D-Fructose-1,6-diphosphate

Determination with Fructose-1,6-diphosphatase

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Principle

The determination of fructose-1,6-diphosphate depends on the following reactions:

(1) Fructose-1,6-diphosphate ⟶ fructose-6-phosphate + phosphate

(2) Fructose-6-phosphate ⇌ glucose-6-phosphate

(3) Glucose-6-phosphate + TPN^+ ⇌ 6-phosphogluconate + TPNH + H^+

Reaction (1) is catalysed by a specific fructose-1,6-diphosphatase[1], (2) by phosphoglucose isomerase (PGI) and (3) by glucose-6-phosphate dehydrogenase (G6P-DH). If great sensitivity is not required, then the inorganic phosphate formed in reaction (1) can be determined colorimetrically[2] since the phosphatase is specific for fructose-1,6-diphosphate. If all three reactions are used for the determination, the increase in optical density at 340 mμ due to the formation of reduced triphosphopyridine nucleotide (TPNH) is a measure of the over-all reaction. One μmole of TPNH is formed for each μmole of fructose-1,6-diphosphate.

Reagents

1. Perchloric acid, sp. gr. 1.67; *ca.* 70% (w/w)
2. Potassium hydroxide, 5 N
3. Tris-hydroxymethyl-aminomethane, tris
4. Hydrochloric acid, 1 N
5. Magnesium chloride, $MgCl_2 \cdot 6H_2O$
6. Ethylene-diamine-tetra-acetic acid, EDTA
   sodium salt, $EDTA\text{-}Na_2H_2 \cdot 2H_2O$
7. Triphosphopyridine nucleotide, TPN
   sodium salt, $TPN\text{-}NaH_2$; commercial preparation, see p. 1029.
8. Glucose-6-phosphate dehydrogenase, G6P-DH
   from yeast; commercial preparation, see p. 975.
9. Phosphoglucose isomerase, PGI
   from yeast; commercial preparation, see p. 993.
10. Fructose-1,6-diphosphatase
    from spinach leaves[1]. For preparation, see Appendix, p. 163.

Purity of the enzyme preparations

The enzyme preparations need not be of the highest purity, since neither sedoheptulose diphosphate nor other phosphate esters form glucose-6-phosphate. However, the preparations must be

[1] *E. Racker* and *E. A. R. Schroeder*, Arch. Biochem. Biophysics *74*, 326 [1958].
[2] *K. Lohmann* and *L. Jendrassik*, Biochem. Z. *178*, 419 [1926].

free from non-specific phosphatases, because hydrolysis of fructose-1,6-diphosphate at the C-6-phosphate group would result in too low values. Glucose-6-phosphate dehydrogenase must be free from 6-phosphogluconic dehydrogenase and TPNH oxidase. The suitability of the fructose-1,6-diphosphatase preparation should be tested by comparing the analytical values obtained with it, with those obtained with the aldolase assay system (see p. 246). The values should be identical.

Preparation of Solutions

I. Perchloric acid (50% w/v):
Dilute 43 ml. 70% $HClO_4$ to 100 ml. with distilled water.

II. Tris buffer (1 M; pH 8.8):
Dissolve 12.1 g. tris-hydroxymethyl-aminomethane in 50 ml., adjust to pH 8.8 with *ca.* 17 ml. 1 N HCl and make up to 100 ml.

III. Magnesium chloride (0.1 M):
Dissolve 2.03 g. $MgCl_2 \cdot 6H_2O$ in distilled water and make up to 100 ml.

IV. Ethylene-diamine-tetra-acetate (1.2% w/v):
Dissolve 1.2 g. EDTA-$Na_2H_2 \cdot 2H_2O$ in distilled water and make up to 100 ml.

V. Triphosphopyridine nucleotide (0.005 M β-TPN):
Dissolve 21.6 mg. TPN-NaH_2 in distilled water and make up to 5 ml.

VI. Glucose-6-phosphate dehydrogenase, G6P-DH (15 units*)/ml.):
Dilute the commercial preparation with distilled water.

VII. Phosphoglucose isomerase, PGI (10 units*)/ml.):
Dilute the commercial preparation with distilled water.

VIII. Fructose-1,6-diphosphatase (70 units*)/ml.):
Dissolve the preparation obtained according to[1] in distilled water.

Stability of the solutions

Glucose-6-phosphate dehydrogenase keeps as an ammonium sulphate suspension for several months at 0° C. Phosphoglucose isomerase can be stored as a suspension in ammonium sulphate solution for several years at 0° C. The fructose-1,6-diphosphatase prepared according to[1] keeps for several years at −20° C.

Procedure

Deproteinization

Trichloroacetic acid inhibits glucose-6-phosphate dehydrogenase and therefore should not be used for deproteinization. Deproteinize the sample by heating for 2 min. in boiling water bath or by adding sufficient perchloric acid solution (I), so that the final perchloric acid concentration in the sample is 5% (w/v). Centrifuge off the protein, adjust the pH of the supernatant to 7.0 with 5 N KOH and allow to stand for 10 min. in an ice bath. Centrifuge off the precipitated $KClO_4$ and use a portion of the supernatant for the assay.

*) A unit is the amount of enzyme which converts 1 μmole of substrate in 1 min.

Spectrophotometric measurements

Wavelength: 340 mμ; light path: 1 cm.; final volume: 1 ml. Read against the control cuvette.

Pipette into the cuvettes:

Experimental cuvette	*Control cuvette*
deproteinized sample (containing 0.01 to 0.08 μmoles fructose-1,6-diphosphate)	deproteinized sample (as for experimental cuvette)
0.10 ml. buffer (soln.II)	0.10 ml. buffer (soln. II)
0.05 ml. $MgCl_2$ soln. (III)	0.05 ml. $MgCl_2$ soln. (III)
0.05 ml. EDTA soln. (IV)	0.05 ml. EDTA soln. (IV)
0.05 ml. TPN solution (V)	
distilled water to 0.94 ml.	distilled water to 0.94 ml.

Read the optical density E_1. Mix into *both cuvettes* (if the deproteinized sample contains TPN, then only into the experimental cuvette):

0.02 ml. G6P-DH solution (VI)

and on completion of the reaction read the optical density E_2. Mix into *both cuvettes*

0.02 ml. PGI solution (VII),

wait for the reaction to stop and then read the optical density E_3. Mix into *both cuvettes*

0.02 ml. fructose-1,6-diphosphatase solution (VIII).

On completion of the reaction read the optical density E_4.

Calculations

$\Delta E_{G6P} = E_2 - E_1$ corresponds to the glucose-6-phosphate content of the assay mixture, $\Delta E_{F6P} = E_3 - E_2$ corresponds to the fructose-6-phosphate content and $\Delta E_{FDP} = E_4 - E_3$ corresponds to the fructose-1,6-diphosphate content. The optical densities E_1 to E_3 must be corrected for the dilution of the assay mixture which occurs on addition of the enzyme solutions:

$$\frac{E_2 - 0.94 \times E_1}{6.22} = \mu\text{moles glucose-6-diphosphate/assay mixture}$$

$$\frac{E_3 - 0.96 \times E_2}{6.22} = \mu\text{moles fructose-6-phosphate/assay mixture}$$

$$\frac{E_4 - 0.98 \times E_3}{6.22} = \mu\text{moles fructose-1,6-diphosphate/assay mixture}$$

where

6.22 = extinction coefficient for TPNH at 340 mμ [$cm.^2$/μmole].

Sources of Error

Samples deproteinized with perchloric acid must be analysed as soon as possible. The sample should not contain glutathione (neither GSSG nor GSH), otherwise TPNH will be re-oxidized by the glutathione reductase which is present in most commercial preparations of glucose-6-phosphate dehydrogenase.

Appendix

Preparation of alkaline (C-1)-fructose-1,6-diphosphatase[1]

To a 100 ml. of the juice expressed from washed spinach leaves add 29.1 g. $(NH_4)_2SO_4$ at 0°C and centrifuge for 15 min. at 18000 g. Add 34.8 g. $(NH_4)_2SO_4$ to each 100 ml. supernatant fluid and then filter through filter paper. Dissolve the solid residue on the filter paper in $^1/_{15}$ of the original extract volume of distilled water. Dialyse overnight, with stirring, against 8 litres distilled water. Centrifuge, dilute the supernatant so that is contains 5 mg. protein/ml. and adjust to pH 5.8. Heat this solution for 15 min. at 62°C, centrifuge and discard the precipitate. Add 2 ml. calcium phosphate gel (15.2 mg. dry weight/ml.) to every 100 ml. of the supernatant. Centrifuge, wash the gel sediment with 100 ml. tris-hydroxymethyl-aminomethane buffer (0.1 M; pH 7.4) and then suspend in 100 ml. 0.05 M potassium phosphate buffer (pH 7.8) and allow to stand for 15 min. Centrifuge and elute the gel with a further 50 ml. buffer. Combine the eluates.

Ammonium sulphate fractionation: To every 100 ml. of the eluate add 39.1 g. $(NH_4)_2SO_4$. Centrifuge off the precipitate (15 min., 18000 g) and discard. Slowly add $(NH_4)_2SO_4$ over a period of 2 hours until the final concentration is 3.05 M and then centrifuge. Dissolve the precipitate in 20 ml. distilled water (fraction I). Add more $(NH_4)_2SO_4$ to the supernatant over a period of 1 hour to give 3.4 M (final concentration). Centrifuge and dissolve the precipitate in 2 ml. distilled water (fraction II). Separate fraction I into four subfractions: discard the precipitate after the addition of 6.3 g. $(NH_4)_2SO_4$. Add to the supernatant 378 mg. $(NH_4)_2SO_4$, collect the precipitate and dissolve in 2 ml. distilled water (subfraction B). Add to the supernatant 70 mg. $(NH_4)_2SO_4$, collect the precipitate and dissolve in 2 ml. distilled water (subfraction C). To the supernatant add 224 mg. $(NH_4)_2SO_4$, collect the precipitate and dissolve in 2 ml. distilled water (subfraction D).

Fraction II and subfraction D have the highest specific activity with about 190 and 120 units/mg. respectively.