A Novel Approach to the Separation of an Enzymatic Protein: Purifying Lactate Dehydrogenase from Chicken Tissue

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Abstract:

The aim of this investigation is to discover how scientists separate a singular protein from animal tissue in order to analyze it. In order to completely understand purification of a protein, lactate dehydrogenase was purified from chicken in order to separate it from the rest of the tissue. Lactate Dehydrogenase (LDH) was chosen as the protein to test, because Lactate Dehydrogenase-A Deficiency currently does not have a cure or additive to assist those who have it. When an individual has lactate dehydrogenase A deficiency, there is no LDH in the body to convert the pyruvate to lactate. This results in the body not being able to produce enough ATP and results in rhabdomyolysis, or the breakdown of muscle tissue, when those individuals exercise. For many with this metabolic muscle disease, the only treatment available is to understand the activities that cause attacks of rhabdomyolysis, while stopping exercising and other strenuous activity. The lack of the ability to successfully complete anaerobic respiration, results in many with this deficiency, becoming overweight from the lack of exercise. Therefore, separating this protein could assist in further research to develop an additive.

Purpose:

The aim of this investigation is to discover how scientists separate a singular protein from animal tissue in order to analyze it. In order to completely understand purification of a protein, lactate dehydrogenase was purified from chicken in order to separate it from the rest of the muscle. Lactate Dehydrogenase (LDH) was chosen as the protein to test, because Lactate Dehydrogenase-A Deficiency currently does not have a cure or additive to assist those who have it. Therefore, separating this protein could assist in further research to develop an additive.

Background:

Purification:

Before a specific protein and its properties can be studied, the protein must be separated from a sample tissue. Many of the medicines developed through modern biotechnology - vaccines, human insulin, cancer treatments - are all entirely based on the extraction of proteins from complex mixtures. It is also extremely beneficial to purify a protein to determine its amino acid sequences and evolutionary relationships. Extraction and purification is how a protein is successfully separated from the animal tissue, as the desired protein is likely in a matrix of other protein molecules and surrounded by non-protein biological elements. To fully understand the process of purification, lactate dehydrogenase was purified from chicken tissue.

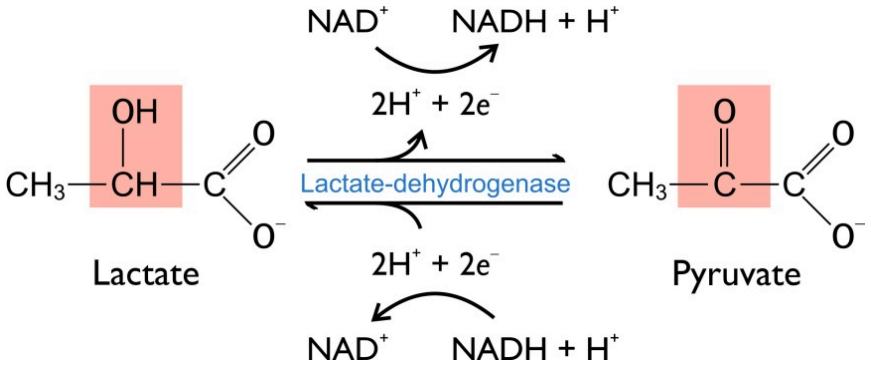
LDH:

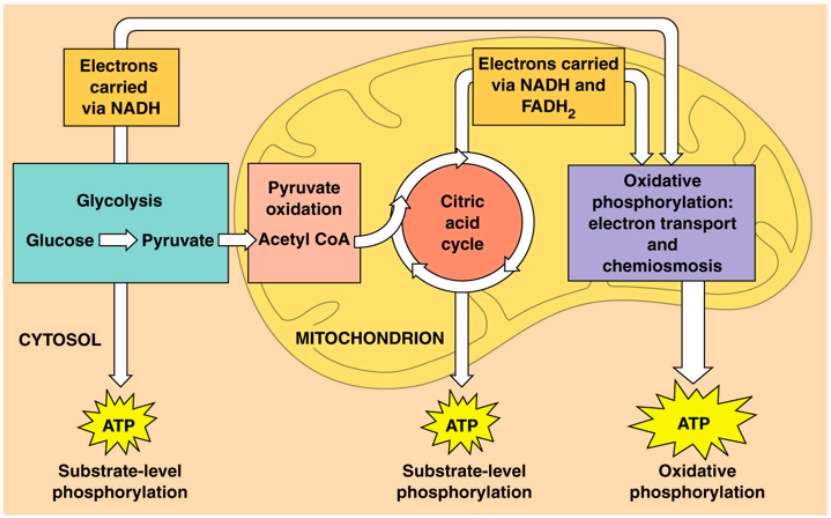
Under normal physiological conditions, pyruvate is generated from glucose by glycolysis. That pyruvate then enters the citric acid cycle in the mitochondria where it then forms acetyl-CoA, which is used to fuel oxidative phosphorylation, generating 36 net more ATP per molecule of glucose. However, when oxygen becomes scarce, such as when one is exercising, cells are unable to use oxidative phosphorylation to generate ATP. Instead they must rely on anaerobic respiration, which consists of glycolysis and fermentation. Once glycolysis is performed, lactate dehydrogenase (LDH) catalyzes the conversion of pyruvate to lactate (lactic acid), as it converts NAD+ to NADH and back. Lactic acid fermentation is how human muscle cells generate ATP. Contrary to the myths about lactic acid being harmful in the muscles, it is actually a defense mechanism, preventing permanent damage to the muscles.

When an individual has lactate dehydrogenase A deficiency, there is no LDH in the body to convert the pyruvate to lactate. This results in the body not being able to produce enough ATP and results in rhabdomyolysis, or the breakdown of muscle tissue. For many with metabolic muscle diseases, the only treatment available is to understand the activities that cause attacks of rhabdomyolysis, while stopping exercising and other strenuous activity. The lack of the ability to successfully complete anaerobic respiration results in many with this deficiency, becoming overweight from the lack of exercise.

Further Uses:

The LDH protein, after isolation, could be grown in plasmids to create an additive for lactate dehydrogenase A deficiency. Those with lactate dehydrogenase A deficiency could effectively complete anaerobic respiration by consuming the derived additive. This allows for the individuals who lack the LDH enzyme to partake in exercise and other strenuous activity.





Research Question:

How do scientists separate a singular protein from animal tissue in order to analyze it?

Materials:

- ❏ 10 mM Tris-HCl (pH 7.4)*
- ❏ 1 mM 2-Mercaptoethanol*
- ❏ 1 mM Phenylmethylsulfonyl fluoride (PMSF)*
- ❏ 1 mM Ethylenediamine tetraacetic acid (EDTA)*
- ❏ Ammonium sulfate (solid)
- ❏ Chicken breast muscle
- ❏ Cheesecloth
- ❏ 50 ml Centrifuge Tubes (four per group)
- ❏ Blender
- ❏ Microcentrifuge Tubes (1.5 ml)
- ❏ Pipet Tips
- ❏ 50-ml Falcon Tubes
- ❏ Tris-HCl (pH 8.6)* * *
- ❏ 2-Mercaptoethanol * * *
- ❏ 1 mM PMSF *
- ❏ Lithium lactate *
- ❏ 1 mM NAD *
- ❏ 1 mM NADH *
- ❏ Econopac desalting column
- ❏ Cibacron Blue column
- ❏ Test Tubes
- ❏ Disposable pipets
- ❏ 120 mM lithium lactate
- ❏ 12 mM NAD+
- ❏ 18 mM NaHCO3
- ❏ 0.5 M NaCl
- ❏ 0.5 mM 2-Mercaptoethanol
- ❏ Cuvette
- ❏ Spectrophotometer
- ❏ 1 mg/ml Bovine serum albumin (BSA)
- ❏ Bradford Coomassie Blue solution
- ❏ H2O

* Extraction Buffer * Tris-PMSF Buffer * NAD Buffer * NADH buffer

Safety Precautions:

Face: Wear goggles to protect the eyes from possible splashing of any liquid or solution.
Hands: Wear gloves to protect the skin from direct contact with the chemicals.
Body: Wear an apron to protect the chest and stomach area from contact with the chemicals.
Apparel: Wear closed toed shoes to prevent any possible contact and protect the skin from direct contact with the chemicals. Tie long hair back.

Procedure:

Purification:

1. Cut 50 g (50.35) of muscle tissue from the tissue source. Cut the tissue into small pieces with scalpel or razor blades. Discard the connective tissue and fat.
2. Place the minced tissue and 75 ml of cold Extraction Buffer in a blender, and put the top on the blender. Disrupt the tissue by homogenizing. Use 4 x 30 second bursts, with at least 10 seconds in between each burst to allow the temperature of the homogenate to decrease.
3. Put the homogenized tissue/buffer mixture into four pre-chilled 50 ml centrifuge tubes. Balance the tubes. Make sure that the tubes are not too full. Centrifuge your homogenate for 20 minutes at 15,000 rpm.
4. Pour the supernatant through two layers of cheesecloth into a pre-chilled beaker. The cheesecloth removes lipids from the solution. Discard the cell debris pellets. Measure and record the volume of the supernatant. Save three 0.5 ml aliquots.
5. Slowly (over a period of ~15 minutes) add 0.39 grams of ammonium sulfate per ml of supernatant to your filtered supernatant on a magnetic stirrer. Stir for an additional 15 minutes after you finish adding the ammonium sulfate.
6. Centrifuge the sample as before. Pour the supernatant into a separate container while keeping the pellet in the centrifuge tube.
7. Add 1 ml of Tris-PMSF buffer to the ammonium sulfate pellet. Gently mix the buffer and the solid material until the pellet dissolves. Keep on ice as much as possible during this procedure.
8. Remove this buffer from the desalting column, and load 3 ml of the mixture on the desalting column. Allow the liquid to drain to the frit. Discard the flow through.
9. Add 4 ml Tris-PMSF buffer to the column, and collect the flow through.
10. Save three 0.1 ml aliquots of the flow through eluent.
11. After saving the aliquots, load the remaining desalted protein solution onto the Cibacron Blue column. Begin collecting 5 ml fractions.
12. When all of the liquid has entered the column, fill the column with Tris-PMSF buffer, and continue collecting 5 ml fractions. Measure absorbance at 280 nm until it is less than ~0.1 above the absorbance of the Tris-PMSF buffer.
13. After removing the remaining Tris-PMSF buffer with a pipet, add 10 ml NAD buffer to the column.
14. When all of the liquid has entered the column, fill the column with Tris-PMSF buffer, and continue collecting 5 ml fractions.
15. After removing the remaining Tris-PMSF buffer with a pipet, add 10 ml NADH buffer to the column. Continue collecting 5 ml fractions.
16. When all of the liquid has entered the column, add Tris-PMSF buffer to the column, and continue collecting 5 ml fractions.

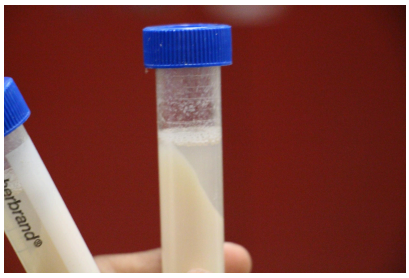
Accuracy Check:

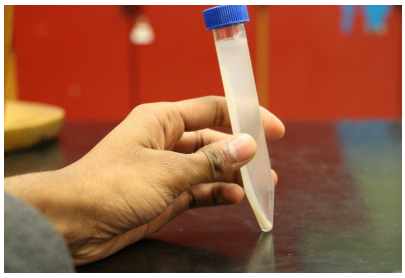
1. Mix 0.6 ml Lactate Stock Solution, 0.4 ml NAD Stock Solution, and 0.2 ml Bicarbonate Stock Solution in a cuvette.
2. Add 10 µl of the solution to be tested for LDH activity, and mix by inverting the cuvette.
3. Place the cuvette in the spectrophotometer to calculate the LDH activity.

Bradford Assay:

1. Make several dilutions of the bovine serum albumin (BSA) stock - 25 µg/ml, 50 µg/ml, 75 µg/ml, 100 µg/ml, 150 µg/ml, and 200 µg/ml solutions.
2. Mix 800 µl of H2O, 200 µl of the Bradford Coomassie Blue solution and 100 µl of the BSA standard dilution together.
3. Measure the absorbance at 595 nm using the spectrophotometer.
4. Plot the absorbance at 595 nm data versus protein concentration to determine the standard curve. Use a linear regression to determine an equation for the standard curve.
5. Dilute the LDH protein to have an absorbance that falls within the standard curve (between 0-1) and assay the sample again.
6. Use the standard curve equation to determine the concentration of LDH using the absorbance of LDH



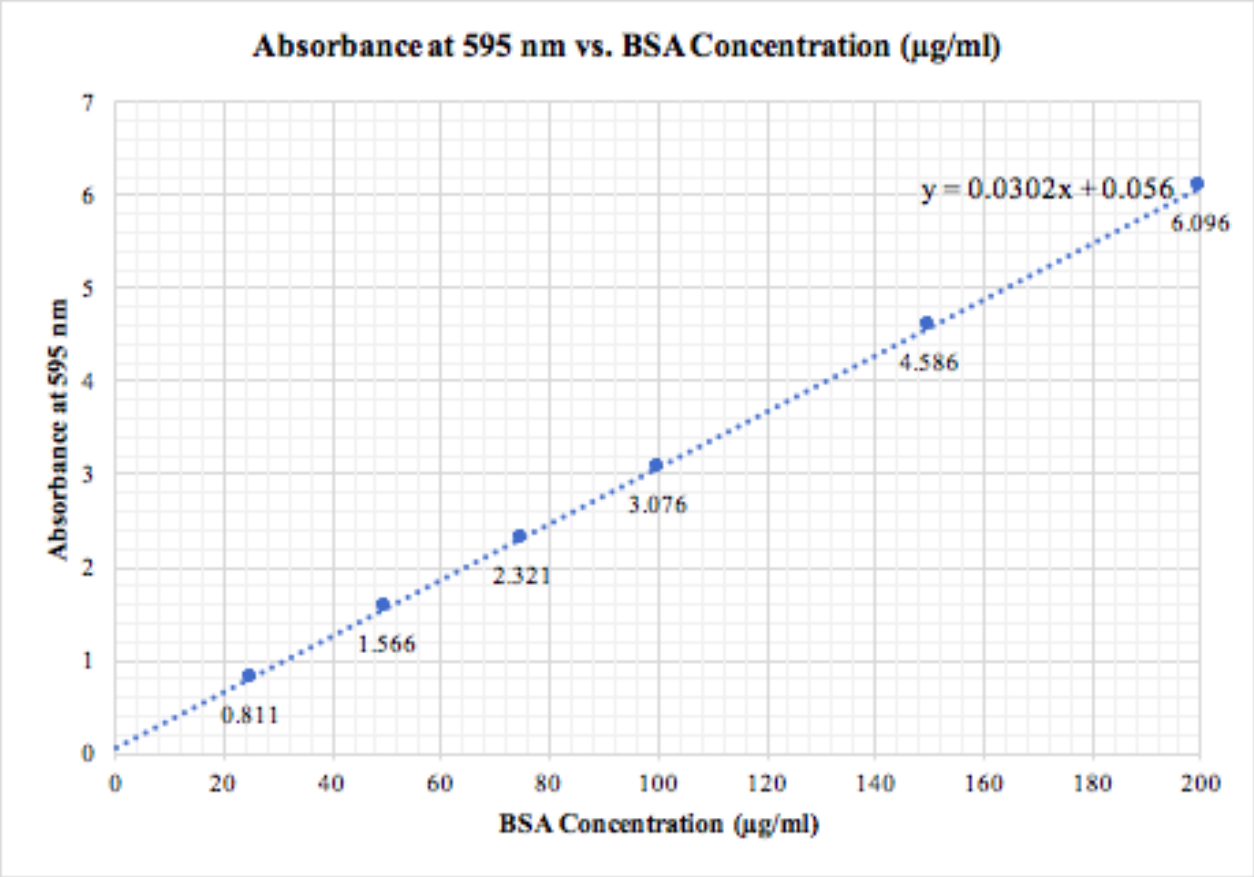



Results:

Bovine Serum Albumin Data

Concentration of BSA (µg/ml)	Absorbance
25	0.811
50	1.566
75	2.321
100	3.076
150	4.586
200	6.096

Standard Curve Graph



LDH Concentration, Purity, and Yield Determination via Bradford Assay

Sample	Crude Homogenate	Post Ammonium Sulfate	Post Tris-PMSF buffer	Post NAD Wash	Post NADH Wash
Dilution	12,500	12,500	125	125	125
Absorbance	0.141	0.065	0.45	0.359	0.613
LDH Activity	75.19	13.11	18.95	38.57	45.86
Diluted Protein Concentration	0.0028	0.0003	0.0130	0.0100	0.0184
Undiluted Protein Concentration	34.810	3.353	1.627	1.250	2.302
Volume	52.0	52.0	7.2	5.5	6.0
Total Protein	1810.099	174.338	11.715	6.877	13.810
Total LDH Activity	3909.88	681.72	136.44	212.135	275.16
Specific LDH Activity	2.160	3.910	11.647	30.846	19.924
Fold Purification	1.00	1.81	5.39	14.28	9.22
% Yield	100.00	17.44	3.49	5.43	7.04

Table 1: The equation of the standard curve determined through the Bradford Assay was: $y = 0.0302x + 0.056$.

Absorbance - Measured using UV-Vis Spectrophotometer

LDH Activity - Measured using the UV-Vis Spectrophotometer, monitoring the production of NADH (umols of NDH per minute per mL)

Diluted protein concentration - determined using the equation of the standard curve

Undiluted Protein Concentration = Diluted Protein Concentration * Dilution Factor

Total Protein = Undiluted Protein Concentration * Volume

Total LDH Activity = LDH Activity * Volume (umols of NDH per minute)

Specific LDH Activity = LDH Activity / Undiluted LDH Concentration. (activity of the enzyme in terms of its purity)

Fold Purification = Specific LDH Activity/Crude Homogenate Activity (shows how pure the protein is)

% Yield = Total LDH Activity/Crude Homogenate LDH Activity (calculates the enzyme activity in terms of the crude homogenate LDH activity)

Discussion:

It was necessary to homogenize the tissue with extraction buffer to prevent changes in pH. The mixture was centrifuged to get rid of larger particles by spinning the mixture to pull the heavier molecules to the bottom of the mixture. Pouring this mixture through the cheesecloth removes the lipids of the mixture, leaving crude homogenate. The protein was then narrowed through ammonium sulfate precipitation, as this process separates proteins by altering their solubility in the presence of a high salt concentration. When the ammonium (NH_4^+) and sulfate (SO_4^{2-}) ions are within the solution, they are attracted to the opposite charges evident on the compound that is being purified. After another round of centrifugation, a pellet that contained the LDH was produced. Tris-PMSF buffer was added to assist in keeping the conditions stable, while dissolving the pellet with LDH. The ammonium sulfate was removed through a desalting column. The columns contains beads with lots of tiny pores of a defined size. Proteins that are small enough can diffuse into the pores and thus travel slowly. The pores catch the LDH and the flow through can be discarded. The LDH was then put under a series of washes to isolate the protein.

In order to test the accuracy of the protein purification, LDH was added to lactate stock solution, and the enzyme's activity was measured using a spectrophotometer. A lamp in the spectrometer provides a source of light at a certain frequency. The beam of light is directed towards the cuvette; a detector measures the transmittance (how much light passes through) and the absorbance (how much light is absorbed) of the sample. Next a Bradford Assay was conducted on the sample to determine the concentration and the purity of the LDH. The Bradford Assay works by using various concentrations of BSA to determine the absorbance at 595 nm. This results in data that can be used to find a standard curve through linear regression, with concentration plotted on the x-axis and absorbance plotted on the y-axis. The equation of the standard curve gives a means for calculating concentration of the LDH protein from the absorbance data provided by the spectrophotometer.

Conclusion:

Scientists separate a singular protein from animal tissue in order to analyze it through a process called purification. However, the methods used during the process of purification will depend on the nature and structure of the protein, as there are vast differences in the properties of each protein.

From the results and data analysis, it can be concluded that the LDH was successfully purified from the chicken tissue. The Bradford Assay provided information about the concentration and purity of the protein. The specific LDH Activity and fold purification measured the purity; the samples with larger values of specific LDH activity and fold purification are purer than samples with lower values. With each step of the purification process, the sample appeared to become more and more pure. Through this process, it was evident that the protein reached its maximum purity after the NAD Wash, even though the highest concentration of LDH was found after the NADH Wash.

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