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Affinity Chromatography for LDH

**Introduction:**

LDH (lactate dehydrogenase), essential in the metabolic processes of glycolysis, the bonding properties of LDH can be studied using affinity chromatography. This type of chromatography separates molecules by passing them through a column of a material with a specific ligand that binds to the enzyme (LDH in this case). Adding NaCl solution breaks the ligand bonds between the Cibacron Blue and LDH and allows the LDH to be eluted, and the resulting solutions can be analyzed using UV-VIS spectrometry. This experiment will investigate the concentrations of NaCl solution that will break the LDH binding. The column material, Trisacryl M, contains the ligand Cibacron Blue.

**Beginning Questions:**

* Why is sodium phosphate used to dilute the NaCl?
* How does the amount of enzyme activity relate to LDH’s ability to function within the body?
* Why is 340 nm absorbance used?

**Procedure:**

5 test tubes were labeled: W1, W2, W3, W4, and W5 using masking tape and a pen. A 6th was labeled “waste.” The stopcock was opened to allow the meniscus to drop just above the column material. The LDH sample was moved into the column with a long Pasteur Pipet and by running it down the sides of the column. The stopcock was opened to elute the volume and allow the LDH to pass through the column and into the “waste”. When the solvent volume was aligned with the top of the column, 2 mL .02 M sodium phosphate buffer was added via Pasteur pipet in the same method previously described. The elution was collected in W1 in the same method of opening the stopcock. Sodium phosphate was added again in this way, and the process was repeated for each of the test tubes W2-W5.

For the 20 enzyme assays, an Erlenmeyer flask was filled and mixed with 38 mL HEPES buffer, 10 mL NAD+, and 10 mL lactate as a “reaction cocktail.” The enzyme activity for W5 was tested to ensure there was no absorbance by mixing .1 mL of W5 with 2.9 mL cocktail, letting it sit for 5 minutes, and measuring the absorbance using the UV-Vis spectrophotometer at 340 nm. Since an absorbance was given, the column was washed with sodium phosphate a 6th time, and the elution was labeled as W6.

2 M NaCl was prepared in 100 mL of .02 M sodium phosphate buffer, and, using the dilution equation (shown in Results), .4 M, .8 M, 1,0 M, and 1.2 M NaCl solutions were prepared. 15 test tubes were labeled from E1 to E15. Another test tube was filled with 2 mL of water to compare to each E-test tube that would be filled. 6 mL of .4 M NaCl was added to the column. 2mL was eluted for E1, 2 for E2, and the remaining for E3. The test tube filled with water served as a measurement of the 2 mL volume for each test tube. This process was repeated with 6 mL of .8 M NaCl for E4-E6, 6 mL of 1.0 M for E7-9, and 12 mL of 1.2 M for E10-15.

The reaction cocktail was assayed for absorbance to set a reference. To assay the column fractions, 15 test tubes were labeled A1-15. 2.9 mL of the cocktail was added with .1 mL for E1 into A1. The same was done for each E-test tube with its respective A-test tube. The absorbance was measured of each of them, one by one, within one minute of making the solution.

**Results:**

Calculation of Volume necessary to dilute NaCl samples:

**Fig. 1**: Absorbance of each LDH sample for our data. The absorbance increased as concentration increased from .4 to .8 M and decreased as concentration increased from 1.0 M through 1.2 M.

|  |  |  |
| --- | --- | --- |
| **Molarity of NaCl** | **Sample** | **Absorbance** |
| .4 M | 1 | .000 |
|  | 2 | .000 |
|  | 3 | .000 |
| .8 M | 4 | .001 |
|  | 5 | .001 |
|  | 6 | .006 |
| 1.0 M | 7 | .042 |
|  | 8 | .032 |
|  | 9 | .027 |
| 1.2 M | 10 | .017 |
|  | 11 | .008 |
|  | 12 | .004 |
|  | 13 | .000 |
|  | 14 | .001 |
|  | 15 | .001 |

**Fig. 2:** Absorbance of each LDH sample for the data of Molly, Sarah, and Olivia. This data was more accurate and shared as the “class” data. This data follows a trend dissimilar to our data.

|  |  |  |
| --- | --- | --- |
| **Molarity of NaCl** | **Sample** | **Absorbance** |
| .4 M | 1 | .0410 |
|  | 2 | .0336 |
|  | 3 | .0341 |
| .8 M | 4 | .1834 |
|  | 5 | .2690 |
|  | 6 | .2676 |
| 1.0 M | 7 | .2267 |
|  | 8 | .2257 |
|  | 9 | .2051 |
| 1.2 M | 10 | .1858 |
|  | 11 | .6493 |
|  | 12 | .9641 |
|  | 13 | .1491 |
|  | 14 | .0241 |
|  | 15 | .0443 |

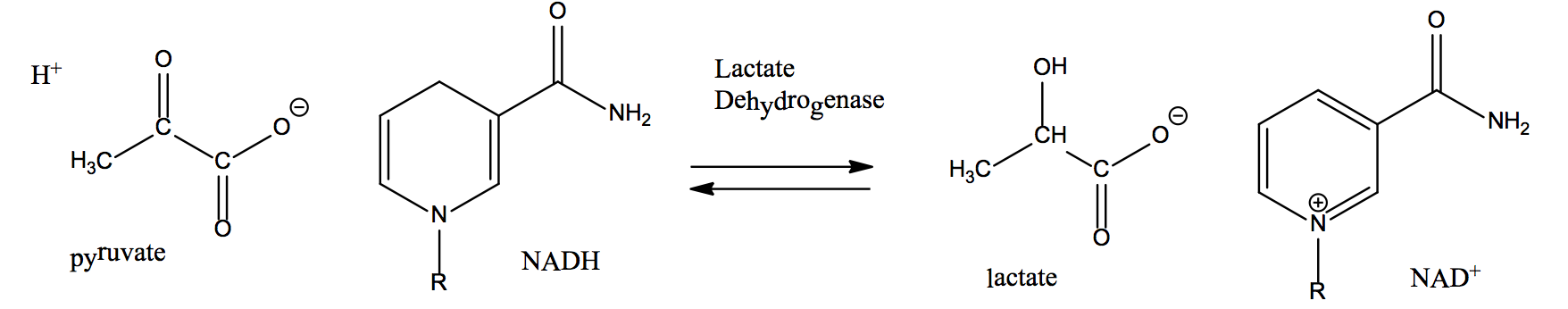
**Fig. 3**: Absorbance values for each sample in graph form. The spike of the class data was unobserved in ours, most likely due to failure to load the enzyme into the sample chamber for absorbance, but our procedure of measuring straight from the spectrophotometer, rather than the computer, most likely made a difference as well. The decreasing values of absorbance indicate enzyme was eluted effectively out of the column in the samples prior to them.

**Discussion:**

This lab investigated the use of assays in validating the scientific results of LDH chromatography. Assays are generally useful due to their lack of difficulty, but can be sensitive to external conditions1. According to the class data, the optimal concentration of NaCl is 1.2 M, but, according to our data, the optimal concentration is 1.0 M. Compared to the class data, our experiment didn’t work very well. However, our results were significantly lower than the class data. This was most likely because the protein was ineffectively loaded into the test tube, so nothing was observed in the UV-VIS spectrophotometer. We didn’t use the computer program to run UV-VIS. Instead, we used the machine itself. Since the rest of the class used the actual computer program, this might have given us a different way for the spectrophotometer to assess the sample and output the data, which would give us different results. Our Cibacron Blue might not have been packed very securely at the bottom of the column. The 12 mL 1.2 M NaCl was added at the end to elute the rest of the LDH from the column. Our last samples failed to show enzyme activity. This means that all or most of the enzyme was eluted before those samples were prepared or they weren’t successfully moved into the test tube.

Column chromatography works by passing a protein sample through a column of liquid buffer with a ligand-dense column below for the protein to attach to the ligands. Properties such as the optimal concentration of salt for removing this protein can be assayed. After adding the LDH, washing the column would ensure that the LDH would elute when concentrations of salt are added.

**Fig. 4:**1 The reaction of pyruvate with NADH, aided by the enzyme LDH. This reaction is universally common among organisms that use glycolysis. Since this experiment tests LDH in the presence of lactate, the reaction will shift to the left, and pyruvate and NADH will form. The UV-VIS absorbance wavelength at 340 nm measures the LDH activity of reducing NAD+ to NADH.



Affinity chromatography has potential applications in molecular biology with the use of gene modification through binding. Measuring the strength of protein binding can determine its structure and/or identity. If the column material were disrupted during the addition of NaCl, LDH, or sodium phosphate buffer, this would reduce the amount of material at the bottom of the column and, thus, reduce the amount of LDH that would be bound to the ligand. For our column, there was some column material that had been stuck to the inner side of the column before the experiment commenced. As the sodium phosphate buffer was squirting down the side of the column, it was aimed in order to “catch” the column material and let it fall down to the bottom. This would correct for error that would have otherwise been caused by this external column material. This experiment could be improved by using additional methods, such as gel electrophoresis and molecular sieve chromatography2, to determine enzymatic activity.

**Conclusion:**

The experiment was somewhat successful, given the class data and comparing it to our data, of showing the absorbance values of LDH at different NaCl concentrations. Sodium phosphate served as a buffer to effectively keep the solution at the right pH. The higher the absorbance value, the better LDH was able to function at that concentration. 340 nm was used because that is the wavelength of light emitted form the reduction of NAD+ to NADH.

References

1. de Souza. *Lab Lecture*
2. Taylor, Susan S., and Jack E. Dixon. "Affinity Chromatography of Lactate Dehydrogenase." *Journal of Chemical Education*. ACS Publication, n.d. Web. 27 Oct. 2013.