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Synthesis of a New Affinity Column for the Specific Binding of Lactate Dehydrogenase

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Background

Purification of enzymes is an important lesson for a biochemist to learn. To teach about purification a common undergrad lab is the purification of Lactate Dehydrogenase (LDH). LDH is an enzyme responsible for the metabolic breakdown of pyruvate to lactic acid for energy in anaerobic conditions. This enzyme has a cofactor of NADH (NAD+).[1] This reaction is illustrated in Figure 1.

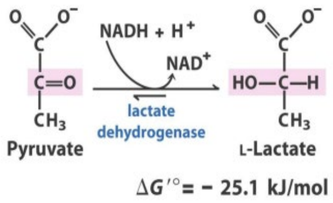


Figure 1: Conversion of Pyruvate to L-lactate by LDH[2]

$\Delta G'^{o} = -25.1$ kJ/mol

The most efficient way to purify this enzyme is through an affinity column, which specifically binds to the enzyme. Cibracron Blue 3G-A (CB) was the leading affinity column on the market which has now been discontinued.[3] Blue Sepharose CL-6B (BS) and oxamate-Sepharose (OS) are two other commonly used columns. BS like CB has been discontinued and OS results were dull in comparison.[4] With few commercial options for an affinity column, this research is focused on synthesizing a low cost, highly effective affinity column for the binding of LDH.

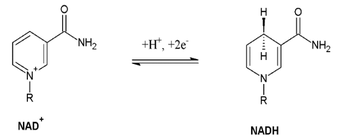


Figure 2: The reduction/oxidation of NADH

LDH has 2 binding sites one for its substrate pyruvate and one for its cofactor NAD+ (figure 2). The cofactor-binding site is the target in this research for the enzyme to chemisorb to the affinity column. This can be achieved by making the column have similar functional groups as NAD+. To do this, affordable organic dye molecules will be attached to an insoluble substrate. The dye molecules will bear similar functionality to NAD+, such as calmagite, and the dye-substrate combinations will be examined for their efficacy in acting as affinity columns for the purification of LDH.

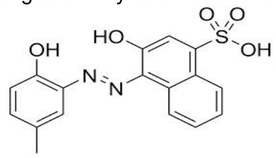
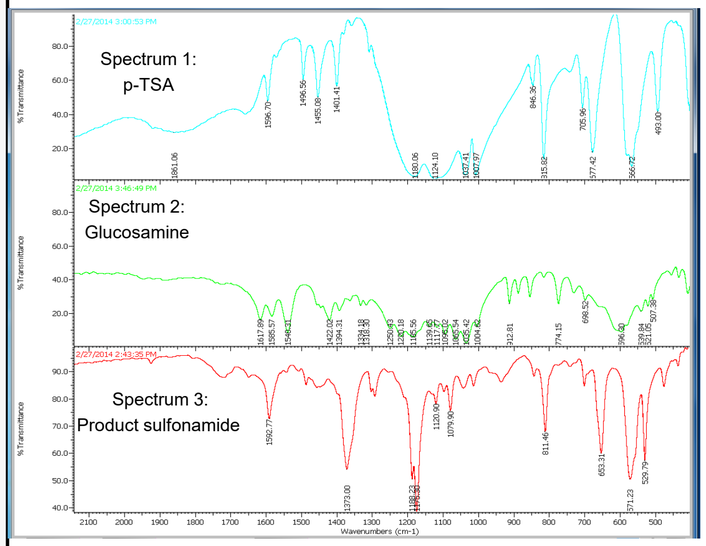


Figure 3: Structure of Calmagite

Calmagite dye was selected based on its similar aromatic structure to NAD+ as well as having a sulfonic acid functional group, which in this experiment will be exploited to form sulfonamide. This will show its potential to be bound to insoluble matrix of chitosan to make the affinity column.

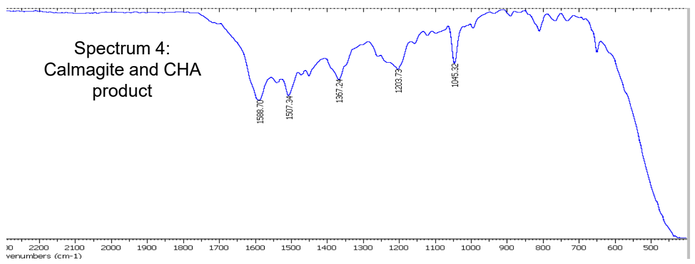
Results and Discussion

Infrared Spectroscopy data:



*Specta 1-3: spectrum reagents and a formed product. Peaks at 1180-1007 cm^{-1} and 1401 cm^{-1} in spectrum 1 are shown shifting in spectrum 3 and are related to sulfone peaks.

Above spectra were made using by crushing KBr salts with samples in a 100:1 by weight ratio. The finely ground mixtures were then pressed into a salt plate for the IR spectrometer. Spectrum 4 was produced by evaporating a thin layer of the product in DCM off the top of a NaCl salt plate.



Infrared spectroscopy Discussion:

Microwave assisted reactions between sulfonic acids and amine groups to form sulfonamides have been explored to model linkage for calmagite and chitosan. A reaction between P-Toluenesulfonic acid (P-TSA) and Glucosamine was focused on to show the true sulfonic linkage. IR spectra for each reactant and the product formed were taken. The spectrum of the P-TSA shows 4 strong broad signals from 1180-1007 cm^{-1} along with a peak at 1401 cm^{-1}, which are indicative of the sulfonic acid. These peaks are then lowered in energy and shown splitting when converted to the sulfonamide due to the addition of the electron rich nitrogen lengthening the S=O bonds.[6] The shifted 4 broad peaks collapsed to a peak at 1175.30 cm^{-1} with a shoulder at 1188.23 cm^{-1} and a lower energy shift from 1401 cm^{-1} to 1373 cm^{-1}. These occurrences suggest proper sulfonamide coupling between the P-TSA and Glucosamine. Spectrum 4 showed peaks both in both of the regions for the S=O regions expected for a sulfonamide indicating linkage but didn't have the same sharp peak as the previous sulfonamides shown. This maybe due to the IR technique used for this 4 spectrum and the concentration of sample on the plate.

Colorimetric analysis:

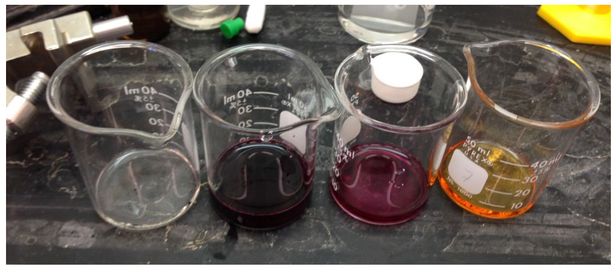


Figure 4:

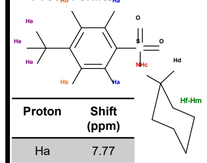
*The above picture is of left to right is Calmagite in DCM, Calmgaite in DI water, Calmagite-CHA Product in acetone, Calmagite-CHA product in DCM

Colorimetric Discussion:

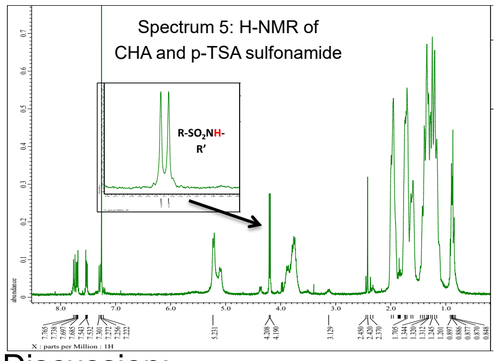
Reaction conditions were also performed using calmagite as the sulfonic acid and cyclohexylamine(CHA) as the amine. This reaction created an emulsion that was hard to separate in a biphasic solution and was even harder to analyze using spectroscopy techniques. This however did show interesting results that shows some form of binding must have occurred due to coloration in a dichloromethane(DMC) solution. Calmagite on the left in figure 4 is insoluble in DCM, where as the product of Calmagite and CHA when introduced to DCM dissolves and is bright orange in color. This indicates a reaction has occurred as now exhibits some solubility in an organic solvent.

Proton-NMR:

Figure 5:
Proton shifts



Proton	Shift (ppm)
Ha	7.77
Hb	7.29
Hc	4.67
Hd	3.12
He	2.42
Hf-Hm	1.74-1.13



Proton-NMR Discussion:

H-NMR was used to determine correct synthesis of the a model reaction between p-TSA and CHA (spectrum 5). This being the least complex sulfonamide created could be easily analyzed using H-NMR. The spectrum was analyzed showing expected synthesis and the sulfonamide proton at 4.67 PPM. The corresponding peaks in the spectrum were marked for their protons in figure 5. This product was also compared to literary spectrum available showing proper peak placement.[7] The H-NMR does however show some unknown peaks, which are believe to be impurities of residual calmagite in the reaction flasks and THF solvent peak.

ACKNOWLEDGEMENTS:

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Experimental

A microwave synthesis was used to attach the sulfonic acid and amine to make a sulfonamide.[5] The sulfonic acid in a 4.9 mmol concentration was dissolved in 10ml of THF in a reaction vessel on a stir plate. In mole-to-mole ratio TCT and Et3N were added to the stirring mixture. The mixture was then microwaved in a MARS at 80 ^{0}C for 20 minutes at a power of 12% of 400 Watts. Then NaOH and amine were then added in 1-1 ratio with the original reactants and were microwaved for 10 minutes at 50 ^{0}C at 12% of 400 Watts. The resulting solution was washed to remove salts and rotovaped. Characterization was performed using IR and H-NMR analysis in Nicolet IR 100 FTIR and JEOL 300 MHz ECX FT-NMR respectively.

Conclusion

Sulfonamides were created using sulfonic acids and amine groups in a microwave assisted reaction. This experiment has shown promising results in creating sulfonamides based on IR analysis, NMR analysis, and colorimetric analysis. These model reaction have shown, for both glucosamine and calmagite, their ability to form sulfonamides with smaller reagents. This indicates that linkage between both glucosamine and calmagite can occur in this same fashion and how to characterize their product.

Further Plans

Additional analytical testing will be preformed to further prove the proper sulfonamide being formed. Upon successful model sulfonamides being synthesized, reactions to coupling the calmagite dye to the insoluble chitosan matrix will be performed. With the column finally produced, enzyme concentration and activity assays can be preformed to determine the value for calmagite as an affinity column.

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