EDULAB FOR INSTRUCTORS: 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).

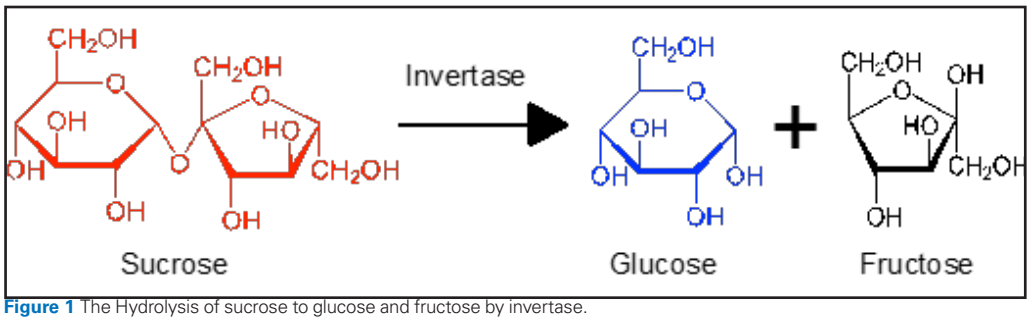

Figure 1 The Hydrolysis of sucrose to glucose and fructose by invertase.

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.

The experiment can be shortened or modified according to the instructors needs: Additional enzyme may be added to reduce the overall time necessary to monitor the reaction, and additional sucrose may be added to reduce the number of scans necessary to obtain usable signal to noise ratios. The amounts of enzyme and sucrose should be increased proportionally to allow students to obtain a decay curve for analysis.

It is strongly recommended that instructors setup the experimental templates prior to the laboratory as this exercise is meant to emphasize basic acquisition/processing and data analysis. Rather than focusing on advanced parameters, students should focus on calibrating and inputting basic parameters such as O1P, P1, DS and NS into prepared templates.

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.

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

Thermal Equilibrium:
The thermal equilibrium in NMR is the state in which the population of nuclear spins is in equilibrium. This is achieved by applying a magnetic field that is strong enough to overcome the forces that cause the population to change

Abbreviations

NMR:
Nuclear Magnetic Resonance

UV-vis Spectroscopy:
Ultraviolet–visible spectroscopy

Experimental Setup:

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

Template setup is done by copying over any previous parameter set, loading the chosen pulse program, and inputting the experimental values outlined below. The students will determine the O1P themselves. The 1D ^{1}H zg experiment should have the same SW and TD as the experiment above but can be run with NS = 1 and DS = 0 for determination of O1P.

Note: it is recommended to perform 20 experiments with 256 scans per experiment (approximately 20 minutes per scans).

1D ^{1}H zg

PULPROG	**zg**
P1 (µs)	spectrometer specific
D1 (s)	1.0
DS	0
NS	1 (recommended)

1D ^{1}H zggpw5

PULPROG	**zggpw5**
TD	4096
SW (ppm)	20
D19 (µs)	800
D1 (s)	5.0
DS	8
NS	256 (recommended)

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 5mm 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.

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
TD:
Number of FID points
SW:
Spectral width
D1:
Relaxation delay
DS:
Dummy scans
NS:
Number of scans
P1:
Length of the ^{1}H excitation pulse
D19:
Delay

Experimental Procedure:

1. Input DS = 0 and NS = 1 into the 1D ^{1}H zg experiment template to obtain the O1P (apex of water frequency) and the P1.

2. Input DS = 8 and NS = 256 into the 1D ^{1}H zggpw5 experiment template as well as the O1P and P1 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). See Edulab "Mixing It Up: Exploring Cocktails with NMR" for template setup parameters (#MixItUp).

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.. Futher information on template setup (for instructor only) is found in the tables under experimental setup.

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. Students may use MNova or Topspin to aid in analyzing the peak areas and stacking kinetics data for display purpose.

The sample data was processed via TopSpin (4.2.0) with a 0.3 Hz line-broadening and zero filled to twice the acquired time domain points. All NMR spectra were phased, and baseline corrected on TopSpin (4.2.0) before extracting integral values. The spectra were exported to MestraNova (MNova 14.0.1) for integral analysis and stacking of spectra for display purposes.

Notes

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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?**

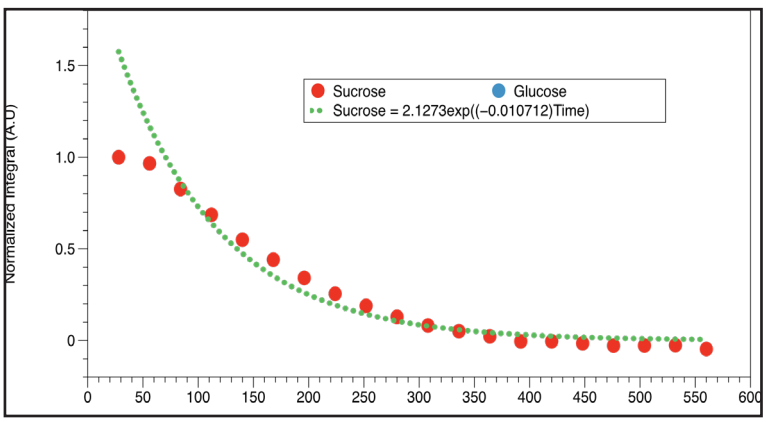
   Hydroxyl groups contain labile protons which will readily exchange with D_2O and H_2O, exchanging and deuterating the site, suppressing the signal.

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

   Absolute quantification could be obtained by using an 'external' standard using ERETIC (Electronic REference To access In vivo Concentrations) or an internal quantitative NMR standard to which signals could be compared to. By comparing signals from other molecular sites to a synthesized or internal standard signal corresponding to a known concentration, the concentration of the remaining molecules can be determined by evaluation of relative peak areas.

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?**

   If the conditions are altered, the reaction rate may be altered. A sample answer is given below.



The time resolution is sufficient for the production of glucose / fructose to be observed (students should refer to their figure; see figure two for an example). Time resolution is limited by sensitivity, since a minimum number of scans are necessary to obtain sufficient signal to noise to quantify the glucose, sucrose, and fructose peaks. To increase time resolution while achieving a comparable sensitivity, a larger concentration of sucrose could be used, a higher magnetic field could be used.

Notes

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

   If these experiments were to be run in H2O, a large residual water signal would be left behind, which may alter the accuracy of the integrals close to the water.

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?**

   Invertase split sucrose into glucose (α-D-glucose) and fructose. Once formed the α-D-glucose will isomerize in solution to a 36:64 ratio of alpha (α) to beta (β) at equilibrium in water. The α-D-glucose signal decreases as it forms the β -D-glucose isomer.

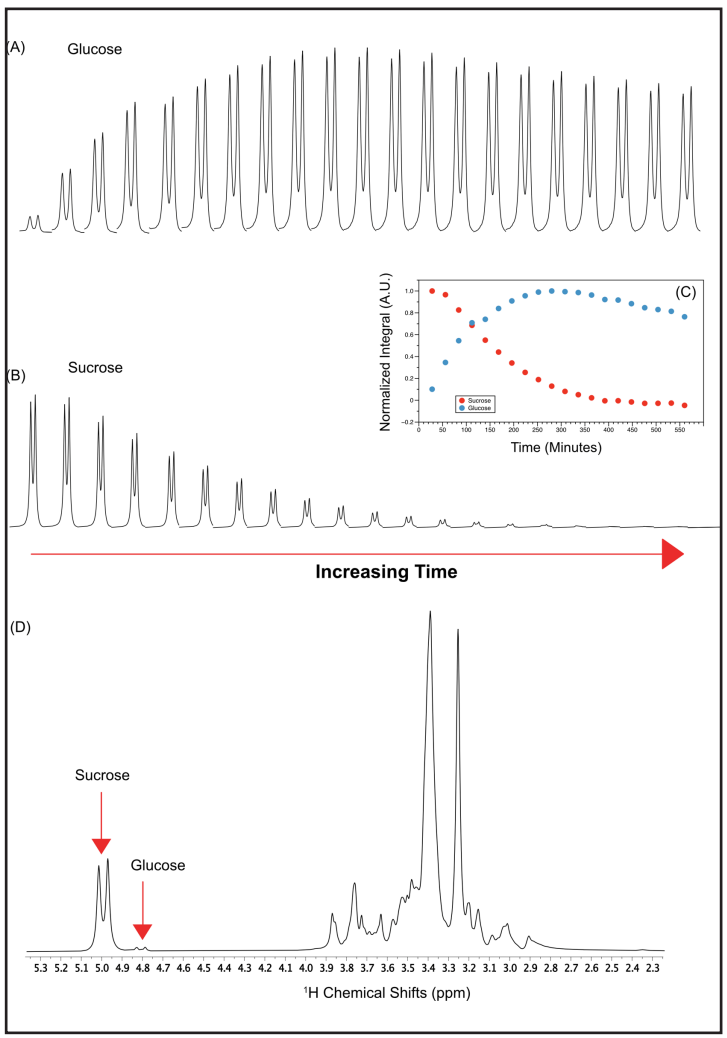


Figure 2. (A-B) A series of NMR spectra as a function of time showing only the anomeric doublet of the (A) glucose and (B) sucrose. (C) A graph of normalized integrals of sucrose and glucose as a function of time. (D) A spectrum showing a mixture of glucose and sucrose during enzymatic hydrolysis reaction.

Notes

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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2. Vang, J.Y.; Her, C.; Krishnan, V.V.; NMR based real-time enzyme kinetics on estimating the inhibitory effect of sucralose in the enzymatic conversion of sucrose. **Biophys. Chem.** 2021, 268, 106495

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