Lactose

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Principle

β-Galactosidase catalyses the hydrolysis of lactose:

(1) $$\text{Lactose} + H_2O \longrightarrow \text{galactose} + \text{glucose}$$

In the presence of hexokinase and adenosine triphosphate (ATP) the glucose is phosphorylated:

(2) $$\text{Glucose} + \text{ATP} \longrightarrow \text{glucose-6-phosphate} + \text{ADP}$$

The oxidation of the glucose-6-phosphate with glucose-6-phosphate dehydrogenase and triphosphopyridine nucleotide (TPN) serves as the indicator reaction:

(3) $$\text{Glucose-6-phosphate} + TPN^+ \longrightarrow \text{6-phosphogluconate} + TPNH + H^+$$

The TPNH formed is measured by the increase of absorption at 340 mμ.

In very dilute solutions the transglucosidase activity of the β-galactosidase preparation does not interfere. In the method described below the concentrations of the reagents have been selected so that reaction (1) is rate-limiting and therefore glucose will be removed by reactions (2) and (3) as rapidly as it is formed.

Reagents

1. Magnesium chloride, $MgCl_2 \cdot 6H_2O$
2. Potassium chloride, KCl
3. Tris-hydroxymethyl-aminomethane, A. R., tris
4. Acetic acid, glacial
5. Adenosine triphosphate, ATP
   disodium salt, ATP-$Na_2H_2 \cdot 3H_2O$; commercial preparation, see p. 1006.
6. Triphosphopyridine nucleotide, TPN
   monosodium salt, TPN-NaH_2; commercial preparation, see p. 1029.
7. Glucose
8. Lactose
9. Hexokinase, HK
   commercial preparation, see p. 983.
10. Glucose-6-phosphate dehydrogenase, G6P-DH
    commercial preparation, see p. 915.
11. β-Galactosidase
    from *Escherichia coli*[1], isolation, see p. 106.

Purity of the enzyme preparations

Crude β-galactosidase preparations contain a nucleotidase which can invalidate the assay. Furthermore, impure preparations usually absorb at 340 mμ and therefore decrease the sensitivity of the determination.

The activity of the hexokinase was 3×10^5 K. M. units*/mg.

*) A K. M. unit is defined according to[2]; see also p. 983.

1) *A. S. L. Hu, R. G. Wolfe* and *F. J. Reithel*, Archives Biochem. Biophysics *81*, 500 [1959].
2) *M. Kunitz* and *M. R. McDonald*, J. gen. Physiol. *29*, 393 [1946].

The glucose-6-phosphate dehydrogenase preparation used was a highly purified and very active preparation from bovine mammary gland[3]. Highly active, commercial preparations (*e.g.* from Boehringer) are also suitable if used in slightly higher concentration.

Preparation of Solutions

I. Salt solution (*ca.* 1 M $MgCl_2$; 1 M KCl):
Dissolve 20 g. $MgCl_2 \cdot 6H_2O$ and 7.5 g. KCl in distilled water and make up to 100 ml.

II. Tris-acetate buffer (0.05 M; pH 8.0):
Titrate 0.1 M acetic acid (dilute 5.75 ml. glacial acetic acid to 1000 ml. with oxygen-free distilled water) with 1 M tris (12 g. tris-hydroxymethyl-aminomethane/100 ml.) to pH 8 (glass electrode) and dilute to twice the original volume with distilled water, so that the solution is 0.05 M with respect to acetate. Remove air from the solution by gassing with nitrogen and store under nitrogen.

III. Adenosine triphosphate (*ca.* 0.5 M ATP):
Dissolve 50 mg. ATP-$Na_2H_2 \cdot 3H_2O$ in 1 ml. distilled water.

IV. Triphosphopyridine nucleotide (*ca.* 0.025 M β-TPN):
Dissolve 20 mg. TPN-NaH_2 in 1 ml. distilled water.

V. Hexokinase, HK (*ca.* 10 mg. protein/ml.):
Dissolve 50 mg. dry powder in 5 ml. distilled water; dilute crystalline suspensions with 3 M $(NH_4)_2SO_4$ solution.

VI. Glucose-6-phosphate dehydrogenase G6P-DH (*ca.* 2 mg. protein/ml.).
If necessary, dilute the stock suspension with 3.3 M $(NH_4)_2SO_4$ solution.

VII. β-Galactosidase (*ca.* 30 mg. protein/ml.):
Dissolve or dilute the enzyme with solution II.

VIII. Glucose (0.02 M):
Dissolve 360 mg. glucose in distilled water and make up to 100 ml. Store, frozen, in polyethylene bottles.

Solutions I—VIII can be used separately as described below or the reagents required for the determination of glucose can be combined as a solution IX.

IX. Mix together, 18.0 ml. buffer (soln. II), 0.2 ml. salt solution (I), 0.4 ml. G6P-DH suspension (VI), 0.4 ml. TPN solution (IV), 0.4 ml. HK solution or suspension (V) and 0.2 ml. ATP solution (III). This mixture is sufficient for 20 determinations.

X. Lactose (0.02 M):
Dissolve 680 mg. lactose in 100 ml. distilled water. Store, frozen, in polyethylene containers.

Stability of the solutions

Store all solutions except I in the frozen state, preferably in polyethylene containers. Allow to stand in an ice bath when in use. Under these conditions all the reagents, including IX, are stable for several weeks.

Procedure

Preliminary treatment of the sample

The presence of large amounts of glucose in the sample interferes with the determination, while small amounts only cause high blanks. It is often convenient to remove the mono-

[3] *G. R. Julian, R. G. Wolfe* and *F. J. Reithel*, J. biol. Chemistry *236*, 754 [1961].

saccharides by adsorbing the sugars on a charcoal-celite column[4] and washing with water. Lactose and other higher saccharides can be eluted with dilute alcohol. This procedure permits the removal of monosaccharides, salts and deproteinizing agents, although extensive water washing is sometimes necessary.

Spectrophotometric measurements

Test of the reagents

For the determination of glucose: Pipette into a silica cuvette (1 ml., 1 cm. light path):

0.980 ml. solution IX

or

0.9 ml. buffer (solution II)
0.010 ml. salt solution (I)
0.020 ml. G6P-DH suspension (VI)
0.020 ml. TPN solution (IV)
0.020 ml. HK solution or suspension (V)
0.010 ml. ATP solution (III).

Allow to equilibrate at room temperature for 2—3 min. Mix thoroughly with a small polyethylene rod. Set the spectrophotometer to read zero at 340 mμ. If the optical density does not change over a period of several minutes, add

0.005 ml. glucose solution (VIII) = 0.10 μmoles glucose.

For this addition use a Lang-Levy micro-pipette*) to which is attached a piece of rubber tubing bearing a mouthpiece. Insert the tip of the micro-pipette almost to the bottom of the cuvette and blow in the contents of the pipette. The optical density at 340 mμ should rise immediately and reach a final value of about 0.620 in 10—15 min., if the solutions for the determination of glucose are in order.

For the determination of lactose: In the same way pipette into a silica cuvette

0.980 ml. solution IX

or

solutions I—VI

in the amounts and order given above. To this mixture add

0.005 ml. lactose solution (X) = 0.10 μmoles lactose
0.020 ml. β-galactosidase solution (VII).

The optical density should again reach a value of about 0.620.

Analysis of the sample

Pipette into a silica cuvette (1 ml., light path 1 cm.)

0.980 ml. solution IX

or

solutions I—VI

in the amounts and order given above. To this mixture add

0.005 ml. sample.

*) Manufacturer: Research Specialities Co., Richmond, Calif., USA.

[4] *R. L. Whistler* and *D. F. Durso*, J. Amer. chem. Soc. *72*, 677 [1950].

This often contains some glucose, therefore wait until the optical density at 340 mμ reaches a constant value E_1. Then mix in

0.020 ml. β-galactosidase solution (VII)

and on completion of the reaction read the optical density E_2 at 340 mμ. The optical density difference $\Delta E = E_2 - E_1$ is used for the calculations.

Calculations

1 μmole of lactose is equivalent to 1 μmole of TPNH (see under "Principle"). According to equation (10) on p. 37 it follows that for the assay volume used here (1.005 ml.) and with a cuvette of 1 cm. light path:

$$\frac{\Delta E \times 1.005 \times 340}{10^{-6} \times 6.22 \times 10^{-6} \times 1 \times 0.005} = \Delta E \times 11 \times 10^3 = \text{μg. lactose/ml. sample}$$

$$= \Delta E \times 11 = \text{mg. lactose/ml. sample}$$

If the light path of the cuvette or the assay volume differs from the values stated here, then it is necessary to correct for this according to equation (10) on p. 37.

Example

The sample was an eluate (50% alcohol) from a charcoal-celite column. The sample was evaporated to a syrup, or to dryness, dissolved in distilled water, made up to 10 ml. and filtered. 0.005 ml. was taken for the determination. V = 1.005 ml., d = 0.970 cm., ΔE = 0.886. Therefore:

$$\Delta E \times \frac{11}{0.970} = 0.886 \times \frac{11}{0.970} = 10.05 \text{ mg. lactose/ml. sample}$$

or: 100.5 mg. lactose/10 ml. sample.

Specificity

Other oligosaccharides containing glucose attached to galactose by a β1→4 linkage will also react, but normally such compounds occur in very small amounts. If their presence is suspected in a sample then it is necessary to check this fact by paper chromatography. They can be separated chromatographically on a charcoal-celite column. Other sugars such as galactosyl-β1→6-glucose can be separated by paper chromatography[5].

Appendix

Isolation of β-galactosidase[1,6]

Escherichia coli is grown on a succinate-peptone-salt solution for 18 hours at 30°C with vigorous aeration. This medium contains in 1000 ml.: 8 g. Na succinate, 1.5 g. Difco-Bactopeptone, 4 g. $(NH_4)_2SO_4$, 27 g. KH_2PO_4, 0.4 g. $MgSO_4 \cdot 7\,H_2O$, 10 mg. $CaCl_2$; 0.4 mg. $FeSO_4 \cdot 7\,H_2O$. From 80 litres of medium is obtained 110–160 g. cells.

The isolation of the enzyme includes the following steps: Grinding the cells in a mortar. Extraction with tris buffer (pH 7) (containing thioglycollic acid and $MgCl_2$). Precipitation with streptomycin (final concentration 2.5%). Precipitation with $(NH_4)_2SO_4$ at 64% saturation. Fractionation with $(NH_4)_2SO_4$ between 28 and 37% saturation. Dialysis. Chromatography on DEAE-cellulose. The enzyme can be crystallized by the addition of $(NH_4)_2SO_4$ to the eluate.

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[5] See, for example, *B. E. Lederer* and *M. Lederer:* Chromatography. Elsevier, New York 1960.

[6] See also *K. Wallenfels et al.*, Biochem. Z. *331*, 459 [1959]; Angew. Chem. *69*, 482 [1957].