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Expression of Lactate Dehydrogenase (LDHA) WT and A320T Mutant

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Abstract

The human genome has a length of approximately 3 billion base pairs, containing a total of over 20,000 genes, which can all be subjected to mutations that cause variations in the genome. Variations in a genome can be potentially either benign or pathogenic, but it can be difficult to distinguish whether a variant is one or the other, leading to tens of thousands being classified as variants of unknown significance (VUS). Our lab was particularly interested in the VUSs in lactate dehydrogenase (LDHA), an oxidoreductase enzyme that catalyzes the reversible conversion of pyruvate to lactate, an important component to anaerobic metabolism. Our lab is focusing on the A320T (alanine to threonine mutation at position 320) VUS, in which we cultured BL21 (DE3) and DH5α *E. coli* cell strains, so that we are able to clone wild type (WT) and A320T mutant enzymes. We eventually expressed and purified the two types of LDHA to observe the activity of each. Through expression and purification of both wild type and mutated forms of LDHA, we can observe the impact that the A320T-mutated VUS will have on the function of the LDHA protein, thus determining whether the VUS is benign or pathogenic to the enzyme function.

Introduction

In our research, we observed the enzyme Lactate Dehydrogenase A (LDHA) and worked towards characterizing Variants of Uncertain Significance (VUS). We were able to do this by expressing mutant and wild-type proteins, and then characterizing the activity of the LDHA enzyme. VUSs are specific mutations in an individual's genome that are potentially benign or deleterious. There are 16 missense VUSs in LDHA alone. Our observations of VUSs in human lactate dehydrogenase (LDHA) may provide insights on their effects on the molecular structure, enzymatic behavior, and behavior of the LDHA protein in comparison to the reference sequences.

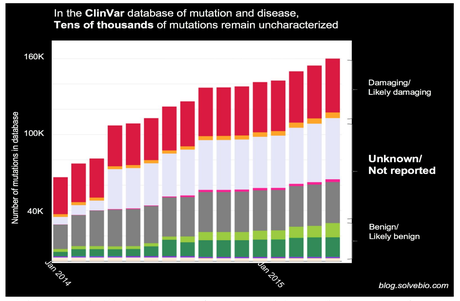


Figure 1 (above): In the ClinVar database of mutation and disease, the proportion of mutations that have an unknown significance has steadily increased since January 2014.

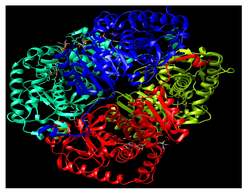


Figure 2: Lactate Dehydrogenase A (LDHA) Chimera view.
Molecular Weight (MW): 35 kDa (35,000 kDa)
Isoelectric Point (pI): 8.44

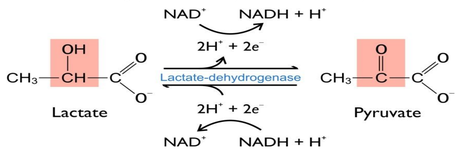
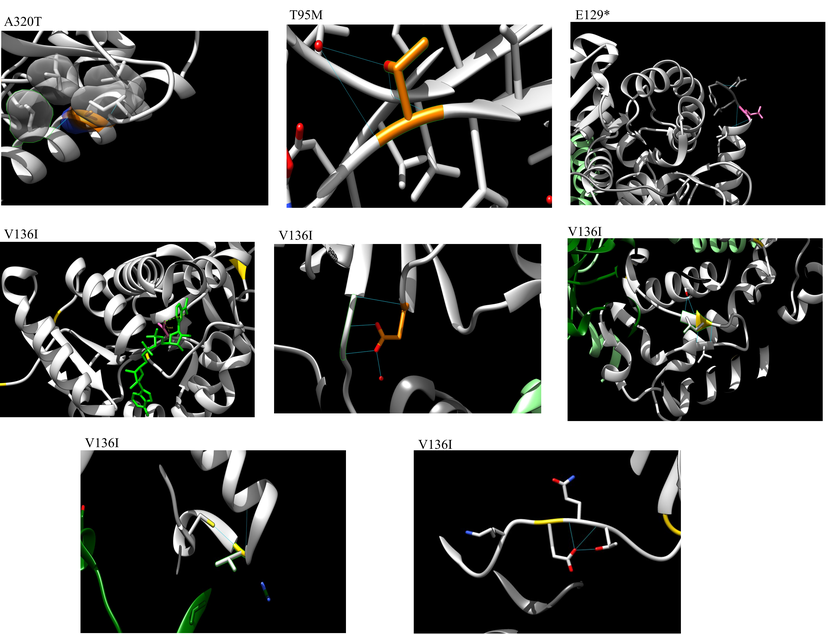
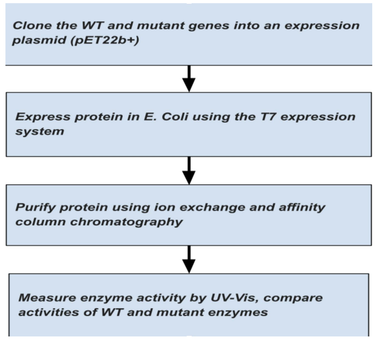


Figure 3: Lactate Dehydrogenase (LDH or LDHA) is an important enzyme that enables the deprotonation of the hydroxyl groupon lactate so that it can become a carbonyl so that it can produce pyruvate with the oxidation of NADH, or vice versa. LDHA is an important enzyme in regards to anaerobic metabolism because when not only will the production of lactic acid in anaerobic respiration, but also for the production of pyruvate needed for the Krebs cycle in aerobic respiration.

Observed Variants of Unknown Significances of Lactate Dehydrogenase A (LDHA)



Methods



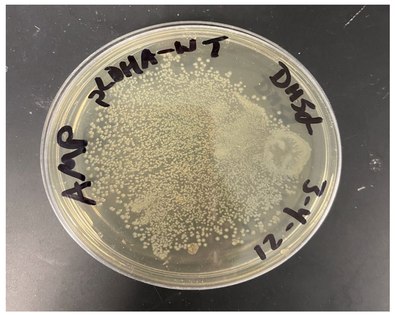


Figure 4:Our plate with our successfully transformed cells .

Results and Discussion

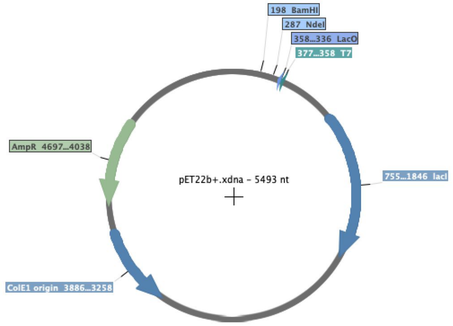


Figure 5: Plasmid map of pET22b+. AmpR encodes for beta lactamase, which provides the cells with resistance to AMP, ColE1 is the origin of replication, lacI codes for the lac repressor, which binds to lacO, the lac operon, and prevents the expression of T7 until induction with IPTG. In our cells, the gene for LDHA is placed between the NdeI and BamHI genes, adding about 996 base pairs to the plasmid.

Figure 6: The agarose gel in which the pET22b+ plasmids from the BL21(DE3) competent cells that have been cultures, enabling us to observe the differences of protein expression between wild type and mutated LDHA proteins.

When the competent cells were transformed, they were put and grown on a plate containing Ampicillin (AMP). Ampicillin is an antibiotic that kills bacteria. In the E.Coli cells injected with pLDHA, they should survive in an environment with AMP due to AMP^r, an antibiotic resistance gene. As a result of having a resistance gene, using AMP allows only cells that were successfully transformed to grow. Once our plated competent cells grew, and we could determine that we had successfully transformed our cells, we were able to move onto plasmid purification. Purifying the plasmid DNA would allow us to run this DNA on an agarose gel to determine different DNA fragment sizes. Before we could run the gel however, we needed to use the restriction enzyme NDE-1. In Figure 5, on the top, there is a gene called NDE. When a restriction enzyme is introduced to the purified plasmid, it will cut the plasmid so that it opens up into a line and is no longer in a circle. This helps to ensure clearer and more accurate gel results. Once the restriction enzyme had been introduced, the agarose gel was run. In order to analyze the gel correctly, we had to determine the base pairs of the bands in column 2-4. Pet22B+ alone has a molecular weight of 5,500 and the LDHA protein weighs around 996 base pairs. Therefore, the molecular weight for the successfully transformed cells should be around 6489 bp. Using computational analysis, we were able to analyze the gel and deduce that these 'unknown bands' had base pair lengths of around 6500-7000. This result meant that we had successfully transformed our cells and isolated the DNA, and could now move on to protein expression.

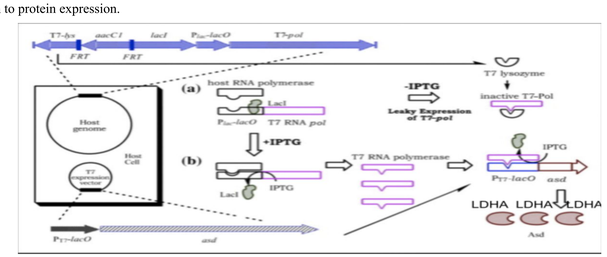


Figure 7: A diagram of the T7 expression system.
Adapted from Kang, Y., Son, M. S., & Hoang, T. T. (2007). One step engineering of T7-EXPRESSION strains for protein production: Increasing the host-range of THE T7-expression system. *Protein Expression and Purification, 55*(2), 325-333.

In our research, we wanted to find the best method for overexpression of the protein in order to get the clearest band possible on the SDS-PAGE gel. We used IPTG (final concentration 500 um) in conjunction with the T7 expression system to try and overexpress the protein. The T7 expression system is used because it allows for high levels of expression in *E. coli*. Using this expression system is important because it can help to allow for easy protein purification as well as detection. T7 is an RNA polymerase, which is an extremely active enzyme. Compared to the rate that RNA of E. coli works, the T7 enzyme works much faster. By using both an upstream expression vector, as well as a heat inducible form of the polymerase, when activated, it will initiate transcription and allow for protein expression. IPTG works in a similar way, by initiating transcription,just through different methods. IPTG is similar to lactose, and therefore it is able to bind to the lac repressor of the operon which induces a conformational change to start transcription. Unlike lactose however, it is not a part of the metabolic pathway and therefore will not be digested within the cell. The BL21-(DE3) cells used carry a gene that codes for the T7 enzyme and therefore, we needed to be able to find a way to express it. Using IPTG as a way to remove the repressor, it will activate the lacUV5 promoter which controls T7 and therefore leads to expression. The first gel trial we ran did not show much difference between the induced and uninduced cells. We next tried inducing cells with fresh IPTG and incubating them for 2,4,8 and 24 hours post-induction to see which time slot had the highest level of expression. We also attempted protein expression in Rosetta(DE3) cells, which are engineered to express higher levels of rare tRNAs. Although we cannot know the ideal expression conditions without further experiments, the results using Rosetta(DE3) cells appear most promising.

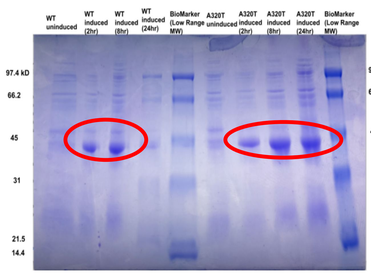


Figure 8: SDS-PAGE analysis of LDHA-WT and LDHA-A320T expression in BL21(DE3) cells. IPTG (500 uM final concentration) was added to induce expression at OD600=0.6-0.8, and gel samples were subsequently taken at indicated time intervals. Low Range MW standards (BioRad) are indicated at far left and right.

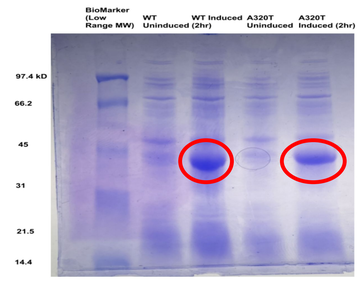


Figure 9: SDS-PAGE analysis of LDHA-WT and LDHA-A320T expression in Rosetta (DE3) cells. IPTG (500 uM final concentration) was added to induce expression at OD600 = 0.4, and gel samples were subsequently taken at 2hrs post induction. Low Range MW standards (BioRad) are indicated at far left.

Future Work

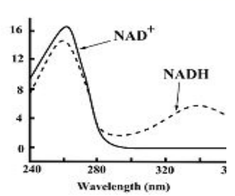


Figure 11: [NADH] can be monitored at 340 nm

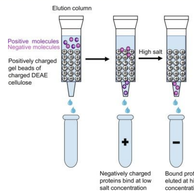


Figure 12: Anion exchange column.(sciencedirect.com)

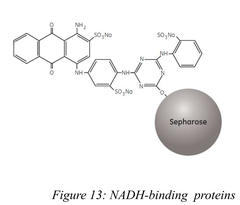


Figure 13: NADH-binding proteins have affinity for Cibacron blue resin (sigmaaldrich.com)

With conditions for overexpression of both WT and mutant LDHA now established, the next step is protein purification. Previous studies have accomplished LDHA purification via anion exchange followed by cibacron blue affinity chromatography. We expect LDHA to elute early in the anion exchange. We calculated the pI to be 8.4, which indicates that LDHA would be net positive at a pH of 7. In an anion exchange column, this positive charge would result in the LDHA protein passing quickly through the column. This is due to the positively charged chemical groups attached to the beads in the anion exchange, and then negatively charged ions are loaded to be attracted to it. This causes the negatively charged compounds in the mixture being passed through the column to exchange with the counter-ions and "stick" to the positively charged groups on the beads. Cibacron blue acts as a mimic of NADH, a substrate of LDHA. Then, using NADH, we can elute our protein of interest (LDHA). Concerning the optimization of future use of LDHA mutants, it is important to analyze the different LDHA VUSs and how they can potentially affect treatments for pathogenic diseases. Our research can contribute to solving the mysteries of these VUS mutations, each of which appear in real people, with real conditions.

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