D-Xylulose-5-phosphate

Efraim Racker

Principle

The determination of xylulose-5-phosphate is based on the following reactions[1]:

(1) Xylulose-5-phosphate + ribose-5-phosphate $\rightleftharpoons$ sedoheptulose-7-phosphate + glyceraldehyde-3-phosphate

(2) Glyceraldehyde-3-phosphate + $DPN^+ \xrightarrow{\text{arsenate}}$ 3-phosphoglycerate + DPNH + $2H^+$

Reaction (1) is catalysed by transketolase, reaction (2) is catalysed by glyceraldehyde-3-phosphate dehydrogenase. The glyceraldehyde-3-phosphate formed in the first reaction is quantitatively oxidized to 3-phosphoglycerate in the presence of arsenate, giving rise to one equivalent of reduced diphosphopyridine nucleotide (DPNH). With excess ribose-5-phosphate, which acts as acceptor aldehyde, the amount of DPNH formed is stoichiometric with the xylulose-5-phosphate present in the sample.

Reagents

1. Glycylglycine
2. Magnesium chloride, $MgCl_2 \cdot 6H_2O$
3. Ethylene-diamine-tetra-acetic acid, EDTA
   disodium salt, EDTA-$Na_2 \cdot 2H_2O$
4. Thiamine pyrophosphate
   commercial preparation, see p. 1028.
5. Sodium arsenate, $Na_3AsO_4 \cdot 12H_2O$
6. Trichloroacetic acid
7. Sodium hydrogen carbonate, $NaHCO_3$
8. Diphosphopyridine nucleotide, DPN
   free acid; commercial preparation, see p. 1010.
9. Ribose-5-phosphate, R-5-P
   barium salt; commercial preparation, see p. 1028.
10. Glyceraldehyde-3-phosphate dehydrogenase, GAPDH
    repeatedly recrystallized in the presence of EDTA[2]. Commercial preparation, see p. 979.
11. Transketolase
    crystalline, from baker's yeast. Preparation, see p. 204.

Purity of reagents and enzyme preparations

The ribose-5-phosphate must not give the carbazole reaction[4] for ketopentose phosphates. If present, ketopentose phosphates can be removed as follows[5]:

Mix

4.5 ml. 0.1 M ribose-5-phosphate solution and

0.5 ml. 10 N NaOH

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[1] *J. Cooper, P. A. Srere, M. Tabachnick* and *E. Racker*, Arch. Biochem. Biophysics *74*, 306 [1958].
[2] *I. Krimsky* and *E. Racker*, J. biol. Chemistry *198*, 721 [1952].
[3] *P. Srere, J. R. Cooper, M. Tabachnick* and *E. Racker*, Arch. Biochem. Biophysics *74*, 295 [1958].
[4] *Z. Dische* and *E. Borenfreund*, J. biol. Chemistry *192*, 583 [1951].
[5] *F. Dickens* and *D. H. Williamson*, Biochem. J. *64*, 567 [1956].

allow to stand for 10 min. at 25° C. Add

0.5 ml. 10 N HCl,

adjust to pH 6.5 and dilute the solution to the desired concentration.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) should be recrystallized 7 times in the presence of EDTA and must be essentially free of α-glycerophosphate dehydrogenase and triose phosphate isomerase. Neither glyceraldehyde-3-phosphate nor dihydroxyacetone phosphate should cause the disappearance of DPNH in the presence of 3.2 units GAPDH (= 0.05 ml. GAPDH solution).

Transketolase must be recrystallized[6] until it is essentially free of ribose-5-phosphate isomerase and xylulose-5-phosphate epimerase. Addition of ribulose-5-phosphate or ribose-5-phosphate to the test system should cause no reduction of DPN.

Preparation of Solutions

Prepare all solutions in doubly distilled water and, if necessary, neutralize before use. Thiamine pyrophosphate and $MgCl_2$ are used as a single solution. When many samples are to be analysed, a reagent mixture composed of the first 6 components of the assay system can be prepared (see "Procedure").

I. Glycylglycine buffer (0.25 M; pH 7.4):
Dissolve 3.30 g. glycylglycine in 70 ml. doubly distilled water, adjust to pH 7.4 (glass electrode) with *ca.* 21 ml. 0.2 N NaOH.

II. Magnesium chloride (0.3 M)/thiamine pyrophosphate (0.5% w/v):
Dissolve 610 mg. $MgCl_2 \cdot 6H_2O$ and 50 mg. thiamine pyrophosphate together in doubly distilled water and make up to 10 ml.

III. Sodium arsenate (0.09 M):
Dissolve 380 mg. $Na_3AsO_4 \cdot 12H_2O$ in doubly distilled water and make up to 10 ml.

IV. Trichloroacetic acid (10% w/v):
Dissolve 10 g. trichloroacetic acid in doubly distilled water and make up to 100 ml.

V. Sodium hydrogen carbonate (1 M):
Dissolve 8.4 g. $NaHCO_3$ in doubly distilled water and make up to 100 ml.

VI. Diphosphopyridine nucleotide (0.1 M β-DPN):
Dissolve 78 mg. DPN in doubly distilled water, adjust to pH 6.0 and make up to 1.0 ml.

VII. Ribose-5-phosphate (0.0015 M R-5-P):
Dissolve 54.83 mg. barium ribose-5-phosphate in doubly distilled water, remove barium ions with a cation exchanger or with $(NH_4)_2SO_4$ solution and make up eluate or filtrate to 100 ml. The amount of barium salt dissolved is equal to 34.5 mg. ribose-5-phosphate (free acid) if the salt is 100% pure. Ribose-5-phosphate must be free of ketopentose phosphates (cf. "Purity of reagents and enzyme preparations", p. 201).

VIII. Glyceraldehyde-3-phosphate dehydrogenase, GAPDH (*ca.* 2.5 mg. protein/ml. = 64 units*)/ml.):
Centrifuge stock suspension, dissolve crystalline precipitate in 0.002 M EDTA solution (pH 7.4) to give 64 units*)/ml.

*) A unit is defined as the amount of enzyme which converts 1 μmole of substrate in 1 minute[1]

[6] *G. de la Haba, I. Leder* and *E. Racker*, J. biol. Chemistry *214*, 409 [1955].

IX. Transketolase (*ca.* 0.5 mg. protein/ml. = 10 units*)/ml.):
Centrifuge the stock solution, dissolve crystalline precipitate in glycylglycine buffer (solution I) to give *ca.* 0.5 mg./ml.

Stability of solutions

In order to prevent bacterial growth store all solutions, with the exception of the two enzymes, at −20°C. Even at −20°C solutions of ribose-5-phosphate slowly form ketopentose phosphates; these should be removed as described above (see "Purity of reagents and enzyme preparations"). Ketopentose phosphate concentrations of less than 1% can be neglected. Store the enzymes at 2°C. GAPDH can be used for at least a year, even if the specific activity is reduced to one half. Transketolase is stable for several years. After storage for longer than one year, the enzyme frequently becomes insoluble but it is redissolved by the amounts of thiamine pyrophosphate/$MgCl_2$ solution used in the assay system.

Procedure

Deproteinization

To each ml. of the sample to be analysed for xylulose-5-phosphate add

1 ml. trichloroacetic acid solution (IV)

mix and centrifuge. Neutralize part of the supernatant with

$NaHCO_3$ solution (V).

A sample of the neutralized supernatant containing 0.01 to 0.08 μmoles xylulose-5-phosphate is taken for the assay.

Spectrophotometric measurements

Wavelength: 340 mμ; light path: 1 cm.; final volume: 1 ml.

Read optical density of the experimental cuvette against the control cuvette.

Into quartz micro-cuvettes pipette sufficient water to bring the final volume of the assay mixture to 1.00 ml. Then pipette into the experimental and control cuvettes:

0.10 ml. glycylglycine buffer (solution I)
0.02 ml. $MgCl_2$/thiamine pyrophosphate solution (II)
0.05 ml. sodium arsenate solution (III)
0.03 ml. DPN solution (VI), *add only to the experimental cuvette!*
0.10 ml. ribose-5-phosphate solution (VII)
0.05 ml. GAPDH solution (VIII); *in case any DPN is present in the sample only add the GAPDH to the experimental cuvette.*

Measure the optical density E_1 at 340 mμ. Add to both cuvettes

deproteinized sample,

read the optical density E_2 at 340 mμ. The change in optical density $E_2 - E_1$ is a measure of the glyceraldehyde-3-phosphate content of the sample. After completion of the reaction add

0.03 ml. transketolase solution (IX)

to both cuvettes and read the optical density E_3 at 340 mμ when the reaction has come to a stop.

*) A unit is defined as the amount of enzyme which converts 1 μmole of substrate in 1 minute[1])

Calculations

If no glyceraldehyde-3-phosphate is present in the sample, then $E_3 - E_2$ is a measure of the xylulose-5-phosphate content of the test mixture. DPNH is formed in the absence of transketolase when glyceraldehyde-3-phosphate is present in the sample. The addition of 0.03 ml. of transketolase results in a 3% decrease in the optical density E_2, therefore $E_3 - E_2$ must be corrected for this amount ΔE_{Tr}.

An increase in optical density of 6.22 corresponds to the reduction of 1 μmole DPN. If glyceraldehyde-3-phosphate is absent from the sample, the content of xylulose-5-phosphate is calculated from the formula:

$$\mu\text{moles xylulose-5-phosphate/ml. test mixture} = \frac{E_3 - E_2}{6.22}$$

If the sample contains glyceraldehyde-3-phosphate then:

$$\mu\text{moles xylulose-5-phosphate/ml. test mixture} = \frac{E_3 - E_2 + \Delta E_{Tr}}{6.22}$$

Sources of Error

Even after seven recrystallizations, GAPDH may still contain traces of lactic dehydrogenase (LDH), which reoxidizes DPNH in the presence of pyruvate. If the sample contains pyruvate, then GAPDH free from LDH (*e.g.* prepared from baker's yeast) should be used, otherwise low values for xylulose-5-phosphate will be obtained. Indications of interference in the assay are: lack of stability in the optical density after completion of the reaction and lack of proportionality between DPNH formation and volume of sample taken.

Appendix

Crystallization of transketolase[3,7]

The isolation and crystallization of the enzyme from baker's yeast includes the following steps: drying the yeast at room temperature (2 or 4–5 days; rapidly dried yeast has a lower initial activity, but the enzyme is brought more rapidly to a high specific activity). Incubation of the crude extract for 2.5 hours at 40° C. Acetone fractionation at −2° C and dialysis overnight. Heating for 15 min. at 55° C. Ethanol fractionation at −6° C. Chromatography on DEAE-cellulose in the cold. Fractionation with $(NH_4)_2SO_4$. The preparation obtained in this way is 10–20% pure. It can be crystallized by the addition of $(NH_4)_2SO_4$ to 50–60% saturation. Even at room temperature the crystal suspension is stable at pH 7.4 to 7.6.

[7] *G. de la Haba, I. G. Leder* and *E. Racker*, J. biol. Chemistry *214*, 409 [1955].