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Exploring enzyme kinetics

Monitoring invertase activity using DNSA reagent

Aim

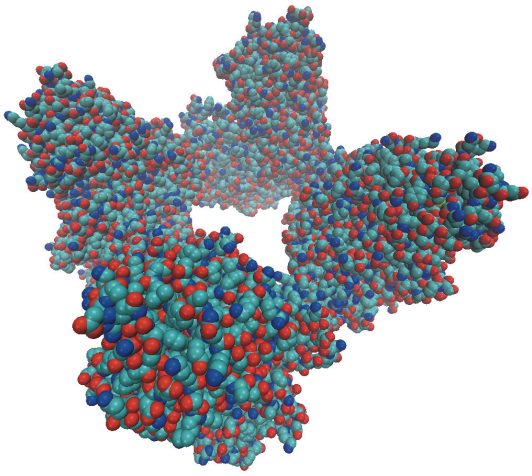
The aim of this practical activity is to investigate the initial rate of reaction of the enzyme invertase. The basic method can be adapted to study the effect of temperature and pH on the activity of the enzyme. The DNSA reagent used (an alternative to Benedict's Quantitative reagent) is also suitable for monitoring other reactions where reducing sugars are formed *e.g.*, the action of amylase or cellulase.

Introduction

Invertase, (β-fructofuranosidase, E.C. 3.2.1.26) is produced intracellularly by baker's yeast, *Saccharomyces cerevisiae*. The enzyme splits the disaccharide sucrose into the monosaccharides glucose and fructose. Both glucose and fructose are reducing sugars, while sucrose is not.

Invertase can be used to demonstrate many features of enzyme activity including the effect of pH and temperature as well as inhibition of the enzyme by the substrate and products. The biochemists Leonor Michaelis and Maud Menten conducted their fundamental studies on enzyme kinetics in 1913 using invertase [1].

The enzyme's activity may be monitored by measuring the production of glucose, using semi-quantitative glucose test strips. These are, however, costly and are becoming increasingly difficult to obtain, as they are being replaced by different test strips that are designed to be coupled with electronic glucose test meters.

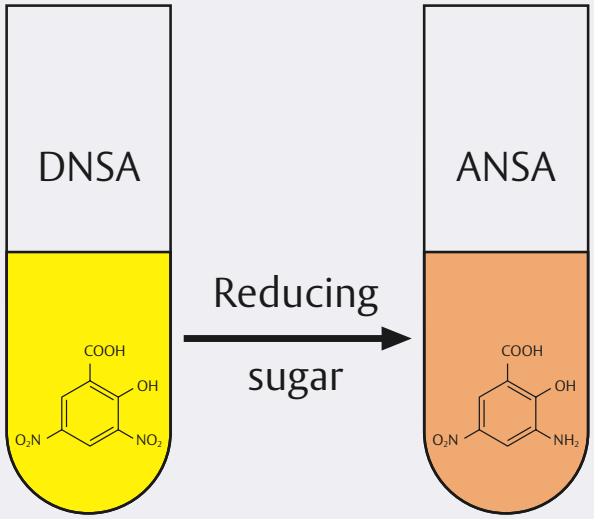


The structure of invertase from Saccharomyces cerevisiae was determined in 2013. The active enzyme is made from four sub-units.
Data from: Sainz-Polo, et al [2]

DNSA reagent

A commonly-used quantitative colorimetric assay of reducing sugars uses 3,5-dinitrosalicylic acid (DNSA). On heating with reducing sugars under alkaline conditions, the yellow-coloured DNSA is reduced to red-coloured 3-amino,5-nitrosalicylic acid (ANSA). This colour change can be measured using a colorimeter at 500–560 nm (that is, with a green light or filter). The ideal wavelength is 540 nm.

The DNSA test can detect concentrations of glucose between 0.5 mM (0.09 g of glucose per 100 cm^3) and 40 mM (0.72 g per of glucose per 100 cm^3).



Safety guidelines

DNSA reagent contains 0.4 M sodium hydroxide (that is, 1.59% NaOH w/v) and has a pH of 13.7. The reagent can therefore cause skin irritation and serious eye irritation, so you MUST wear eye protection throughout the practical procedure. In addition, DNSA reagent is harmful if ingested.

Equipment and materials

Needed by each student or group

Equipment

- Eye protection (safety spectacles or goggles)
- Water bath at 35 °C
- Kettle for boiling water
- Large beaker *e.g.*, 500 cm^3, to stand 11 test tubes in
- 2 x 1 cm^3 syringes, calibrated at 0.1 cm^3 intervals or more frequently
- 10 cm^3 syringe
- Beaker of near-boiling water
- Permanent marker pen or paper labels
- 11 test tubes
- Stopclock or timer *e.g.*, on a mobile phone (if permitted!)

Materials

- DNSA reagent, 5 cm^3 (0.3 cm^3 is needed per test)
- 0.4 M sucrose solution, 10 cm^3 (0.4 M is 13.69 g of sucrose in 100 cm^3 of distilled water)
- 1 cm^3 of 0.01% solution of invertase (*Bioinvert*® from the NCBE — if you use invertase from another source you will have to conduct trial experiments to discover how much is needed)
- Distilled water, 50 cm^3

Procedure

1 Label the 11 tubes 0 to 10.

2 Using a clean 1 cm^3 syringe, add 0.3 cm^3 of DNSA reagent to each tube.

3 Using a clean 1 cm^3 syringe, add 0.3 cm^3 of sucrose solution to tube '0'. This is a 'blank' to which no invertase will be added, and it will be used to 'zero' the colorimeter.

4 Stand the remaining sucrose solution in the water bath for a few minutes so that it warms to the reaction temperature.

5 Using a clean 1 cm^3 syringe, add 1 cm^3 of the invertase solution to the sucrose solution. Stir with the syringe to mix and simultaneously start the timer.

6 After 60 seconds, use the syringe to take 0.3 cm^3 of the reaction mixture and add it to tube '1'. *Adding the reaction mix to the DNSA reagent, which contains 0.4 M sodium hydroxide, stops the enzyme action.*

7 Every 60 seconds, take a 0.3 cm^3 sample of the reaction mixture and add it to one of the successively numbered tubes until you have 10 samples, each in a separate, numbered tube.

8 Stand all 11 test tubes in a large beaker of just-boiled water for 5–10 minutes until the contents have changed colour (the contents of tube '0' will be bright yellow; the contents of tube '10' will be dark red).

9 Using a clean 10 cm^3 syringe, add 3 cm^3 of distilled water to each test tube. Flick the tubes gently with a finger to mix their contents.

10 Zero the colorimeter using sample '0', then measure the absorbance of the 10 samples at approximately 500 nm (green light; anywhere between 500 nm to 550 nm, depending upon the type of colorimeter you have, although 540 nm is the ideal wavelength). *If the later samples are too dark and beyond the range of the colorimeter, you will have to dilute them with distilled water.*

11 Plot a suitable graph of the results.

Additional investigations

1 Prepare a series of glucose solutions of known concentrations, mix 0.3 cm^3 of each with 0.3 cm^3 of DNSA reagent, heat each sample for 5–10 minutes, then measure the absorbance using a colorimeter. This will allow you to produce a calibration curve. (Suitable concentrations of glucose would be 0, 0.5, 1, 2, 4, 6, 8, 10 and 12 mM).

2 Try the experiment with water baths at different temperatures to investigate the effect of temperature on the action of invertase.

3 Prepare the sucrose solution in buffers of different pH values to investigate the effect of pH on the action of invertase. Note that strong acids will hydrolyse sucrose, so that if the enzyme is denatured by the acid, you may obtain a positive result. (What 'control' treatment(s) would you need to do to check this?)

Teacher's and technician's notes

Background

Invertase, (β-fructofuranosidase, E.C. 3.2.1.26) is produced intracellularly by baker's yeast, *Saccharomyces cerevisiae*. The enzyme hydrolyses the disaccharide sucrose into the monosaccharides glucose and fructose.

Sucrose + Water ⟶ Glucose + Fructose

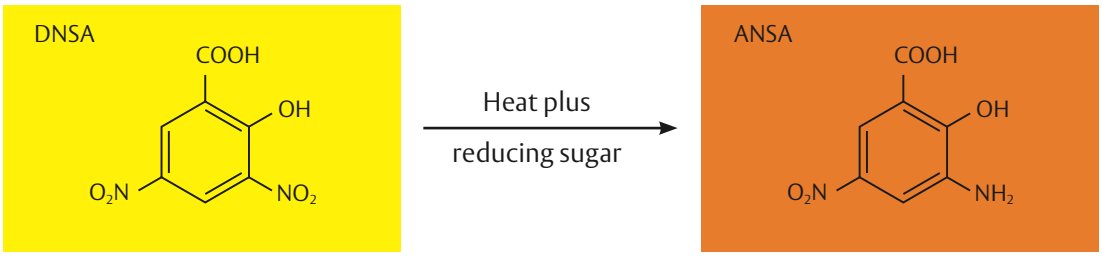
Both glucose and fructose are reducing sugars, while sucrose is not.

Invertase can be used to demonstrate many features of enzyme activity including the effect of pH and temperature as well as inhibition of the enzyme by the substrate and products. The biochemists Leonor Michaelis and Maud Menten conducted their fundamental studies on enzyme kinetics using invertase [1].

Invertase activity may be monitored by measuring the production of glucose. This can be done using semi-quantitative glucose test strips. These are, however, costly and are becoming increasingly difficult to obtain, as they are being replaced by different test strips that are designed to be coupled with electronic glucose test meters.

The DNSA test

On heating with reducing sugars, the 3-nitro (NO_2) group of DNSA is reduced to an amino (NH_2) group. The colour of the reagent changes from yellow to orange or red, depending upon the concentration of reducing sugar present.



3,5-dinitrosalicylic acid

3-amino-5-nitrosalicylic acid

The DNSA test is more sensitive than Benedict's reagent and can detect concentrations of glucose between 0.5 mM (0.09 g of glucose per 100 cm^3) and 40 mM (0.72 g per of glucose per 100 cm^3).

The DNSA reagent used for this work is suitable for monitoring other reactions where reducing sugars are formed *e.g.*, the action of amylase or cellulase [3].

Preparation and timing

The DNSA reagent can be prepared in advance; it will keep on the shelf at room temperature for at least 12 months. If they are well-organised, the practical activity will take students about 30 minutes to complete.

Troubleshooting

If the concentration of the glucose in the samples is too great, they will need to be diluted before the absorbance is measured.

Safety

DNSA reagent

DNSA reagent contains 0.4 M sodium hydroxide (that is, 1.59% NaOH w/v) and has a pH of 13.7. The reagent can therefore cause skin irritation and serious eye irritation, so you MUST wear eye protection throughout the practical procedure. In addition, DNSA reagent is harmful if ingested.

IF ON SKIN: Wash with plenty of soap and water. If skin irritation occurs seek medical advice/attention.
EYE CONTACT: Check for and remove contact lenses if present. Rinse opened eye immediately with running water, also wash under the eyelids, for several minutes. Seek medical advice if irritation persists.
INGESTION: Rinse out mouth with water, then drink plenty of water. Do not induce vomiting. Seek medical attention.

Additional investigations

Preparation of a calibration curve

This allows you to convert absorbance measurements from the colorimeter into glucose/fructose concentrations.

Prepare a set of standard solutions of glucose, ranging from, for example, 0.5 to 12 mM. (The molar mass of glucose is 180.16 g, so a 10 millimolar solution would contain 1.8016 g of glucose per litre.)

Add 0.5 cm^3 of DNSA reagent to 0.3 cm^3 of each glucose sample, then stand the tubes in a beaker of boiled water for five minutes.

Dilute each sample with 3 cm^3 of distilled water and flick each tube gently to mix the contents.

Measure the absorbance of the liquids at 500–550 nm (540 nm is the ideal wavelength if your colorimeter has a filter or light source of that wavelength.)

Note that when invertase breaks down sucrose, two sugars, glucose and fructose, are produced. Therefore a more accurate calibration curve would be made using a mixture of these two sugars rather than glucose alone. However, the results produced when either pure glucose or pure fructose are reacted with DNSA are virtually identical [4], so the accuracy of the calibration curve is not significantly affected.

DIY invertase

Invertase is easy to isolate from dried or fresh baker's yeast. 'Fast action' yeasts, sold for use in bread-making machines, are said to produce far more invertase than normal baker's yeast [5].

Equipment and materials

Needed by each person or group

Equipment

- Water bath at 35 °C
- Kettle for boiling water
- Large beaker *e.g.*, 500 cm^3, to stand 11 test tubes in
- 2 x 1 cm^3 syringes, calibrated at 0.1 cm^3 intervals or more frequently
- 10 cm^3 syringe
- Beaker of near-boiling water (from the kettle)
- Permanent marker pen or paper labels
- 11 test tubes
- Stopclock or timer *e.g.*, on a mobile phone (if permitted!)

Materials

- DNSA reagent, 5 cm^3 (0.3 cm^3 is needed per test)
- 0.4 M sucrose solution, 10 cm^3 (0.4 M is 13.69 g of sucrose in 100 cm^3 of distilled water)
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- Distilled water, 50 cm^3

Suppliers

DNSA reagent and invertase

DNSA reagent and invertase (*Bioinvert*®) are available from the NCBE.

Colorimeters

Biochrom
A colorimeter suitable for school use is the WPA CO7500 from Biochrom Ltd, 22 Cambridge Science Park, Milton Road, Cambridge CB4 0FJ. **www.biochrom.co.uk/product/19/biochrom-wpa-co7500-colorimeter.html** This colorimeter has a digital readout, it is simple to use, reliable, robust and the results are repeatable.

Mystrica
An inexpensive alternative is the *Mystrica* colorimeter, available from Mystrica Ltd, 39 Charterhall Road, Edinburgh, EH9 3HS. **www.mystrica.com**

Both colorimeters are supplied with software and can be linked to PC computers. Additionally, the *Mystrica* colorimeter can link to an iPhone or iPad or similar Android device *via* Bluetooth 4.0.

References

1. Johnson, K. A. and Goody, R.S. (2011) The original Michaelis constant: Translation of the 1913 Michaelis-Menten paper *Biochemistry* **50**, 8264–8269.
This commentary is available online at:
http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3381512/
The translation of the 1913 paper is available here:
http://pubs.acs.org/doi/suppl/10.1021/bi201284u

2. Sainz-Polo, M.A. *et al* (2013) Three-dimensional structure of *Saccharomyces* invertase. *Journal of Biological Chemistry* **288**, 9755–9766. *Note: The Protein Data Bank accession code for this molecule is: 4EQV.*

3. Miller, G.L. (1959) Use of the dinitrosalicylic acid reagent for determination of reducing sugar. *Analytic chemistry* **31**, 426–428.

4. Saquib, A.A.N. and Witney, P.J. (2011) Differential behaviour of the dinitrosalicylic acid (DNS) reagent towards mono- and disaccharide sugars. *Biomass and Bioenergy* **35**, 4748–4750.

5. Timmerman, A. P. (2012) The isolation of invertase from baker's yeast. In: *Protein Purification*, by Rizwan Ahmad [Ed] pp 29–51. Intech. ISBN: 978 953 307 831 1. Available from: **http://www.intechopen.com/books/protein-purification/the-isolation-of-invertase-from-baker-s-yeast-an-introduction-to-protein-purification-strategies**