Name: Zhiyu Wei
Date: 11/15/2022
Lab Partner: Crina Maria Curca

Measuring the Activity of Lactate Dehydrogenase Obtained from Purified Muscle Protein

Introduction

Glycolysis is an indispensable metabolic pathway in organisms that converts glucose into pyruvates. Adenosine triphosphate (ATP) is the form of energy that is produced during glycolysis. When oxygen is scarce, especially in muscle cells during exercise, the pyruvates produced in glycolysis are transformed into lactate to continue the production of energy in the form of ATP. Lactate dehydrogenase (LDH), a tetrameric protein, is required to catalyze the process of transforming pyruvate into lactate. 5 possible types of LDH: H_4, H_3M, H_2M_2, HM_3, and M_4 can be formed by the 2 different types of subunits, heart type (H), and muscle type (M). In an anaerobic environment, such as muscle tissue, there is a major proportion of M subunits. In aerobic environments, such as the heart tissue, there is a major proportion of H subunits. The main difference between the 2 types of subunits is that they are composed of different ratios of glutamic acid (Glu) and aspartic acids (Asp). This results in the difference in charges of the 2 subunits under physiological conditions. This feature allows the separation using native gel electrophoresis to visualize the different isoforms of LDH in samples. [1]

Along with the conversion of pyruvate to lactate, NADH is oxidized to NAD^+. The transitioning from pyruvate to lactate is reversible which means both the production of NAD^+ and NADH are observable for the purpose of measuring enzyme activity.[2] In this experiment, the conversion of NAD^+ to NADH is measured. NADH absorbs light at a wavelength of 340 nm while NAD^+ does not absorb at this wavelength.

"Ordered Bi Bi" is the mechanism of the conversion from lactate to pyruvate. NAD^+ would first bind to LDH followed by the binding of lactate. After both substrates are bound to LDH, pyruvate is released as one of the products of the reaction followed by the release of NADH from LDH. The reverse reaction works in the same way but first starts with the binding of NADH to LDH. H subunits have a higher affinity for NAD^+, which causes it to favor the conversion from lactate to pyruvate whereas M subunits would favor the transformation from pyruvate to lactate given that the M subunits have higher affinity of NADH.

Methods

Pork homogenate was the source of protein that was used in this experiment. The partially thawed porcine was first cut into small pieces then homogenized by the homogenization buffer. The homogenization buffer contains benzamine and phenyl-methyl-sulfonyl fluoride (PMSF) that are serine protease inhibitors to preen proteolytic degradation of enzymes. β -mercaptoethanol (β -ME) was the reducing agent in the buffer to maintain a reducing

---

[1] Heinová D, Kostecká Z, Csank T. Separation of turkey lactate dehydrogenase isoenzymes using isoelectric focusing technique. Electrophoresis. 2016 Jan;37(2):335-8. doi: 10.1002/elps.201500407. Epub 2015 Dec 15. PMID: 26471476.

[2] Read JA, Winter VJ, Eszes CM, Sessions RB, Brady RL. Structural basis for altered activity of M- and H-isozyme forms of human lactate dehydrogenase. Proteins. 2001 May 1;43(2):175-85. doi: 10.1002/1097-0134(20010501)43:2<175::aid-prot1029>3.0.co;2-#. PMID: 11276087.

environment for the LDH and to preserve the activity of LDH. The pork homogenate sample needed to be purified to be used for the subsequent analysis LDH.

During Day 1, an affinity chromatography exploiting the difference in affinity of NAD^+ and NADH to purify LDH was performed. The resin, cibacron blue, used in the column resembles the dinucleotide fold of NAD^+. Thus, targeted isoforms of LDH could bind to the resin and be eluted from the column by increasing the concentration of NAD^+ or NADH. Based on the buffer used for eluting the sample factions, different compositions of protein were resulted in the fractions. A dialysis was performed afterward to get rid of the unwanted small proteins in each fraction.

On Day 2, a native gel electrophoresis was performed to examine the types of LDH isozymes and the abundance of each type. Based on the length of migration toward the anode, we could tell which LDH isozymes were present in each fraction. The color of each band indicated the abundance of LDH isozymes in the fractions. A bicinchoninic acid (BCA) assay was performed to visualize the concentration of protein in each fraction.

On Day 3, an activity assay was performed to measure the activity of LDH in each fraction by setting the spectrometer to measure the absorbance of the wavelength at 340 nm. The change in absorbance could be calculated to the rate of production of NADH. The gel for sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was prepared.

On Day 4, SDS-PAGE was performed using the fraction samples to see what other proteins were present in each fraction.

On Day 5, kinetic analysis was performed using the rate of the production of NADH to propose the mechanism and constants of the reactions catalyzed by LDH.

Results

Fraction of F1 – F10

Fraction #	Avg abs	[Protein] (mg/mL)	Specific Activity (U LDH/mg)
F1 (1:25 dilution)	0.2760	0.38837196	0.0001154
F2	0.2735	0.38216884	0
F3 (1:5 dilution)	0.2230	0.25686573	0
F4	0.5345	1.02977500	0
F5	0.3985	0.69232504	0
F6	0.3035	0.45660633	0
F7	0.3195	0.49630632	0
F8	0.3260	0.51243444	0.00048714
F9	0.3200	0.49754695	0.00038971
F10	0.3225	0.50375007	$4.8714*10^{-5}$

Table 1 Protein Concentration and Specific Activity

F1 and F2 were diluted to make sure the protein concentration was not too high in order to avoid big absorbance numbers. In

Name: Zhiyu Wei
Date: 11/15/2022
Lab Partner: Crina Maria Curca

the calculation process, the dilution was corrected by dividing the concentration of protein by the dilution ration). The protein concentration was calculated using the equation obtained from the BCA assay (figure 1).

Fraction	Abs @ 340nm (min^{-1})	LDH activity (U LDH/mL)	% Recovery (%)
F1 (1:20 dilution)	0.0006	0.00974277	8.69565217
F2	0	0	0
F3	0	0	0
F4	0	0	0
F5	0	0	0
F6	0	0	0
F7	0	0	0
F8 (1:10 dilution)	0.0011	0.01786174	15.942029
F9 (1:10 dilution)	0.0005	0.00811897	7.24637681
F10	0.0003	0.00487138	4.34782609

Table 2 LDH Activity and Recovery

The absorbance values in the table were obtained from the BCA assay activity assay from Day 3. The LDH activity was calculated based on the absorbance values and the total volume of each fraction added into the cuvette (Equation 1). The % recovery was calculated by using the total LDH units in F1. Sample calculations (Equation 2) can be found below.

Equation 1 LDH activity

$$LDH\ Activity = \frac{(\frac{\Delta\ abs}{\alpha\ l})(V\ in\ cuvette) * \frac{1\ Unit\ of\ LDH\ activity}{1\ \mu mol\ NADH\ generated}}{Volume\ of\ sample\ added}$$

$$LDH\ Activity\ (F9\ 1:10) = \frac{\frac{0.0005\ min^{-1}}{(6220\ M^{-1}cm^{-1})(1cm)} * (1010\ \mu L) * \frac{1\ Unit\ of\ LDH\ activity}{1\ \mu mol\ NADH\ generated}}{0.01\ mL} = 0.00812\ \frac{U\ LDH}{mL}$$

Equation 2 LDH % Recovery

$$LDH\ \%\ recovery = \frac{Units\ Enzyme\ Activity}{Milligram\ Protein} * 100$$

$$LDH\ \%\ Recovery\ (F8\ 1:10) = \frac{\left(\frac{Units}{mL}\right)(V\ F9\ 1:10)}{\left(\frac{Units}{mL}\ F1\right)(\ Total\ volume\ added)} * 100 = \frac{0.0179\ \frac{U\ LDH}{mL} * 0.01mL}{\left(0.112\ \frac{units}{mL}\right)(0.01mL)} * 100 = 15.9\ \%$$

LDH did bind to the affigel blue resin given that F2 to F4, which were the fractions eluted using the wash buffer, contained no LDH at all.

F1 might have all possible isoforms of LDH. It also had the most abundant H_4 isoform of LDH which the F1 sample rapidly converted NAD^+ into NADH according to the table 2. F2 to F4 had mostly the untargted proteins that were washed through the column using the wash buffer. F5 to F7 all predominantly contained the M_4 isoforms that mainly facilitate the conversion of lactate to pyruvate. The rate of NADH production shown in the table revealed that the isoforms in the samples had a higher affinity for pyruvate which mainly facilitated the conversion from pyruvate

to lactate along with the conversion of NADH to NAD^+. F8-F10 had a high concentration of H_4 which the NADH production rates for the 3 fractions were high compared to the other fractions.

Based on the percentage recovery data presented in table 2, the LDH recovery rate was high. The % recovery of LDH for the diluted F8 and F9 samples was high. The faction that contained pure LDH should be F10 since it was the final fraction eluted using the NADH solution down the column. The % recovery for F10 is 4.35%.

The purity of the samples looked good. They all had a high concentration of protein in them. The recovery rate calculated for the fractions that contained LDH showed the presence of LDH in the fractions, which also proved the purity of the samples.

Standard Curve of the BCA

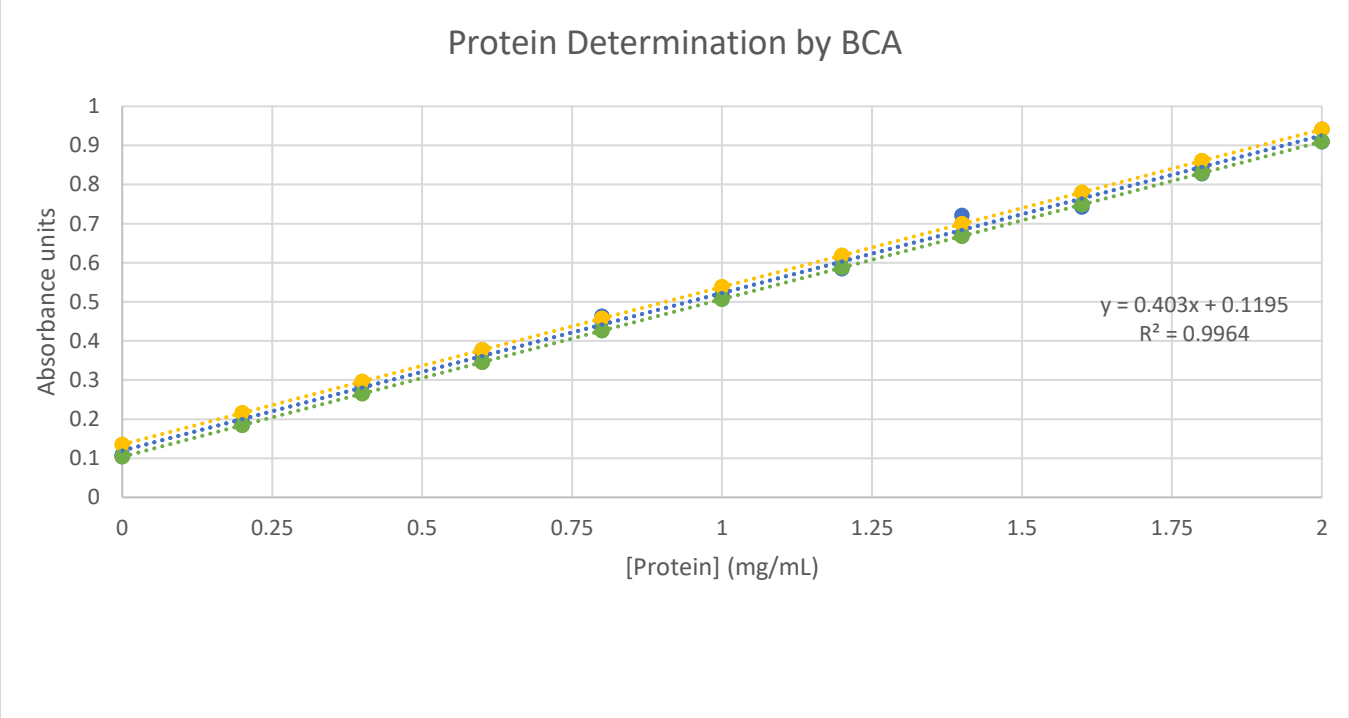

Figure 1 Best fit lines determined through BCA assays protein concentration
The absorbance values are the y values, and the protein concentration was the x values. Best fit lines were drawn for 3 equations: [Protein] vs. absorbance value, [Protein] vs. absorbance values + 1 standard deviation, and [Protein] vs. absorbance values – 1 standard deviation. R^2 value was calculated and put on the top right of the graph. The equation above the R^2 value was the best fit line of [Protein] vs. absorbance values. Sample calculation of the standard deviation is shown below (equation 3)

Equation 3 Standard deviation calculation based on observed absorbance values and absorbance values obtained from equation of best fit

$$Standard\ Deviation = \sqrt{\frac{\sum(y_i - y_l)^2}{n}} = \sqrt{\frac{0.00518}{21}} = 0.0157$$

Name: Zhiyu Wei
Date: 11/15/2022
Lab Partner: Crina Maria Curca

Native Gel

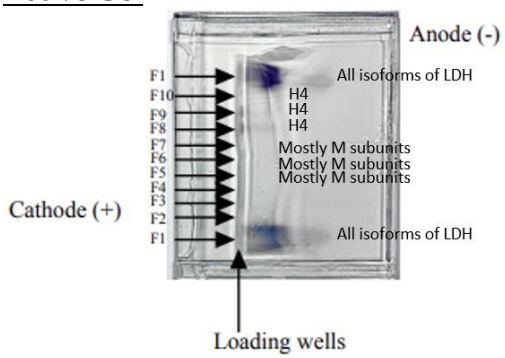

Figure 2 Native Gel ran using all fractions to determine main isoforms of LDH present in each fraction. *The top and the bottom were labeled with the cathode (+) and the anode (-) that the protein could run toward. The bands are labeled on the left to show which fraction it is. The different isoforms of LDH are labeled on the figure right next to the bands.*

Both lanes that were loaded with F1 samples migrated faster than the other factions toward the anode. F1 had all the possible isoforms of LDH including the H4 isoform of LDH that resulted the migration. Fraction 5-7 had mainly the M subunits that would not migrate as fast as H4 toward the anode. F8-F10 migrated toward the anode faster than F5-F7 which indicated F8-F10 contained predominantly the H4 isoforms of LDH.

The LDH Native Gel Substrate Solution reaction has 3 coupled reactions that resulted in the NBT form of the protein that would appear in color. On the other hand, the difference between the pKas in the different subunits resulted in different speed of each sample migrating toward the anode. Isozymes that predominantly contained the H subunits would migrate faster toward the anode compared to the other isozymes.

SDS-PAGE

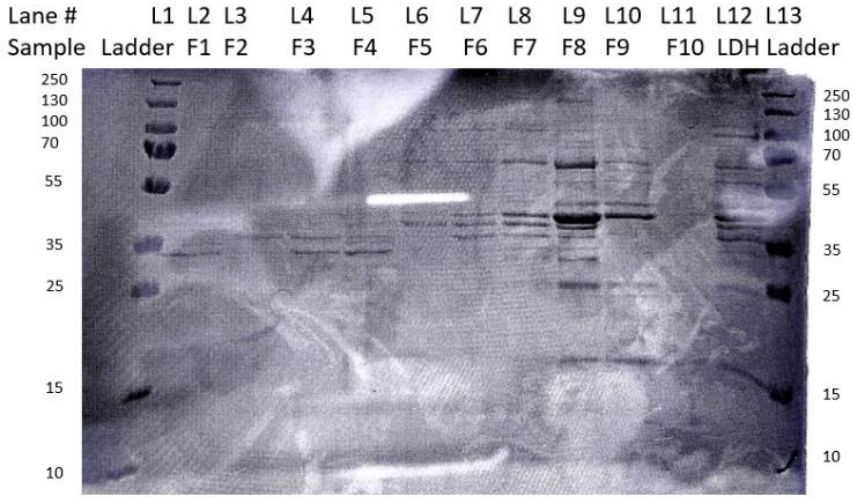

Figure 3 SDS-PAGE ran using all fractions, protein ladder, and LDH standard. *F1 and F4 (L2 and L5) were diluted in order to not overstain the gel. Lane numbers and sample names are labeled on the top of gel. Protein ladder length is labeled on both the right and left sides of the gel.*

Name: Zhiyu Wei
Date: 11/15/2022
Lab Partner: Crina Maria Curca

According to the bands illustrated on the SDS-PAGE, F8 had the highest purity, which all the bands ran by F8 corresponded to the LDH standard in L12.

The data presented by the SDS-PAGE corresponded to the other previous data. F8 and F9 should have the most abundant LDH. This was shown in the fraction tables (table 1 and table 2).

Lineweaver-Burk Plot

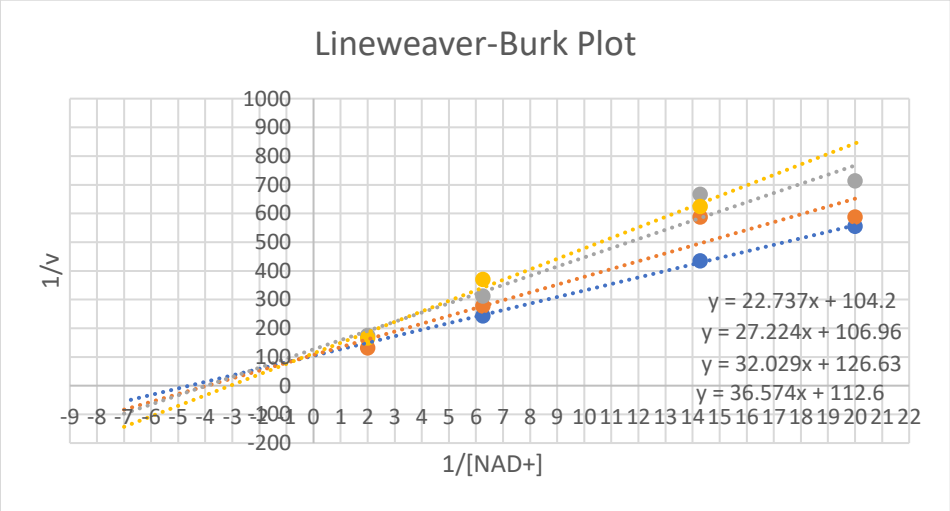

Figure 4 The Lineweaver-Burk Plot obtained by creating the best fit lines from the equations. *The 4 best fit equations are presented under the 4 lines of best fit.*

[lactate] (mM)	1/[Lactate] (1/mM)	slope	intercept	equation
200	0.005	22.737	104.2	y=22.737x+104.2
50	0.02	27.224	106.96	y=27.224x+106.96
35	0.02857143	32.029	126.63	y=32.029x+126.63
25	0.04	36.574	112.6	y=36.574x+112.6

Table 3 Equation values from best fit lines plotted in Lineweaver-Burk Plot. *The values from the table were used to construct the secondary plots.*

The 4 lines did intersect after tossing out a few data points that seemed unreasonable. The lines intersect around the point (-1.2, 80).

Secondary and Intercept Plots

The x value of the intersecting point is $-\frac{1}{K_a}$. Visually, the 4 best fit lines intersect at the point that has the x-value -1.2.

Equation 4 K_a calculation based on best fit lines displayed in figure

$$-\frac{1}{K_a} = -1.20$$

Name: Zhiyu Wei
Date: 11/15/2022
Lab Partner: Crina Maria Curca

$$K_a = \frac{1}{1.2} = 0.833$$

The velocity (V) of the reaction is obtained by plotting the slopes and the intercept against 1/[Lactate].

Using the slope from the secondary plot of intercept vs. 1/[Lactate], the velocity of the reaction is calculated below:

$$slope = \frac{K_a}{V}$$

$$377.5 = \frac{0.833}{V}$$

$$V = \frac{0.833}{377.5} = 0.00221$$

Using the intercept from the secondary plot of intercept vs. 1/[Lactate], the velocity of the reaction is calculated below:

$$Intercept = \frac{1}{V}$$

$$103.8 = \frac{1}{V}$$

$$V = \frac{1}{103.88} = 0.00963$$

Using the intercept from the secondary plot of slope vs. 1/[Lactate], the velocity of the reaction is calculated below:

$$Intercept = \frac{K_a}{V}$$

$$20.2 = \frac{0.833}{V}$$

$$V = \frac{0.833}{20.2} = 0.0412$$

The purity of the samples was quite high, which they added up to be close to a hundred when using F1's LDH activity as the reference. However, there were a couple of limitations in the experiment. First, the elution of the fractions could possibly lead to loss of samples. Students were required to manually stop elution of the column by putting the cap on the bottom of the column. This caused some impurity in the fractions after collecting the eluted samples. During the activity assay and the kinetic assay, the cuvette was required to be quickly set into the spectrophotometer after the substrates were added. Delaying the time would result in inconsistent data points.

Temperature was also a big factor that should be counted as one of the limitations. The experiment lasted for a total of 3 weeks and the samples were kept in a cold room after each day of the experiment. However, when transferring the samples for different analysis purposes, there was a gap of time between when the samples were taken off from the ice bucket and when the samples were put back. This gap of time could result in a possible loss of LDH activity.

Name: Zhiyu Wei
Date: 11/15/2022
Lab Partner: Crina Maria Curca

When dilution was done using the samples, loss of LDH could also be resulted. Many of the dilution ratio was small that caused the measurement of LDH activity to be small as well. Since the % recovery was calculated based on the measurement of LDH activity, some % recovery of LDH was also quite small.

Observed from the activity assay, no LDH activity was observed from F2 to F7. However, the activity suddenly went up from F8 and slowly declined going to F10.

The Kinetic analysis made sense since the rate of change in absorbance declined as the concentration of NAD+ and lithium lactate declined. Unfortunately, there were a few data points in the kinetic assay that were unexpected. They did not follow along the trend of declining absorbance rate. These values could be resulted by the in the gap of time between when the fraction sample was added and when the spectrophotometer started measuring the change in absorbance. The data points omitted were either having the same value as the previous measurement that had different concentration or it had a higher value than the previous data point that had a higher concentration.

In the step of performing the staining of native gel, the LDH Native Gel Substrate Solution was not properly vortexed to remove the precipitate before use. It might result in the vague staining of the native gel that could be seen in figure 2. The band for F10 was not obvious. Some adjustments in the color of the figure were to better visualize all the bands.

If the experiment is repeated for a second time, the activity assay procedure and the kinetic analysis should be put on the same day. This could make a higher efficiency when doing both analyses and make the data points for both analyses done when the samples are under the same conditions.

Based on the results obtained from this experiment, it would be better if another experiment that measures the production of NAD^+ could be done. This experiment would require setting the spectrophotometer to measure the absorbance of the wavelength that only NAD^+ absorbs but NADH does not. It would be ideal if the two experiments (experiment 1 and this experiment) could be done together at the same time to make sure that the samples and fractions are under the same conditions. Results from both experiments should be compared to make a higher precision of conclusions.

Name: Zhiyu Wei
Date: 11/15/2022
Lab Partner: Crina Maria Curca

References

Heinová D, Kostecká Z, Csank T. Separation of turkey lactate dehydrogenase isoenzymes using isoelectric focusing technique. Electrophoresis. 2016 Jan;37(2):335-8. doi: 10.1002/elps.201500407. Epub 2015 Dec 15. PMID: 26471476.

Read JA, Winter VJ, Eszes CM, Sessions RB, Brady RL. Structural basis for altered activity of M- and H-isozyme forms of human lactate dehydrogenase. Proteins. 2001 May 1;43(2):175-85. doi: 10.1002/1097-0134(20010501)43:2<175::aid-prot1029>3.0.co;2-#. PMID: 11276087.