EDULAB FOR STUDENTS: FOURIER 80

Munching on Sweets: The monitoring of catalytic hydrolysis of sucrose via invertase through benchtop NMR

An enzyme kinetic experiment with a sweet taste!

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Experiment Hashtag #: #MunchingOnSweets #Educate2Resonate

Keywords:

Enzyme, Catalysis, Kinetics

Target group:

Undergraduate, Analytical Chemistry, Biochemistry, Food Chemistry

Objectives:

- The determination of the rate of reaction via benchtop NMR.
- The understanding of enzyme catalyzed reactions.
- The processing and interpretation of NMR spectra.
- Introduction to relative quantitation by NMR.

Background of the Experiment:

Invertase is an enzyme that catalyzes the hydrolysis of sucrose into an equimolar mixture of glucose and fructose. This process is commonly used in the food industry to manufacture 'invert syrup' (Figure 1).

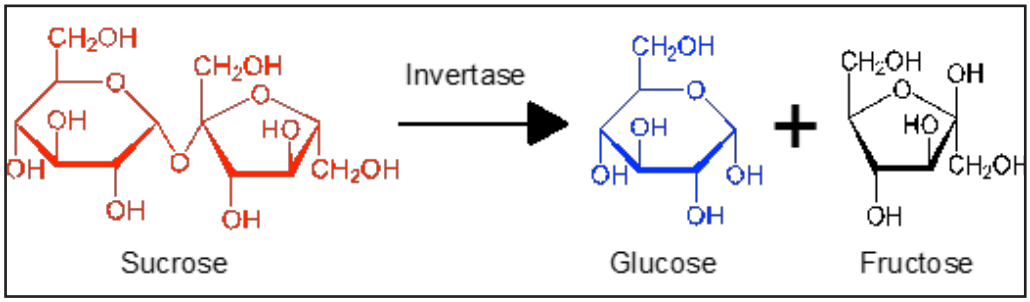

Figure 1 The Hydrolysis of sucrose to glucose and fructose by invertase.

Since NMR is both non-selective and time-resolved, it is an ideal platform for non-invasive reaction monitoring, especially in the case where the products or reactants are not detectable by other techniques (e.g., UV-vis spectroscopy). Since benchtop NMR systems do not require cryogens, this permits them to be sited in a fume hood or directly attached to a chemical reactor to monitor chemical reactions. This has made it a potent tool for monitoring reaction progress and characterizing the processes and mechanisms of a reaction.

NMR is inherently quantitative: each signal is proportional to the number of nuclei in the sample, and thus the concentration. To obtain absolute quantitation, all of the nuclei must be given the time to return to thermal equilibrium which can be a time consuming process. A more convenient approach to reaction monitoring is relative quantitation, in which relative changes in peak areas can be monitored over time. This allows for rate constants to be obtained within shorter time periods. In this experiment, the hydrolysis of sucrose by invertase will be tracked by relative quantitation.

Preparation & Perquisite

This lab is designed to take ~ 6 hrs as a two-week experiment, and it is assumed that students have basic knowledge of 1D NMR and basics of spectral interpretation. The experiments are designed to be ideally completed in groups of 3-6 students. This investigation aims to demonstrate key NMR concepts, including interpretation of 1D spectra, relative quantitation, and first order kinetics. Prior to carrying out these experiments, it is strongly recommended to be familiar with basic 1D NMR processing.

This information is readily available in the version 001 Fourier EduLab Students Guide, which can be found on the USB stick delivered with the Fourier 80. In addition, having a basic understanding of processing software such as MestreNova is strongly recommended.

To perform this experiment, a properly installed and adjusted Fourier 80 system with TopSpin Software is required. Fourier 80 equipped with pulse field gradient is optional. In addition, a 10 mL volumetric flask & stopper, 10 uL micropipettes, 1000 uL micropipettes and vortex should be available.

Experimental Setup:

- Food grade invertase, purchased online
- Sucrose
- 5 mm disposable NMR tube with cap
- Pulse program: zg, zggpw5

Glossary

Hydrolysis:
Hydrolysis is any chemical reaction in which a molecule of water breaks one or more chemical bonds

NMR:
Spectroscopic analytical technique based on radio frequency-induced transitions between energy levels that atomic nuclei adopt in an external magnetic field as a result of their own magnetic moment

UV-vis spectroscopy:
UV spectroscopy or UV–visible spectrophotometry refers to absorption spectroscopy or reflectance spectroscopy in part of the ultraviolet and the full, adjacent visible regions of the electromagnetic spectrum

Abbreviations

NMR:
Nuclear Magnetic Resonance

UV-vis Spectroscopy:
Ultraviolet–visible spectroscopy

Sample Preparation:

Preparation of stock sucrose solution (200 mM):

- Obtain ~ 684 mg of sucrose.
- Transfer the sucrose to a 10 mL volumetric flask.
- Fill the volumetric flask to the appropriate height with H_2O such that the total volume is 10 mL and mix thoroughly.

Preparation of stock invertase solution:

- Transfer 990 µL of water to the 1.5 mL Eppendorf tube.
- Using a 10 µL micropipette, dispense 10 µL of food grade invertase in the Eppendorf tube.
- Using a vortexer, mix the solution and ensure complete mixing of this solution.

Preparation of NMR sample:

- To a 5 mm NMR tube, add 100 uL of stock sucrose solution and 490 µL of D_2O and mix well.
- Dispense 10 uL of invertase from the stock solution to this NMR solution.
- Shake the NMR tube vigorously before placing it in the NMR spectrometer.
- Flick the tube and ensure there are no bubbles in the sample before inserting it into the NMR spectrometer.

Experimental Procedure:

1. Prepare a 1D ^{1}H zg experiment to obtain the O1P (apex of water frequency).

2. Prepare a 1D ^{1}H zggpw5 experiment (parameters found below) using the O1P and 90-degree pulse lengths found in step 1. W5 is a simple and very effective water suppression approach but requires pulse field gradients. If your spectrometer is not fitted with gradient then use zgcppr and ~66dB for PLdb9 (presaturation power). If additional parameters are needed for zgcppr see Edulab "Mixing It Up: Exploring Cocktails with NMR".

3. Select the ^{1}H NMR experiment created in step 2, and run it using "multizg", when prompted, enter the number of experiments you would like to run, and they will automatically be generated for you. Example Conditions provided below.

4. Process the NMR spectra in accordance to [chapter 1, Fourier Edulab version 1] using the parameters provided below.

Data Processing:

Phase and baseline correct the NMR spectrum as necessary, using a 0.3 Hz line-broadening (EM window function) prior to integration and analysis. May use MNova or TopSpin software to aid in analyzing the peak areas and stacking kinetics data for display purpose.

Notes:

Glossary

Quantitation:
NMR is quantitative by nature because the intensity of the signal is directly proportional to the concentration of the molecule that produces the signal. Quantitation is the process of measuring the intensity of a NMR signal and calculating the concentration of the molecule that produces the signal.

Relative quantitation:
Process of comparing the intensity of a signal from a molecule in one sample to the intensity of a signal from a molecule in another sample. This is done by normalizing the signals to a common reference.

Internal referencing:
Process of measuring the intensity of a signal against a standard molecule of known concentration that is inside the sample.

External referencing:
Process of measuring the intensity of a signal against a standard molecule that is outside the sample.

Abbreviations

O1P:
O1 (or O1P for the value in ppm) is the carrier frequency used for the hard pulses
D1:
Relaxation delay
DS:
Dummy scans
NS:
Number of scans
P1:
Length of the ^{1}H excitation pulse

Results & Discussion:

To complete the exercise, answer the following questions:

1. The protons attached to the alpha carbon were used to track the progress of the reaction. Why weren't hydroxyl groups chosen to monitor the reaction?

2. Aside from the relaxation delay (D1), what changes could be made to the experiment conditions to obtain absolute quantitation of glucose and sucrose?

3. Fit the results to a first-order kinetics decay curve. Is the time resolution sufficient to establish a rate constant? What could be changed about the experiment to enhance the time resolution while obtaining a comparable sensitivity?

4. These experiments were run in 100% D_2O. What would happen if these experiments were to be run in pure H_2O?

5. Advanced Question. Sample results of the invertase experiment are shown in Figure 2. After production the glucose anomeric peak decreases. Can you work out why? A hint the peak plotted and monitored is the peak for α-glucose. The peak for β-glucose (if applicable) would be under the water. Do your experimental results match the sample data? If not, why?

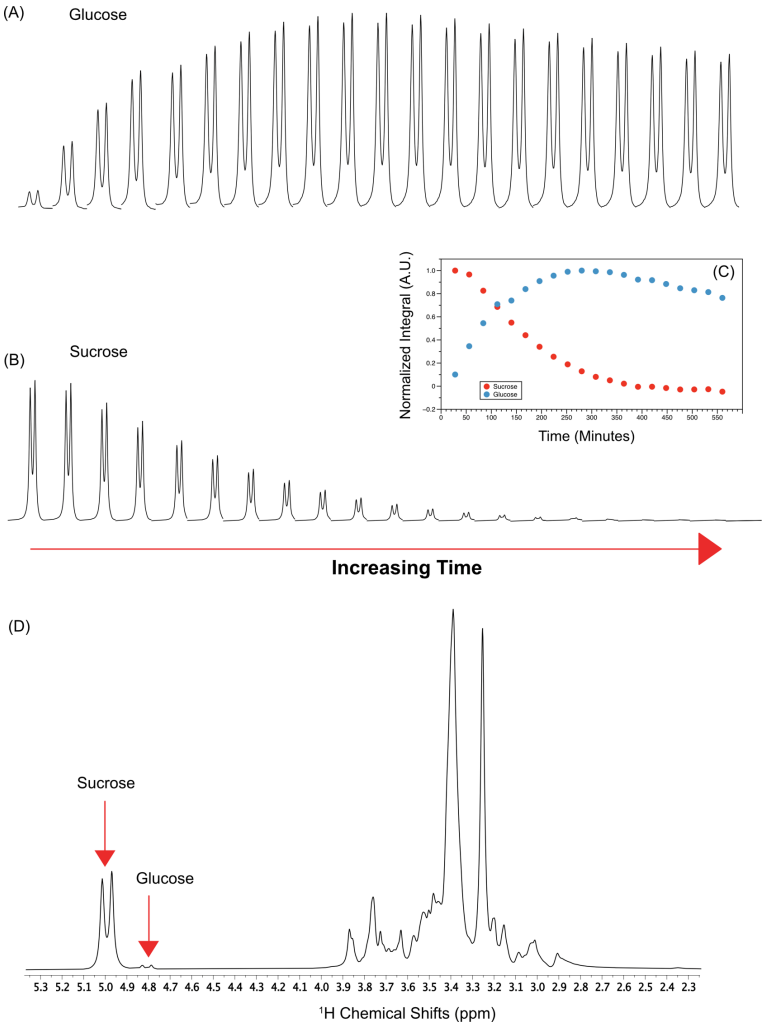

Figure 2. (A-B) A series of NMR spectra as a function of time showing only the anomeric doublet of the (A) glucose and (B) sucrose. (C) A graph of normalized integrals of sucrose and glucose as a function of time. (D) A spectrum showing a mixture of glucose and sucrose during enzymatic hydrolysis reaction.

Key Take Home Messages:

This experiment demonstrates the ability of NMR to monitor an enzymatic reaction. In this case, the enzymatic hydrolysis of sucrose into glucose via invertase. Through this experiment, you learned the following NMR concepts:

- Processing of NMR spectra and integration of NMR resonances.
- Analysis of NMR spectra.
- Using relative quantitation to determine kinetic parameters by NMR.

References:

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