D-Xylulose and D-Xylose

Determination with D-Xylose Isomerase

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The cysteine-carbazole reaction[1], in conjunction with D-xylose isomerase[2], can be employed for the determination of D-xylose and D-xylulose. The enzyme is readily purified from extracts of *Lactobacillus plantarum*. A correction is necessary if the sample contains L-arabinose or L-ribulose, as even the best D-xylose isomerase preparations still have L-arabinose isomerase activity (see p. 178).

Principle

D-Xylose isomerase catalyses the reaction:

(1) D-Xylulose $\rightleftharpoons$ D-xylose

The equilibrium lies in favour of the aldopentose[3], since the equilibrium constant is 4.55 at 23°C. In the presence of excess isomerase 82% of the D-xylulose is converted to D-xylose. D-Xylose does not react in the cysteine-carbazole test. Therefore if the colour test is carried out before and after incubation with D-xylose isomerase the difference in colour intensities is equivalent to 82% of the D-xylulose present in the sample. The method is standardized with crystalline ribulose-*o*-nitrophenylhydrazone[4], which reacts quantitatively as ketopentose in the colour test. A similar procedure is used for the determination of D-xylose, except that the reaction is carried out in borate buffer at pH 8.2 instead of tris buffer pH 7.5 (at pH 8.2 the equilibrium of the reaction is in favour of the ketopentose). Crystalline D-xylose serves as a standard.

Reagents

1. Tris-hydroxymethyl-aminomethane, tris
2. Hydrochloric acid, A. R., 0.2 N
3. Cysteine hydrochloride
4. Carbazole, purified by sublimation
5. Sulphuric acid, conc., A. R.
6. Boric acid, H_3BO_3
7. Borax, $Na_2B_4O_{12} \cdot 10H_2O$
8. Perchloric acid, sp. gr. 1.54, 60% (w/w)
9. Amberlite, MB-3 or MB-4 *)
10. D-Xylose
    crystalline, $[\alpha]_D^{20} = +19°$, if necessary, recrystallize from 95% ethanol. Commercial preparation, see p. 1034.
11. L-Ribulose-*o*-nitrophenylhydrazone
    prepared according to[4].
12. Ethanol, absolute
13. D-Xylose isomerase
    preparation, see p. 199.

*) Mix-bed ion exchange resin from Rohm & Haas, Philadelphia, USA.

1) *Z. Dische* and *E. Borenfreund*, J. biol. Chemistry *192*, 583 [1951].
2) *S. Mitsuhashi* and *J. O. Lampen*, J. biol. Chemistry *204*, 1011 [1953].
3) *D. P. Burma* and *B. L. Horecker*, J. biol. Chemistry *231*, 1053 [1958].
4) *T. Reichstein*, Helv. chim. Acta *17*, 996 [1934].

Preparation of Solutions

I. Tris buffer (0.05 M; pH 7.5):
Dissolve 2.42 g. tris-hydroxymethyl-aminomethane in 100 ml. distilled water, adjust the pH to 7.5 with *ca.* 80 ml. 0.2 N HCl and dilute to 400 ml. with distilled water.

II. Cysteine hydrochloride (1.5% w/v):
Dissolve 1.5 g. cysteine hydrochloride in distilled water and make up to 100 ml.

III. Carbazole (0.12% w/v):
Dissolve 0.12 g. carbazole in ethanol and make up to 100 ml.

IV. Sulphuric acid:
Add 70 ml. conc. H_2SO_4 to 30 ml. distilled water.

V. Borate buffer (0.1 M; pH 8.2):
Dissolve 1.24 g. boric acid in 100 ml. distilled water, add 14.6 ml. borax solution (19.177 g. $Na_2B_4O_{10} \cdot 10H_2O$/100 ml.) and dilute the mixture to 200 ml. with distilled water.

VI. D-Xylose, standard solution (2×10^{-3} M):
Dissolve 30 mg. D-xylose in distilled water and make up to 100 ml.

VII. L-Ribulose-*o*-nitrophenylhydrazone, standard solution (2×10^{-3} M):
Dissolve 28.5 mg. L-ribulose-*o*-nitrophenylhydrazone in absolute ethanol and make up to 50 ml.

VIII. D-Xylose isomerase (1.8 mg. protein/ml.):
Dilute the enzyme solution prepared according to p. 199 with 0.05 M tris buffer (solution I).

Stability of the solutions

The enzyme solution should be stored at $-16°C$ and all other solutions should be kept in a refrigerator. If the tris buffer becomes turbid it should be filtered. The cysteine solution must be prepared freshly every two weeks.

Procedure

Experimental material

Deproteinize the sample by addition of 1/30th volume perchloric acid (60% w/w), centrifuge and deionize the supernatant by passing through a mixed-bed ion exchange resin (Amberlite MB-3 or MB-4). Concentrate dilute solutions *in vacuo* at 40°C. Solutions containing ketopentose must not be alkaline.

Standardization of the cysteine-carbazole reaction

Pipette into a test tube:

0.95 ml. distilled water
0.05 ml. solution VII (containing 0.1 μmoles L-ribulose-*o*-nitrophenylhydrazone)
6 ml. H_2SO_4 (solution IV)
0.2 ml. cysteine solution (II)
0.2 ml. carbazole solution (III).

Mix thoroughly after each addition. Allow the mixture to stand 1 hour at room temperature and then read the optical density at 540 mμ ($E_{Standard}$) in a 1 cm. cuvette.

Determination of D-xylulose

Pipette into a small test tube with a conical tip:

0.30 ml. tris buffer (solution I)
0.04 ml. sample (containing about 2 μmoles D-xylulose)

Mix, remove 0.05 ml. and add

0.01 ml. D-xylose isomerase solution (VIII)

to the remainder of the mixture. Incubate at 23°C and remove 0.05 ml. samples at 20 min. intervals. Add to these 0.05 ml. samples (S_0, S_1, S_2 .....)

0.95 ml. distilled water
6 ml. H_2SO_4 (solution IV)
0.2 ml. cysteine solution (II)
0.2 ml. carbazole solution (III),

mix thoroughly after the addition of each reagent. Allow the mixtures to stand 1 hour at room temperature, then pour into 1 cm. cuvettes and read the optical densities (E_0, E_1, E_2..... E_{Final}) at 540 mμ. All samples taken after 60 min. should have the same optical density (E_{Final}).

Calculations

The D-xylulose content is calculated according to the formula:

$$\frac{E_0 - E_{Final}}{E_{Standard}} \times 0.10 \times \frac{0.35}{0.05} \times 1.22 = \text{μmoles D-xylulose/enzymatic incubation mixture}$$

The factor 0.1 allows for the cysteine-carbazole reaction being standardized with 0.1 μmole L-ribulose-*o*-nitrophenylhydrazone. The factor $\frac{0.35}{0.05}$ is to correct for the portion of the enzymatic incubation mixture taken for the colour test, while the factor 1.22 is to correct for the fact that only 82% of the D-xylulose is converted to D-xylose.

Determination of D-xylose

Pipette the following solutions into three test tubes:

	Experimental	*Control*	*Standard*
borate buffer (solution V)	0.15 ml.	0.15 ml.	0.15 ml.
sample (containing about 0.1 μmoles D-xylose)	0.05 ml.	0.05 ml.	—
D-xylose solution (VI, corresponding to 0.1 μmoles D-xylose)	—	—	0.05 ml.
enzyme solution (VIII)	0.01 ml.	—	0.01 ml.
distilled water	—	0.01 ml.	—

Mix and incubate 1 hour at 37°C. To all three tubes add

0.8 ml. distilled water
6 ml. H_2SO_4 (solution IV)
0.2 ml. cysteine solution (II)
0.2 ml. carbazole solution (III),

mix thoroughly after the addition of each reagent. Allow the tubes to stand 2 hours at room temperature and then read the optical density at 540 mμ.

Calculations

The D-xylose content of the experimental tube is calculated according to the formula:

$$\frac{E_E - E_C}{E_S - E_C} \times 0.1 = \mu\text{moles D-xylose/experimental tube}$$

where

E_E = optical density of experimental tube
E_C = optical density of control tube
E_S = optical density of standard tube

Sources of Error

The method cannot be used for the determination of D-xylose if L-arabinose is present, nor for the determination of D-xylulose in the presence of L-ribulose, since the enzyme preparation contains some L-arabinose isomerase. In such cases, the sample must first be treated with L-arabinose isomerase and then with D-xylose isomerase when the first reaction is complete. In this way the same reaction mixture can be used for the successive determination of L-arabinose and D-xylose or of L-ribulose and D-xylulose (determination of L-arabinose and L-ribulose, see p. 178).

The values found for D-xylulose are too low if substantial amounts of D-xylose are present (−19% if D-xylulose : D-xylose is 1 : 1), because the equilibrium of the xylose isomerase is displaced.

Specificity

The method is specific for D-xylulose and D-xylose providing that the sample does not contain L-arabinose or L-ribulose.

Appendix

Preparation of D-Xylose Isomerase

Reagents

Difco yeast extract *)
Difco nutrient broth *)
Sodium acetate
D-Xylose
Glucose
Magnesium sulphate, $MgSO_4 \cdot 7H_2O$
Sodium chloride
Ferrous sulphate, $FeSO_4 \cdot 7H_2O$
Manganous sulphate, $MnSO_4 \cdot 4H_2O$
Sodium hydrogen carbonate, $NaHCO_3$
Ammonium sulphate
Tris-hydroxymethyl-aminomethane, tris

Preparation of solutions

I. Sodium hydrogen carbonate (0.02 M): Dissolve 1.68 g. $NaHCO_3$ in distilled water and make up to 1000 ml.

II. Manganous sulphate (1 M): Dissolve 22.3 g. $MnSO_4 \cdot 4H_2O$ in distilled water and make up to 100 ml.

III. Tris buffer (0.05 M; pH 7.5): Dissolve 2.42 g. tris-hydroxymethyl-aminomethane in 100 ml. distilled water, adjust pH to 7.5 with *ca.* 80 ml. 0.2 N HCl and dilute with distilled water to 400 ml.

Procedure

Strain of bacteria: Lactobacillus plantarum, strain 124−2 (ATCC 8041).

Growth medium: Contains 0.4% yeast extract; 1% nutrient broth; 1% sodium acetate; 1% D-xylose; 0.1% glucose; 0.02% $MgSO_4 \cdot 7H_2O$; 0.001% NaCl; 0.001% $FeSO_4 \cdot 7H_2O$ and 0.001% $MnSO_4 \cdot 4H_2O$. For stab cultures use the same medium containing 2% agar. Sterilize the sugars separately as a 20 times more concentrated solution and add aseptically to the rest of the sterile medium.

*) from Difco Laboratories, Inc., Detroit 1, Mich., USA.

Culture of bacteria: Maintain the bacteria in stab culture and transfer frequently. Prepare successive subcultures of 2, 10 and 100 ml. of medium, incubate for 24 hours at 37° C each time and inoculate each successive subculture with the whole of the previous one. Add the final subculture to 3 l. of medium in a 3 l. flask. Incubate for 18—24 hours at 37° C without aeration until a fine sediment of cells settles out. Harvest the cells at 2° C with a Sharples supercentrifuge and wash with about 100 ml. $NaHCO_3$ solution (I). The cell paste can be stored for months at −16° C.

Preparation of the extract: Suspend 1 g. of cells (fresh weight) in 4 ml. $NaHCO_3$ solution (I) and extract in a *Nossal* shaker[5] with 4 g. glass beads (Superbrite). Shake 3 times for 30 sec., remove the container each time and cool in ice. Dilute the suspension with 3 ml. $NaHCO_3$ solution (I) and centrifuge at 15000 g. Keep the supernatant, suspend the precipitate in 3 ml. $NaHCO_3$ solution (I) and recentrifuge. Discard the precipitate. Add 5.5 ml. $MnSO_4$ solution (II) to the combined supernatants and allow to stand for 30 min. at 0° C. Then centrifuge and discard the precipitate. Add 4.35 g. $(NH_4)_2SO_4$ to the supernatant (10 ml.) and centrifuge. Dissolve the precipitate in 2 ml. tris buffer (solution III) and store at −16° C.

[5] *P. M. Nossal*, Australian J. exp. Biol. med. Sci. *31*, 583 [1953].