Investigation of Enzyme Inhibition Mechanism

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Abstract
Alcohol abuse is a worldwide issue that affects all generations, and efforts to treat or monitor alcoholism have been a focus of the medical community for many years. A widely accepted, accurate, unbiased method to monitor alcohol abstinence has yet to be established. An up and coming approach is to monitor the iron-transfer protein "transferrin", which appears in a carbohydrate deficient form over time in chronic alcohol abusers. However, no mechanism for inhibition of the process by which the protein is sialylated has been suggested in literature. We have considered the possibility that ethanol directly inhibits the sialyltransferase enzyme responsible for the formation of the complete form of sialyltransferase. The purpose of this research was to investigate methods of enzymatic inhibition using alcohol dehydrogenase, and analyze the ability to evaluate sialyllactose activity using liquid chromatographic techniques. Lactose, the carbohydrate used specifically for this investigation, was analyzed, as well as its sialylated forms, 3'-sialyllactose or 6'-sialyllactose. Sialylation was evaluated via incubation of rabbit serum, lactose, and the sialyl-donor, cytidine monophosphate-N-Acetylneuraminic acid (CMP-Neu5Ac). Samples were analyzed using liquid chromatography-mass spectroscopy with a triple quadrupole analyzer, as well as high performance liquid chromatography. No method was able to be validated at the completion of this project.

Introduction

Alcoholism and alcohol abuse are worldwide issues that can affect all generations. Many people try to control their alcoholism in an effort to maintain their personal relationships, improve their health, and retain a job. Alcohol abuse is difficult to treat and control, and a widely accepted, accurate, unbiased method to monitor alcohol abstinence has yet to be identified. One approach currently being utilized in some locations to monitor alcoholism is evaluation of the iron-transfer protein "transferrin", which appears in a carbohydrate deficient form with chronic alcoholism. However, the mechanism by which alcohol affects the glycosylation/sialylation of transferrin, yielding carbohydrate deficient transferrin is unclear. An increase in the number of sialic acid-deficient isoforms of transferrin in the serum may represent a sensitive indicator of alcoholism.

Transferrin is the primary iron transport protein in the body. It's synthesis involves, in addition to the underlying 679- amino acid core, a "glycosylation" process wherein multiple sialyl (N-acetylneuraminic acid; Neu5Ac) residues are added to the protein. Alcoholics, or habitual drinkers characteristically exhibit some degree of diminished efficiency in that process, and "carbohydrate deficient transferrin" (CDT) has been proposed as a marker for chronic alcoholism. CDT is currently being used clinically as an indicator of compliance, or lack thereof, with alcohol-treatment programs in Europe. CDT levels are being considered for their possible applicability in various ways within the criminal justice system of the United States as well.

Testing for alcoholism is difficult and the contemporary methods are imperfect. Such techniques as breath alcohol analysis and blood tests only evaluate the blood alcohol level at the time of sampling. Alcoholism is currently recognized and diagnosed by identifying physical symptoms or changes in personality. Such methods are highly subjective and potentially insensitive and inaccurate. Measurement of carbohydrate deficient transferrin is currently being utilized and evaluated for this process. An alcoholic cannot skew these results to ensure that the carbohydrate deficient transferrin does not appear in a test. Using carbohydrate deficient transferrin as a means to identify a chronic alcoholic could be an efficient method.

Despite the apparent correlation between carbohydrate deficient transferrin and alcoholism, there has been no mechanism proposed for how this occurs on a molecular level. We hypothesize that ethanol acts as a direct, competitive inhibitor of the enzymatic system responsible for adding sialyl residues to the transferrin molecule, the "sialyltransferases". Sialic acid, shown in Figure 1 below, is a cyclic nine-carbon sugar analog. When attached to cytidine 5'-triphosphate (CTP), sialic acid yields the activated substrate

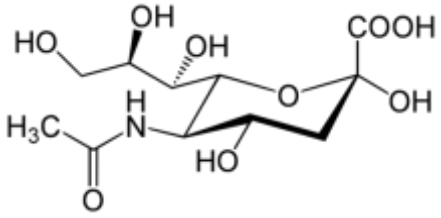

Figure 1: Structure of N-acetylneuraminic acid

CMP-Neu5Ac, which may then be taken up in the Golgi apparatus as a substrate for sialyltransferase activity, transferring the sialyl residue to the unconjugated protein.

Enzyme inhibition can be determined in the laboratory, including the mechanism by which it occurs. There are three different types of inhibition: competitive, non-competitive and uncompetitive. In the "competitive" case, the inhibitor competes with the normal substrate of the enzyme for the active, or "binding site" of the enzyme. The presence of a competitive inhibitor can be inferred experimentally by observing a change in the ability of the enzyme to bind the substrate (Km) in the presence of the supposed inhibitor. Non-competitive inhibitors act on the

enzyme themselves, affecting the ability of the molecule to catalyze the specific reaction, without affecting substrate binding. Experimentally, this can be observed as a change in the maximal rate of reaction of the enzyme (Vmax). Finally, uncompetitive inhibition occurs as the inhibitor interacts with the enzyme-substrate complex, resulting experimentally, in changes in both substrate binding and maximal velocity (Km & Vmax). The nature of inhibitor action can be determined using a plot of reaction rates as substrate concentration is varied, allowing evaluation of the reaction kinetic variables.

Initial experiments on enzyme kinetics and inhibition, including substrate-velocity experiments, and calculations of Km, Vmax and Ki in a competitive-inhibitory system were carried out using rat cytosolic alcohol dehydrogenase, and pyrazole as an inhibitor. Because of difficulties anticipated in working with transferrin, and transferrin sialyltransferase, the model system chosen was a rabbit cytosolic lactose sialyltransferase. In this system, the sialyltransferase catalyzes the sialylation of lactose, yielding sialyllactose, in a manner analogous the sialylation of transferrin, as shown in Figure 2, below.

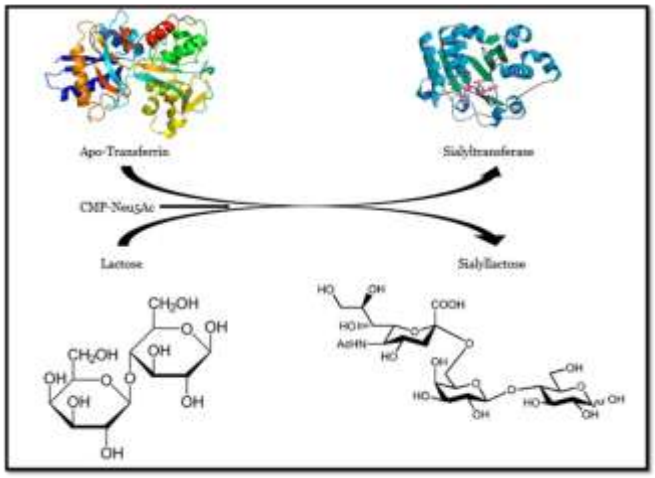

Figure 2: Shows the similarities between sialylation of transferrin and lactose in the presence of CMP-Neu5Ac

Materials and Methods

Evaluation of Inhibition of Alcohol Dehydrogenase

Alcohol dehydrogenase (EC 1.1.1.1) activity was measured using Shimadzu PharmaSpec UV-1700 Spectrophotometer, with a diode array detector set to 340nm. Enzyme incubations were made with 500 µL of 0.05M phosphate buffer, pH 8.5; 100 µL 0.015M beta-nicotinamide adenine dinucleotide hydrate (NAD); 100 µL 20mg/mL rat cytosol; 30 µL of pyrazole (Aldrich); 3.0M ethanol (Sigma Aldrich) in amounts of 0, 5, 10, 25, 50, and 100 µL; and deionized water (obtained using the Millipore System) to volume of 3.0 mL. Analysis was performed in triplicate for each ethanol concentration. Absorbance was measured for 120 seconds, with a lag time of 45 seconds, and in a recording range from 0.00A to 0.60A. Results were given by the change in absorbance (ΔA) per minute. Incubations, which were run only at room temperature, were allowed to run for the entire time of analysis, and stopped and discarded upon their removal from the spectrophotometer.

Results from analysis were interpreted and analyzed for Michaelis Menton kinetics using Microsoft Excel. This was performed by first plotting ΔA/min retrieved from the spectrophotometer versus the concentration of ethanol. Averages of each ΔA/min were used, since analysis was performed in triplicate. This plot, the Michaelis Menton plot, was then linearized by taking the inverse of both x and y value. This yielded the Lineweaver Burk plot, which was used for direct analysis of enzyme kinetics.

Evaluation of Sialyllactose

Enzyme assays were performed followed the procedure outlined in "Simple Assay for Sialyltransferase Activity with a New Fluorogenic Substrate" (Sato et. al.). The total volume for all incubation solutions was 40µL. 100mM Tris-HCl buffer was prepared by dissolving Trizma base salt (Sigma) in deionized water (obtained using Millipore System), then using and adjusting to pH 6.75 with 0.1M HCl (Sigma-Aldrich) and NaOH (Fisher). 2.4 mM Lactose and CMP-Neu5Ac solutions were each prepared in Tris-HCl using D-lactose monohydrate (Fisher) and CMP-Neu5Ac powder (Sigma).

Enzyme incubations were performed according to the following schedule:

Blank:	10 µL serum, buffer to volume
Substrate:	10 µL serum, 15 µL, 2.5 mM lactose, buffer to volume
Cofactor:	10 µL serum, 15 µL 2.5 mM CMP-Neu5Ac, buffer to volume
Full:	10 µL serum, 15 µL each; 2.5 mM Lactose, 2.5 mM CMP-Neu5Ac

Each assay solution prepared was either stopped at time zero with 200µL of 0.100M acetic acid (VWR Scientific), or allowed to incubate at 40 degrees Celsius in a water bath for one hour, then killed using 200µL of acetic acid. Incubations were done using HotShaker water bath. All solutions were stored at 4 degrees Celsius after incubations.

Analysis of all incubations was performed on a high performance liquid chromatography/triple quadrupole mass spectrometry instrument, shown in Figure 3 on the next page, with a hydrophilic interaction liquid chromatography (HILIC) column (Luna 3u HILIC 200A 50x3.3mm, Phenomex). Further analysis of lactose and a 3'-sialyllactose standard (Sigma) was performed using Agilent 1100 series high performance liquid chromatography instrument, shown on next page, figure 4.

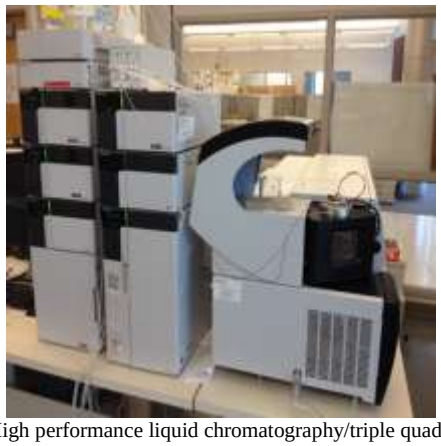

Figure 3: High performance liquid chromatography/triple quadrupole mass spectrometry instrument, located in Connecticut State Crime Laboratory

Figure 4: High performance liquid chromatography instrumented, located in Forensic Science department

Results

Preliminary experiments using alcohol dehydrogenase as a basis for demonstrating competitive inhibition via substrate-velocity and Lineweaver Burk plots using rat cytosol were straightforward, and without complication. The analogous experiments with cytosolic sialyllactose are awaiting final analysis because of time and instrument issues with the Triple-Quadrupole Mass Spectrometer.

We have as yet failed to establish a method for the analysis of 3'-sialyllactose using high performance liquid chromatography. We are currently utilizing a gradient mobile phase system, transitioning from 10% acetonitrile to 50% acetonitrile in 10 mM ammonium acetate at 0.5 mL/min. Diode array detector was set at 250 nm, with 50 µl injection volume.

Discussion

Evaluation of an enzyme assay and determination of a classic Ki for pyrazole/alcohol dehydrogenase, performed as a training exercise for the evaluation of inhibition of sialyltransferase was achieved without complication.

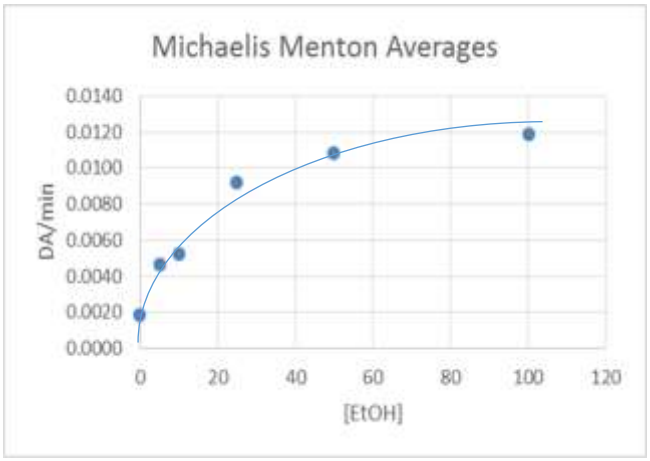

Figure 5: Example of Michaelis Menton plot with ethanol as an inhibitor of alcohol dehydrogenase

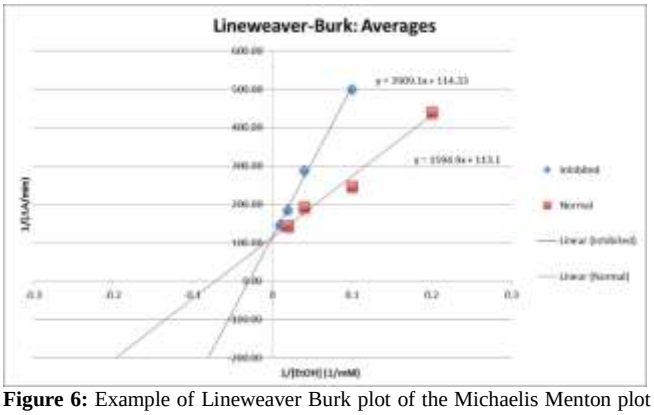

Figure 6: Example of Lineweaver Burk plot of the Michaelis Menton plot shown above

Competitive inhibition, as shown above in Figure 6, was showcased for this enzyme system. This was determined via analysis of Michaelis Menton kinetics, shown above in Figure 5, which lent its way to a Lineweaver Burk plot, shown in Figure 6, with intersection at approximately the y-axis. This intersection implies competitive inhibition. The two plots intersecting are a superimposition of the analysis let run with and without the inhibitor. The data from the analysis with the inhibitor is shown with the blue data points, and the uninhibited analysis is shown with the red data points.

Development of a direct analytical method for sialyllactose by HPLC in incubation samples was not resolved by the completion of the project. Nevertheless, the remaining analytical issues are expected to be resolved, allowing direct analysis of incubation samples by HPLC. Similarly, we are awaiting results from LC-MSMS instrumentation. Although these results will not be available for the completion of this portion of the research, they will

ideally become available for the next portion of this research. While routine analysis of incubation samples is expected to be performed on HPLC, the combination of HPLC and triple quadrupole mass spectrometry (LC-MSMS) allows for a higher degree of sensitivity, which may be important during the process of assay optimization.

Conclusions and Future Work

In this study, we have failed to develop a method for analysis of sialyllactose and components of an enzymatic assay using solely high performance liquid chromatography. There is a possibility that these components may be detected using high performance liquid chromatography/triple quadrupole mass spectrometry, however, that information is not available at the time of completion of this paper. Finally, a proper understanding of enzyme kinetics and competitive inhibition has been achieved through work with a graduate student.

Further analysis following this project will continue to evaluate ethanol as an inhibitor on the sialylation of lactose (as a model for transferrin sialylation). This project was an important stepping stone for these further analyses. A link between carbohydrate deficient transferrin and ethanol has been establish and accepted, yet no mechanism for this enzymatic inhibition has been proposed. There are not many other components of this reaction that could cause the inhibition, so ethanol is an obvious first step. Although present in low quantities, ethanol has an effect on all proteins, and it is possible that this specific protein is especially sensitive to ethanol. Further analysis is especially important because it difficult to justify and accept a parameter for being a valid indicator, when there is no mechanism proposed or accepted for that action.

This project will be continued as part of an honors thesis, beginning with the continued method development that began in this investigation. The project will then begin looking at the inhibition of lactose by ethanol, with the goal being to ultimately analyze transferrin directly.

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Biography

Lauren Ebersol is a Forensic Science and Chemistry double major from Lancaster, Pennsylvania, who will be graduating in May 2016. She is an organic and general chemistry teaching assistant for the Chemistry and Chemical Engineering department, as well as a tutor for the Forensic Science Living Learning Community. Following her graduation from the University of New Haven, Lauren plans to attend graduate school for biochemistry to work towards a career as a forensic toxicologist. She plans to continue this research through the next year for her honors thesis.