecting the fact that this respiratory protein is inherently “sticky” to other proteins, and is in very high concentration in the extracellular fluids of the crabs’ open venous system. Previous studies have shown that glycolytic proteins interact with the cytoskeleton in a way that allows for the compartmentalization of highly organized multi-enzyme complexes (Glass-Marmor and Beitner, 1997). Among these glycolytic enzymes identified in our study and that have been shown to form supramolecular complexes with other metabolic enzymes and cytoskeletal elements are phosphoglycerate mutase (PGM), fructose-bisphosphate aldolase C (ALDO), and enolase. PGM converts 3-phosphoglycerate to 2-phosphoglycerate in the eighth step of glycolysis (Kowalski et al.,

2009). In rabbit striated muscle, PGM has been shown to interact with cytoskeletal elements and other glycolytic proteins, with LDH hypothesized as a linker between the actin-tropomyosin complex and PGM (Kowalski et al., 2009). ALDO has been shown to have a high affinity for actin and other glycolytic enzymes, such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Waingeh et al., 2006), and has electrophoretic co-mobilization with LDH, tubulin, and other glycolytic enzymes (Knull and Minton, 1996). ALDO converts fructose 1,6-bisphosphate to glyceraldehydes 3-phosphate and dihydroxyacetone phosphate in the fourth step of glycolysis (Freemont et al., 1988). Enolase, a metalloenzyme that catalyzes the conversion of 2-phosphoglycerate (2-PG) to phosphoenolpyruvate in the ninth step of glycolysis (Peshavaria, 1991), interacts with the cytoskeleton and other metabolic enzymes to form a supramolecular complex (Masters, 1992).

Structural proteins identified in our study that could form the “backbone” of an organizing center for the metabolic enzymes included actin, myosin, tropomyosin, and four and a half LIM domain proteins. Tropomyosin is an actin binding protein that is involved in the regulation of actin mechanics, connections between actin and myosin, as well as the motor functionality of myosin (Kee et al., 2009). Tropomyosin has been shown to be involved in the interactions between the cytoskeleton and glycolytic enzymes in studies using affinity chromatography, where LDH, enolase, ALDO, PGM and other glycolytic enzymes were shown to associate with actin-tropomyosin complexes (Beeckmans et al., 1993).

Our study found protein interaction complexes consistent with metabolon theory; groups of enzymes within a metabolic pathway that are organized by the cytoskeleton interact to form highly arranged enzyme complexes (Velot et al., 1997). In addition to other glycolytic enzymes, we observed arginine kinase, the enzyme that could be involved with transfer of ATP produced by substrate-level phosphorylation to the phosphoarginine phosphagen. Prior studies indicate the stability of porcelain crab LDH is sometimes enhanced by protein-protein interactions (Stillman and Somero, 2001), and thus metabolons may do more than just offer maximally efficient metabolite transfer, they may also offer protection from denaturation under extreme environmental conditions such as high temp, low pH, and hypoxia. Thus, biochemical adaptation of structure-function relationships in enzymes may be due to protein-protein interactions as well as interactions with the cellular solutes, solvents, and changes within the enzymes themselves (Hochachka and Somero, 2002).

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Figure 1.

Analysis of proteins present during steps of the co-immunoprecipitation procedure. All gels are 12% SDS-PAGE stained with Coomassie Blue. (A) Analysis of proteins present during pre-clearing and washing steps of the co-immunoprecipitation procedure. Lane contents are 1: molecular weight markers, 2: RIPA buffer, 3: muscle tissue homogenate supernatant, 4: pre-clearing #1 supernatant, 5: pre-clearing #1 pellet, 6: pre-clearing #2 supernatant, 7: pre-clearing #2 pellet, 8: pellet wash #1, 9: pellet wash #2, 10: pellet wash #3 showing that nearly all non-specific proteins were gone by the third pellet wash. See methods for details on sample preparation. (B) Controls for co-immunoprecipitation procedure. Lane contents are 1: molecular weight markers, 2: fourth pellet wash (wash #4) of Co-IP procedure done

without LDH IgG showing that there was no non-specific carry over of proteins, 3: pellet wash #4 of Co-IP procedure done with LDH only showing that LDH stuck to the pellet and was not in the wash, 4: Co-IP pellet; procedure done with muscle homogenate but without LDH IgG indicating that there was no non-specific binding to the protein-A sepharose beads, 5: Co-IP pellet; procedure done with LDH only showing what banding pattern looks like with only binding of the primary antibody target, 6: Co-IP pellet; procedure done with muscle homogenate and LDH IgG showing bands representing specific binding to the primary antibody target, 7: purified LDH, 8: purified LDH IgG. (C) Results of co-immunoprecipitation procedure. Lane contents are 1: molecular weight markers, 2: wash #4: of Co-IP procedure done LDH IgG showing that all non-specific binding had been washed away, 3: Co-IP pellet; procedure done with muscle homogenate and LDH IgG showing bands representing specific binding to the primary antibody target, 4: purified LDH, 5: purified LDH IgG.

Figure 2.

Two dimensional (2D) SDS-PAGE gel electrophoresis by isoelectric focusing (1st dimension) and size separation (2nd dimension) of proteins from co-immunoprecipitation (Co-IP) experiments. Increasing isoelectric point (pI) is indicated along top of gel, and molecular weight markers indicated on the right side of the images. (A) Co-IP experiment made with no muscle homogenate supernatant indicating proteins present in the LDH-IgG antibodies used to target LDH, and in the proteinA-sepharose. (B) Co-IP experiment made with muscle homogenate supernatant. Black spots represent the proteins that were co-immunoprecipitated along with LDH, as well as from the LDH-IgG antibodies used to target LDH, and the proteinA-sepharose. (C) Overlay of (A) in purple and (B) in green to identify

proteins that were co-immunoprecipitated from muscle homogenate. Black indicates that the protein features were present in both gels, purple indicates that they were only found in the LDH-IgG+ProteinA sample, and green indicates proteins that were unique to the muscle homogenate. Proteins of interest (n=71), indicated by red circles, were excised from the gel for peptide mass fingerprint analysis.

Figure 3. LDH activity assays of elution fractions from two size exclusion chromatography experiments. Fraction volumes were the same between experiments, but in (A) the flow rate was 1 drop/19 sec, and in (B) the flow rate was 1 drop/10 sec. Y-axis values represent the percentage of total activity across all fractions that were observed in each fraction, thus the values sum to equal 1.

Size Exclusion Chromatography Fraction

Figure 4.

Western blots of LDH-containing proteins from size exclusion column chromatography fractions (from Fig. 3). 12% SDS-PAGE stained using ECL Fraction numbers are indicated at top of the blots. Blots in (A) were from the column in Figure 3A, and blots in B were from the column in Figure 3B.

Table 1

Peptide mass fingerprint homology results for co-immunoprecipitated proteins identified using 1D gel electrophoresis.

Band Size ¹	EST ²	Protein
190 kDa	1237_5_CAYC_CAYF_CCAG	Myosin
90 kDa	266_1_CAYC_CAYF_CCAG	Actin
85 kDa	266_1_CAYC_CAYF_CCAG	Actin
80 kDa	1725_2_CAYC_CAYF_CCAG	Hemocyanin
45 kDa	15_14_CAYC_CAYF_CCAG	Actin

¹. See Figure 1 for details.

². Contig (Clustered consensus sequence) with highest % coverage of peptide mass fragments indicated.

Table 2

Peptide mass fingerprint homology results for co-immunoprecipitated proteins identified using 2D gel electrophoresis.

Spots ¹	Contig ²	Protein
1	188_2_CAYC_CAYF_CCAG	Phosphoglycerate mutase
2,4,38	4594_1_CAYC_CAYF_CCAG	Fructose-bisphosphate aldolase C
6,7,8,9,22,70	178_2_CAYC_CAYF_CCAG	Arginine Kinase
54,55	15_8_CAYC_CAYF_CCAG	Actin
19	3261_1_CAYC_CAYF_CCAG	No match
29,30,31,32,33,34,35,36,37,40,42,43,44,46,47,48	1725_1_CAYC_CAYF_CCAG 1148_1_CAYC_CAYF_CCAG 534_1_CAYC_CAYF_CCAG	Hemocyanin
23	13_1_CAYC_CAYF_CCAG	Tropomyosin
20	2861_1_CAYC_CAYF_CCAG	Enolase
5	1223_5_CAYC_CAYF_CCAG	Four and a half LIM domain protein

¹. See Figure 2 for details

². Contig (Clustered consensus sequence) with highest % coverage of peptide mass fragments indicated.