Purification and Computational Analysis of Bovine *Lactate Dehydrogenase*

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Lactate Dehydrogenase was purified under reducing conditions using ammonium sulfate washes, IEX, and affinity chromatography. Gel filtration chromatography, SDS-PAGE, and Western Blot techniques helped confirm the size of the ~37,500Da LDH subunits. Kinetic analysis also served to characterize LDH activity at a pH of 10. The V_{max} showed to be 0.1371 $\mu mol\ NAD^+/min$ whereas the K_m was 0.7134 mM. The k_{cat} was calculated to be 247.08 min^{-1}, which is higher than the expected k_{cat} of roughly 20 min^{-1} (3). The purification and kinetic results suggest that LDH is not the only protein present within the samples, and that further purification is needed. Computational analysis using a 1i0z.pdb Human LDH protein sequence revealed the structure of the LDH dimer and monomer as well as the ARG169 and HIS193 residues involved in the reaction mechanism interconverting lactate to pyruvate.

Lactate dehydrogenase (LDH) is an enzyme found among bacteria, plants, and animals and plays a central role in metabolism – specifically the final step in glycolysis, interconverting lactate and pyruvate. Properties and activities of each of the five LDH isoenzymes change under pathological conditions. In the heart, increased LDH activity commonly results in myocardial infarctions. For this reason, the purified enzyme is useful in biomedical analysis to make calibration curves for measurements of LDH activity in tissues. In this experiment enzyme assays were performed on protein precipitation ammonium sulfate (AS) washes, ion-exchange chromatography (IEX), affinity chromatography, and gel filtration protein purification techniques. The isoenzyme chosen was LDH from bovine heart tissue, given its importance in current biomedical research concerning heart health (1). Previous experiments suggest a sigmoidal relationship of LDH with respect to pyruvate (4), thus kinetic analysis was performed in order to study the chemical properties of LDH, as well as a stability under different conditions. Following kinetic analysis, the SDS-PAGE was performed in order to electrically separate proteins based on size, whereas the Western Blot allowed for a visualization and localization of LDH in a mixture of proteins. The techniques together help assess the overall purity following the protein purification process. In addition, computational analysis was performed in order to analyze the structure and active site of bovine and human LDH. The computational analysis will allow for confirmation of the amino acid residues involved at the active site of LDH. The active site includes a histidine and an arginine – the histidine is used in the proton shift and the arginine helps stabilize the lactate as the forward reaction produces NADH + H^+ + pyruvate. The forward reaction is used in a CAPS buffer pH 10 because the protons produced in the forward reaction would make the solution more acidic – however the buffer keeps the solution at a basic pH, continuing the forward reaction.

Experimental Procedures

Purification

To begin the purification, a bovine heart was homogenized using a blender and the solution containing LDH was precipitated with 40% AS followed by centrifugation. The supernatant of the 40% wash was used for the 75% AS wash. Following the 75% AS wash, the sample was re-centrifuged, and the pellet was kept and dissolved in 0.03M Bicine buffer at a pH of 8.5. Once the pellet was re-dissolved, it was dialyzed in 0.03M Bicine buffer to remove the salt from the AS washes. Enzyme activity assays were completed after each AS wash. The 75% re-suspended pellet was dialyzed, run through an

IEX column prepared with 40mL of Q-Sepharose resin, and washed with 0.03M Bicine buffer at pH 8.5. During the elution phase, 3mL fractions were collected with increasing concentrations of NaCl, beginning with 15mL of 0.2M NaCl and increasing in increments of 0.2M until the final wash of 0.03M Bicine-1.0M NaCl was performed. A Bradford Assay was completed with each fraction of the sample wash and elution using Coomassie Blue G-250 dye (absorbance ~595nm). Activity assays were performed on the fractions, with the highest three LDH activity samples pooled and tested again for activity. Finally, the pooled sample was set up and dialyzed against 2L 20mM sodium phosphate pH 7.0 to remove excess salt. The post-dialysis IEX sample was run through an affinity chromatography column filled with Cibacron Blue F3G resin and washed with phosphate buffer. During the elution phase, 2mL fractions were collected with increasing concentrations of NaCl, beginning with 10mL of 0.2M NaCl and increasing in increments of 0.2M until the final wash of phosphate-1.0M NaCl was performed. Activity assays were performed on the fractions, with the highest three LDH activity samples pooled and tested again for activity.

Characterization of LDH

Varying concentrations of NAD^+ assay cocktails were made with a constant concentration of 25mM lactate and 150mM CAPS buffer. Enzyme activity was determined from absorbance data and used to plot [NAD+] versus V_0. The assays were then repeated with 10mM oxamate inhibitor. A Michaelis-Menten curve with a hyperbolic fit was generated to determine K_m and V_{max} for the inhibited and non-inhibited LDH, and a Lineweaver-Burk plot was generated to determine the type of enzyme inhibition. Each plot was made in *SigmaPlot* software. A column, filled with 24mL using gel filtration Superose 12, was used to elute proteins following FPLC methods. The data was used to generate a chromatogram plotting Abs_{280nm} vs. elution volume (mL) for each protein standard and LDH. Elution volume was used to calculate K_{avg} which was plotted against logMW of the protein standards to generate a calibration curve – from which the molecular weight of LDH was calculated. A BSA standard curve was prepared using increasing amounts of BSA stock, and 1mL of Bradford reagent. The linear equation of the standard curve was used to calculate the amount of protein for each LDH purification sample. These data were used in calculating specific activity, fold purification, V_{max}, and k_{cat}. Four of the purification samples were mixed with loading dye containing β-mercaptoethanol, heated at 95°C for 5 minutes, and spun in a centrifuge. Two trials of protein standard ($10\mu L$) and each of the four samples ($15\mu L$) were loaded into the gel and run for 1 hour at 150V. Afterwards, half of the gel was placed in Coomassie blue, while the other was placed against a nitrocellulose membrane and transferred for 1 hour at 100V. The membrane was incubated in Blotto solution, washed three times with 15-20mL TBST, followed by a wash with 1:2000-fold secondary antibody, and three more TBST washes. The membrane, soaked in 10mL TMB solution, was stopped with water when blue bands appeared. The Coomassie gel was soaked in destain solution until the bands were distinguishable. Purity of each sample was analyzed using a Laemmli plot.

Computational Analysis

Computational analysis was performed using human and bovine LDH sequences. The sequences were aligned using Clustal Omega tool, from which %identity and %similarity could be determined. The pI, molecular weight, and extinction coefficient under reducing conditions were gathered from Protparam. Protein structure was visualized using a protein data bank (.pdb) file uploaded in *SwissPDB Viewer* software. First, the structure of the LDH dimer with ligands, the structure of the LDH monomer, the alpha-helix and beta sheet structures in ribbon and ball-stick representations, and the structure of the active site were all explored. The distance between

residues was determined in Angstroms, allowing for spatial analysis of the LDH sequence.

Results

Purification: AS Wash

The purification table (Table 1) shows the final purification table for the protein purification steps of the experiment, and outlines the procedures used for LDH purification. The procedure was successful in obtaining a high fold-purification as the second highest fold was the final, affinity-pooled sample Results gathered from the ammonium sulfate wash reflect the ability of AS salt to separate proteins based on their hydrophilic strength. LDH, a hydrophilic molecule, had a low recovery (68.7%) for the 45% AS wash, compared to the 75% resuspended pellet (91.7%). The percent recovery from the 75% supernatant showed very little activity, confirming that the LDH protein was correctly located in the pellet resuspended for further experimentation.

Purification: Ion Exchange Chromatography

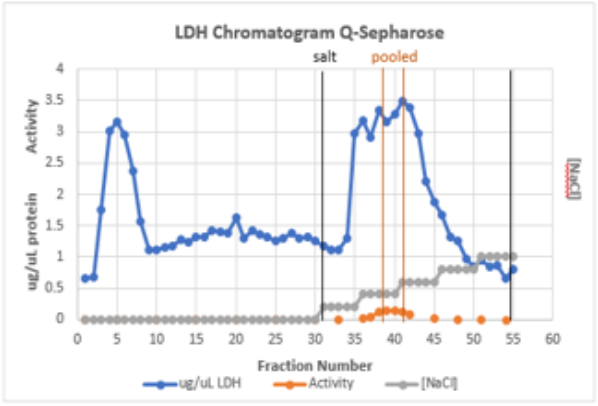
Figure 1. presents the chromatograms following IEX (1a) and affinity (1b) chromatography. The Q-Sepharose proved to be a successful anion-exchange resin as the charge of LDH at pH 10 is above its pI, and therefore is negatively charged. The results of the experiment show that some of the protein activity was lost during the dialysis phase of the purification. However, the pooled samples of the IEX-Chromatography purification show an activity level similar to the 75% post-dialysis activity. This indicates that the LDH purification through IEX retained much of the LDH protein from the post-dialysis sample.

Purification: Affinity Chromatography

The affinity chromatography column filled with Cibacron Blue F3G resin and washed with phosphate buffer showed successful separation

Table 1. Purification Table for each LDH purification step

Sample	Activity (U)	Dilution Factor	Relative Activity (U/mL)	Vol. (mL)	Total Activity (U)	Protein Conc. (mg/mL)	Tot. Protein (mg)	Specific Activity (U/mg)	% Recovery	Fold Purification
Crude	0.0218	100	8.72	100	871.5	40.55	4055.0	0.2149	100	--
40% AS	0.0328	100	5.99	100	598.7	167.91	16791.0	0.0351	68.7	0.16
75% Supernatant	0.0204	70	0.816	104.7	85.43	7.68	804.1	0.1062	9.8	0.49
75% resuspended pellet	0.0333	1	53.27	15.0	799.2	1.59	23.85	33.509	91.7	155.93
75% resuspended pellet, post-dialysis	0.0282	50	22.55	17.1	384.1	28.17	481.71	0.7974	44.1	3.71
IEX pre-dialysis	0.0335	100	26.82	11.4	305.7	6.94	79.12	3.8639	35.1	17.98
IEX post-dialysis	0.0472	100	18.87	10.7	201.9	9.32	99.72	2.0247	23.2	9.42
Affinity-pooled	0.0190	1	15.16	5.2	75.84	2.08	10.82	7.0118	8.7	32.63



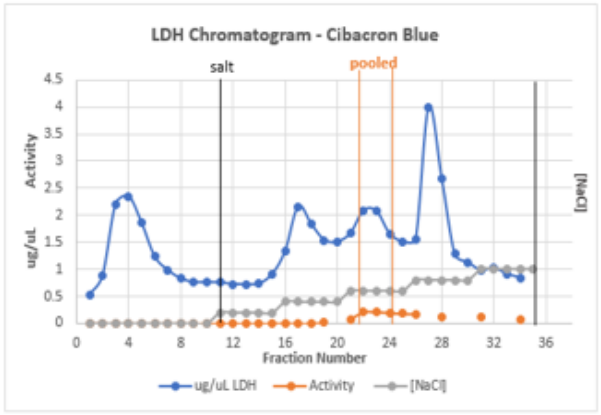
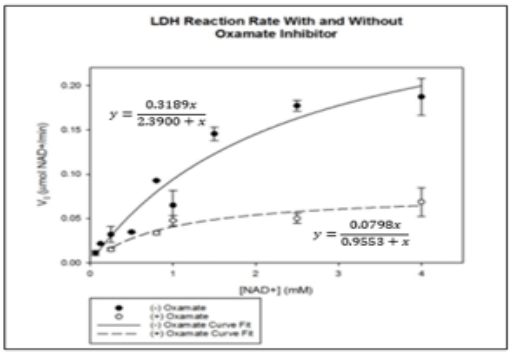


Figure 1. Chromatogram for concentration of LDH, activity units of LDH, and concentration of NaCl in each fraction of the Bradford Assay after (a) IEX chromatography with Q-Sepharose resin, and (b) Affinity chromatography using Cibacron Blue resin. The black lines indicate salt gradient washes, and the orange lines indicate the pooled samples with the highest activity.

of proteins based on specific protein-ligand interactions. The anion-exchange column was necessary in ridding proteins based on charge that may have had a similar affinity for water in the ammonium sulfate wash. However, the post-affinity sample of the protein showed a large loss of LDH. This indicates that the LDH purification through affinity chromatography may not have been completed. A higher concentration of NaCl wash may have been necessary to pull more LDH from the ligand-resin binding column.

Characterization of LDH: Gel Filtration

Gel Filtration Chromatography, employed to fractionate LDH from other proteins in the



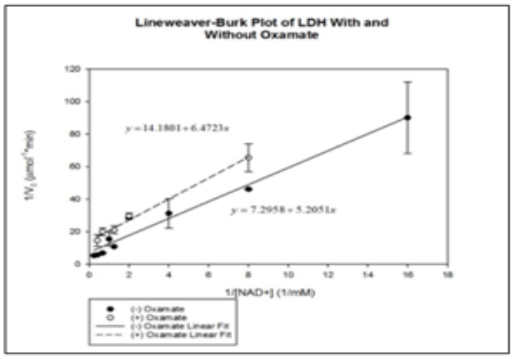


Figure 3. Kinetic analysis of LDH (a) Substrate concentration curve of LDH reaction rate with and without 10mM oxamate Inhibitor (b) Lineweaver-Burk Double Reciprocal plot of LDH reaction rate with and without 10mM oxamate Inhibitor.

sample based on size and shape, was used to create a chromatogram in order to analyze elution profiles. The chromatogram of protein standards and LDH is shown in Figure 2. The chromatogram elution profile shows that LDH and aldolase have a similar elution volume. From aldolase's protein standard data, a molecular weight of around 150,000 Da can be expected since proteins of similar size have similar elution volumes. Based on the amino acid sequence, the LDH polypeptide is 37,500 g/mol. The difference in weight given by the amino acid sequence and the gel filtration calibration curve can be explained by the structure of LDH. This protein generally has four subunits – therefore the 37,500 g/mol can also be defined as the weight of one subunit.

$$37{,}500\frac{g}{mol} \times 4 \; = 150{,}000\frac{g}{mol} = 150{,}000 \; Da \; LDH$$

Therefore, the elution profiles of gel filtration chromatography seem to be accurate measures of the size of the entire protein.

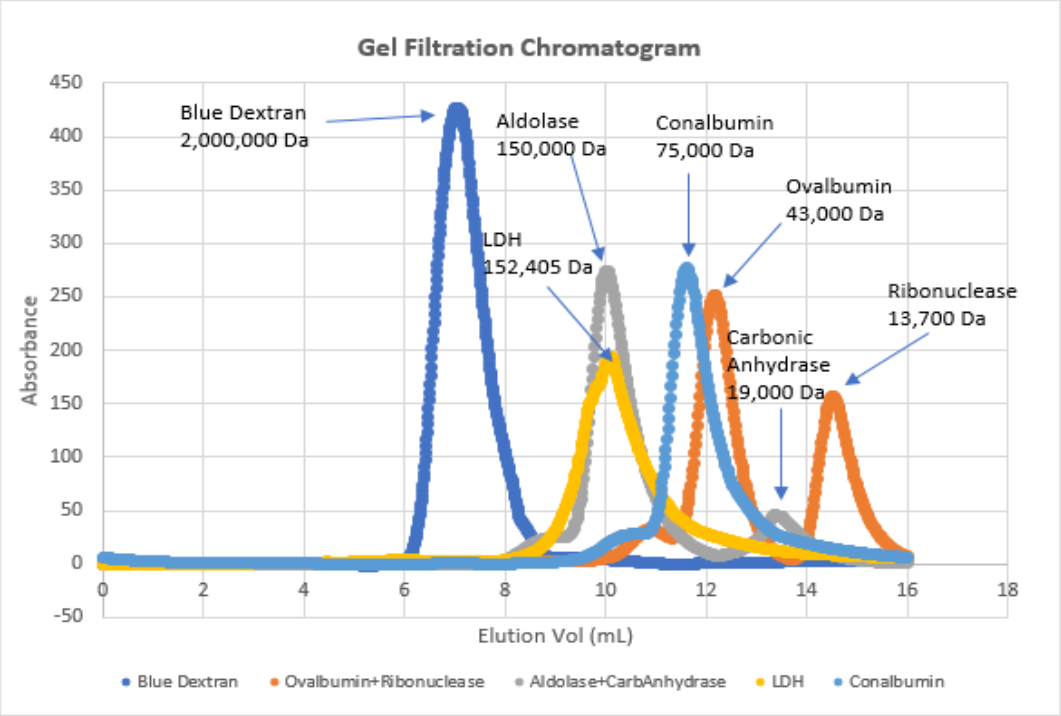


Figure 2. Chromatogram of elution profiles of protein standards and LDH in gel filtration chromatography using 24 mL Superose-12 resin.

Characterization of LDH: Michaelis-Menten Kinetics

Catalytic activity of LDH was studied through Michaelis-Menten kinetics at different concentrations of LDH with and without the presence of Oxamate inhibitor. The enzyme inhibition between LDH and Oxamate could then be predicted from a Lineweaver-Burk plot. Figure 3. shows the substrate saturation curve and Lineweaver-Burk plot.

From the graphs in Fig. 3, the K_m, or the concentration of NAD^+ at which the enzymatic rate is half of it's V_{max} – was determined. These data are shown in Table 2. Because the V_{max} is never reached, the Lineweaver-Burk plot was more accurate in describing the V_{max} of the reaction as the double reciprocal plot linearized the data. The data shows a smaller V_{max} and K_m for both experimental conditions in the Lineweaver-Burk plot than the Substrate Curve, suggesting that the linearized data portrays noncompetitive inhibition between LDH and oxamate in this reaction.

Characterization of LDH: Coomassie Stain and Western Blot

The Western Blot shows the presence of LDH in each sample of protein purification whereas the Coomassie stain shows the molecular weight of LDH and other proteins in the sample (Figure 4.). As the samples move from right to left, the LDH band in the Coomassie stain becomes darker, as well as other proteins being removed

Table 2. V_{max} and K_m values from Saturation Curve and Double Reciprocal Plot with and without 10mM Oxamate. Values were determined by the curves generated during kinetic analysis

	(-) Oxamate		(+) Oxamate	
Saturation Curve	V_{max} ($\mu mol\ NAD^+/min$)	0.3189	V_{max}	0.0798
	K_m (mM)	2.3900	K_m	0.9553
Double Reciprocal Plot	V_{max} ($\mu mol\ NAD^+/min$)	0.1371	V_{max}	0.0705
	K_m (mM)	0.7134	K_m	0.4564

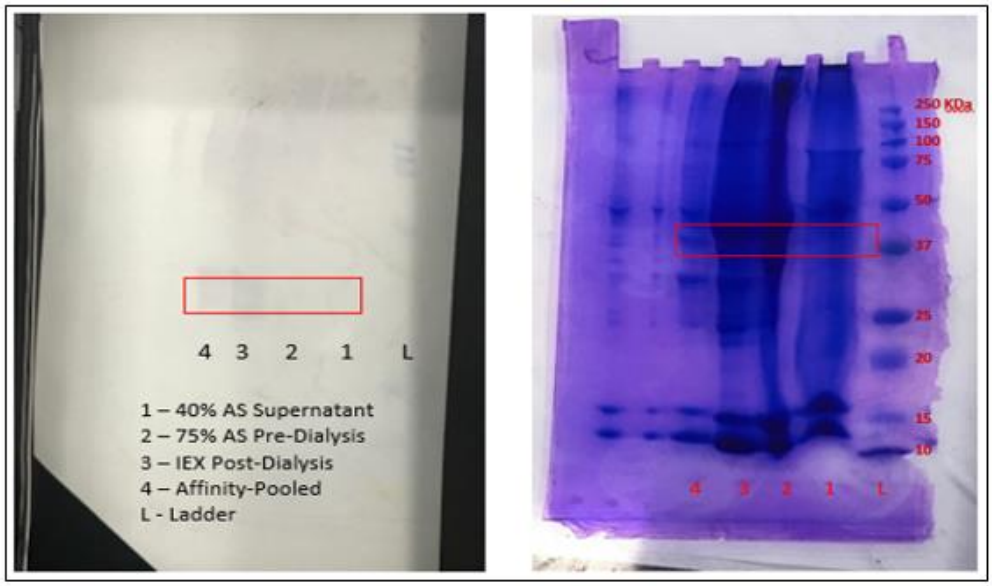


Figure 4. Electrophoretic motility and location of purified LDH. Red boxes highlight LDH location. (a) Western Blot of purification samples. (b) Coomassie stained gel; lanes are the same numbered samples from (a).

from the gel. This indicates that the purification steps were helpful at separating LDH from other proteins. From a Laemmli plot, we calculated the molecular weight of LDH to be 32.63 KDa. Our data from gel filtration showed the molecular weight of each subunit to be 38.10 KDa.

Computational Analysis of LDH

Figure 5. depicts the alignment data from the .pdb LDH isoenzyme protein sequences. Based on the sequence alignment data, the %similarity, 98.5%, suggests that it is reasonable to use the structure of human LDH (1i0z) to understand the activity and mechanism of bovine LDH. The size of the LDH (37,477.13 g/mol), is roughly similar to the 37,500 g/mol we estimate for one subunit of the LDH quaternary structure. The isoelectric point (5.38) of the human LDH is similar to the working pI of bovine LDH throughout the purification process at 6.3. Both of these are acidic pIs, where the molecule is negatively charged above pI 7.0. Representations of LDH dimer and monomer, and active site 3D model are shown in Figure 6.

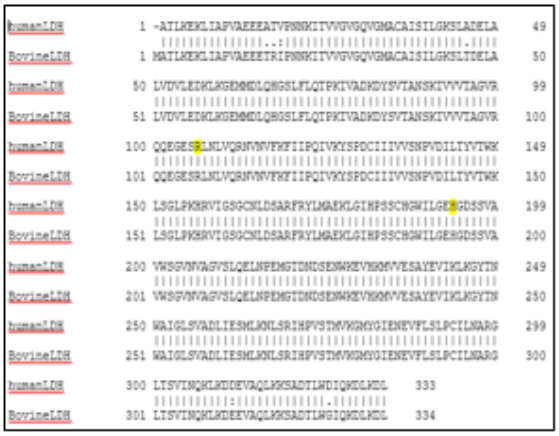


Figure 5. Human and Bovine heart LDH sequence alignment. PDB ID for human LDH sequence is 1i0z. Highlight residues are ARG169 and HIS193 involved in active site mechanisms.

Discussion

LDH is an enzyme that converts lactate into pyruvate and NADH at a pH of 10. Results indicate that the 150mM CAPS buffer solution has a high buffer capacity, and was a sufficient buffer to run LDH enzyme assays with because the pH stays at 10 longer – despite excess protons being produced alongside NADH.

Purification: Ammonium Sulfate Wash

At low concentrations of AS, proteins which are not very hydrophilic were “salted out” as the

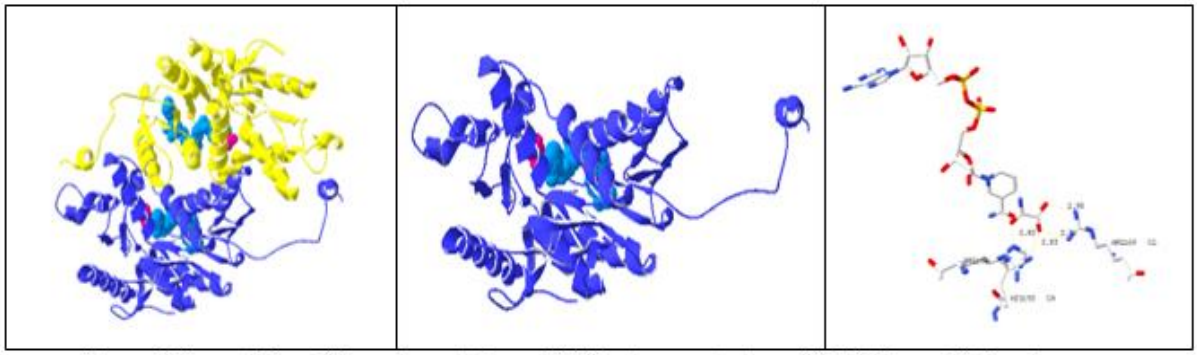


Figure 6. Space-filling ribbon representations of (a) Quaternary structure of LDH dimer with ligands Oxamate (pink) and NADH (light blue), and (b) Tertiary structure of LDH monomer with ligands Oxamate (pink) and NADH (light blue). The active site (c) indicates the closest arginine (ARG169, 2.83 Å) and histidine (HIS193, 2.82 Å) involved in reaction mechanism.

few water molecules surrounding it will be more strongly attracted to the AS salt. At higher concentrations of AS, more hydrophilic molecules were washed out as an increased amount of salt ions in the solution were able to outcompete hydrophilic proteins in binding water. The results gathered from this experiment reflect the ability of an AS wash to separate proteins based on their hydrophilic strength.

Purification: Ion-Exchange Chromatography

The anion-exchange column was necessary in ridding proteins based on charge that may have had a similar affinity for water in the ammonium sulfate wash performed in beginning stages of the purification. Therefore, the IEX column was effective in further separating LDH from other proteins in the sample that may have had similar properties as LDH.

Purification: Affinity Chromatography

The post-affinity sample of the protein showed a large loss of LDH. This indicates that the LDH purification through affinity chromatography may not have been completed. However, the NaCl gradient was the most effective way to pull LDH from the resin because it causes minimal harm to the protein. So although the percent recovery was lower in this step, it provided the largest recovery of LDH relative to other methods of elution.

The method of using a standard curve to quantify protein concentration in each purification step of LDH allowed us to assess specific activity and fold purification of each sample. Based on the data, the 75% re-suspended pellet had the highest specific activity (33.95U/mg) and fold purification (155.93). The affinity-pooled sample had the second highest specific activity and fold purification. This suggests that the 75% re-suspended pellet contained much LDH protein, and that some may have been lost during the second dialysis of the pellet. Additionally, the affinity pool having a high fold purification suggests that the method of purifying LDH based on affinity was a successful method in purification without damaging much LDH protein. The least effective purification step was the 40% AS wash with a specific activity of 0.0351 U/mg and a fold purification of 0.16. This is not surprising though, as we expected more LDH to come off of a purification column at higher concentrations of ammonium sulfate. The 40% AS wash was not concentrated enough to separate much LDH from the lysate.

Characterization of LDH: Gel Filtration

Gel Filtration showed to be an accurate measure of protein size based on elution profiles of protein standards. LDH eluted similarly in fractions near the molecular weight of aldolase, around 150,000 Da. The quaternary structure of LDH is estimated to be 150,000 Da; therefore,

this data makes sense with the theory that gel filtration chromatography is a non-destructive purification technique that can provide information on the quaternary structure of proteins like LDH.

Characterization of LDH: Michaelis-Menten Kinetics

Kinetic analysis of LDH suggested that the linearized data portrays noncompetitive inhibition between LDH and oxamate in this reaction. Because the differences in V_{max} and K_m can be visualized as points of intersection, the changes between each value in the presence of inhibitor can be more easily determined; therefore, the Lineweaver-Burk plots tend to be more advantageous when determining V_{max} and K_m. Our k_{cat} number was 247.08 min^{-1}. This value seems to be smaller than literature reported values, which state a value of 17.4 s^{-1} (3). This may be due to a struggle in experimentation between yield and purity, errors in calculation, or damage to LDH proteins through several purifications, dialysis, and length of time in the refrigerator.

Characterization of LDH: Coomassie Stain and Western Blot

The Western Blot shows some bands of protein in the sample; however it is important to note that the transfer was not completely successful – this is indicated by only a partial ladder transfer on the right side of the membrane. Another Western Blot would be useful in more accurately determining the location of LDH. As the samples move from right to left, the LDH band in the Coomassie stain becomes darker, as well as other proteins being removed from the gel. This indicates that the purification steps were helpful at separating LDH from other proteins. It is notable to mention that the final purification sample, the affinity-pooled sample, still shows other proteins in the Coomassie stain. This indicates that while the LDH band is showing enrichment, further purification is needed in order to remove the rest of the proteins from the sample.

Computational Analysis of LDH

Computational analysis confirmed that the active site includes a histidine and an arginine – the histidine is used in the proton shift and the arginine helps stabilize the lactate as the forward reaction produces NADH + H^+ + pyruvate. The forward reaction is used in a CAPS buffer pH 10 because the protons produced in the forward reaction would make the solution more acidic – however the buffer keeps the solution at a basic pH, continuing the forward reaction.

These experiments have been useful in learning the techniques of protein purification, as well as assessing LDH activity and structure. The structure of LDH active site gives insight to the activity of the enzyme under acidic and basic conditions – and can therefore be used to study the pathological effects of overactive LDH isoenzymes in a biomedical context (2).

References

1. Araújo, R. C. A. F. F. D., Porto, T. S., Martins, D. B. G., Dutra, R. F., Porto, A. L. F., and Filho, J. L. D. L. (2011) Partitioning of lactate dehydrogenase from bovine heart crude extract by polyethylene glycol–citrate aqueous two-phase systems. *Fluid Phase Equilibria*. **301**, 46–50
2. Fosmire, G. J., and Timasheff, S. N. (1972) Molecular weight of beef heart lactate dehydrogenase. *Biochemistry*. 11, 2455–2460
3. Place, A. R., & Powers, D. A. (1979). Genetic variation and relative catalytic efficiencies: lactate dehydrogenase B allozymes of Fundulus heteroclitus. *Proceedings of the National Academy of Sciences*, *76*(5), 2354-2358.
4. Tarmy, E. M., & Kaplan, N. O. (1968). Kinetics of Escherichia coli B D-lactate dehydrogenase and evidence for pyruvate-controlled change in conformation. *Journal of Biological Chemistry*, *243*(10), 2587-2596.