β-Mercaptopyruvate

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Principle

β-Mercaptopyruvate is split by transsulphurase according to equation (1) to give sulphur and pyruvate[1–3]. The latter is determined colorimetrically as the 2,4-dinitrophenylhydrazone.

(1) β-SH-Pyruvate $\xrightarrow{\text{transsulphurase}}$ pyruvate + sulphur

(2) Pyruvate + O_2N–C$_6$H$_3$(–NO_2)–$NHNH_2$ ⟶ pyruvate-2,4-dinitrophenylhydrazone

It is not possible to determine the pyruvate formed enzymatically with lactic dehydrogenase (LDH, see p. 253) because β-mercaptopyruvate also reacts with LDH[4]. Theoretically any residual mercaptopyruvate could be removed with cadmium acetate, but excess Cd^{2+} inhibits LDH.

Reagents

1. Potassium dihydrogen phosphate, KH_2PO_4, A. R.
2. Cadmium acetate·$3H_2O$, A. R.
3. 2-Mercaptoethanol, A. R.
4. 2,4-Dinitrophenylhydrazine
5. Hydrochloric acid, 2 N
6. Toluene, A. R.
7. Sodium carbonate, $Na_2CO_3 \cdot 10H_2O$
8. Sodium hydroxide, 1.5 N
9. Sodium pyruvate*)
   crystalline, prepared according to[5]; commercial preparation, see p. 1027.
10. Transsulphurase
    isolation, see p. 265.

Purity of the enzyme preparation

A preparation having a specific activity of 40–50 units**)/mg. is satisfactory. The contaminating enzyme activities do not interfere with the determination.

Preparation of Solutions

I. Phosphate buffer (0.02 M; pH 7.45):
Dissolve 0.348 g. K_2HPO_4 in distilled water, adjust to pH 7.45 with 2 N HCl and make up to 100 ml.

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*) The purity of the pyruvate preparation must be determined (Pyruvate Determination, see p. 253).

**) A preparation has a specific activity of 1 unit, when 1 mg. forms 1 μmole pyruvate from β-mercaptopyruvate at 30° C in 10 min., under the conditions stated in this chapter. The reaction is linear for the first 20 min. and the rate is proportional to the amount of enzyme.

1) *E. Kun* and *D. W. Fanshier*, Biochim. biophysica Acta *27*, 659 [1958].
2) *E. Kun* and *D. W. Fanshier*, Biochim. biophysica Acta *32*, 338 [1959].
3) *E. Kun* and *D. W. Fanshier*, Biochim. biophysica Acta *33*, 26 [1959].
4) *E. Kun*, Biochim. biophysica Acta *25*, 135 [1957].
5) Biochemical Preparations. Wiley, New York 1952, Vol. II, p. 22.

II. Cadmium acetate solution (saturated):
Dissolve *ca.* 160 g. cadmium acetate·$3H_2O$ in *ca.* 100 ml. hot distilled water, allow to cool and use the supernatant.

III. 2-Mercaptoethanol:
Use undiluted.

IV. 2,4-Dinitrophenylhydrazine solution (0.1%):
Dissolve 100 mg. 2,4-dinitrophenylhydrazine in 100 ml. 2 N HCl. Keep in a dark bottle at 4°C.

V. Na_2CO_3 solution (10% w/v Na_2CO_3):
Dissolve 27 g. $Na_2CO_3 \cdot 10H_2O$ in distilled water and make up to 100 ml.

VI. Sodium hydroxide (1.5 N):
Dissolve 6 g. NaOH in distilled water and make up to 100 ml.

VII. Pyruvate standard solution (0.02 M):
Dissolve 22 mg. (or more according to the purity of the preparation) sodium pyruvate in distilled water and make up to 10 ml.

VIII. Transsulphurase solution (*ca.* 10 mg. protein/ml.):
Dissolve 10 mg. dry preparation in 1 ml. phosphate buffer (solution I).

Stability of the solutions

Keep NaOH and Na_2CO_3 solutions well stoppered. Prevent bacterial growth in the phosphate buffer by storing at 4°C. Prepare the 2,4-dinitrophenylhydrazine solution freshly each week and store at 4°C. The enzyme solution keeps at −15°C for 3−6 weeks.

Procedure

Experimental material

β-Mercaptopyruvate is a labile compound. For preparation of the ammonium salt, see[4, 6]. This salt is stable indefinitely in a desiccator at 4°C.

Pyruvate standard curve

Prepare two standards of 10 and 20 μmoles pyruvate:

0.5 ml. solution VII + 0.5 ml. distilled water or
1.0 ml. solution VII.

Add

2.0 ml. 2,4-dinitrophenylhydrazine solution (IV)

and proceed as described under "Spectrophotometric measurements". Plot a standard curve with the values (optical density at 520 mμ against amount of pyruvate).

Incubation mixture

A reagent blank with water instead of enzyme solution is prepared for each determation. Pipette successively into a test tube standing in ice-water:

1.5 ml. phosphate buffer (solution I)
0.5 ml. sample (50 μmoles freshly dissolved β-mercaptopyruvate)
0.1 ml. 2-mercaptoethanol (solution III)
0.3 ml. distilled water
0.1 ml. transsulphurase solution (VIII).

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[6] *W. D. Kumler* and *E. Kun*, Biochim. biophysica Acta *27*, 464 [1957].

Start the reaction by placing the tubes in a water bath at 30° C. After 10 min. stop the reaction by the addition of

1 ml. saturated cadmium acetate solution (II).

Shake vigorously, allow to stand 10 min. at room temperature, centrifuge (3000 r.p.m.) and decant supernatant fluid from the precipitate.

Spectrophotometric measurements

Into a clean test-tube pipette:

1 ml. supernatant

2 ml. 2,4-dinitrophenylhydrazine solution (IV).

Incubate for 20 min. at 30° C. Extract the pyruvate dinitrophenylhydrazone with

3 ml. toluene

in a small separating funnel; extract the toluene layer with

5 ml. carbonate solution (V)

and filter the carbonate extract. For colorimetric measurements pipette into a clean test-tube:

3 ml. carbonate extract

5 ml. 1.5 N NaOH (solution VI).

After 5 min. measure the optical density at 520 mμ.

Calculations

Obtain from the standard curve the pyruvate value corresponding to the measured optical density. According to equation (1) 1 μmole pyruvate corresponds to 1 μmole β-mercaptopyruvate. To correct for the dilution of the 0.5 ml. sample in the above procedure the pyruvate value must be multiplied by 7 to obtain μmoles β-mercaptopyruvate/ml. sample.

Sources of Error

Transsulphurase is the most specific reagent for β-mercaptopyruvate; K_s for β-mercaptopyruvate [2] is 2.7×10^{-3}. Lactic dehydrogenase (LDH) reduces β-mercaptopyruvate in the presence of DPNH to β-mercaptolactate. K_s of LDH from heart muscle for β-mercaptopyruvate is 8.2×10^{-4} compared to 5.4×10^{-5} with pyruvate[4]. Pyruvic carboxylase decarboxylates β-mercaptopyruvate to give CO_2 and mercaptoacetaldehyde[7].

Appendix

Isolation of transsulphurase [1-3]

The preparation of the enzyme includes the following steps: preparation of an acetone powder from rat liver (stable for 3—5 months at −30° C); extraction of the dry powder with phosphate buffer; fractionation with ammonium sulphate; lyophilization of the protein precipitated between 0.2 and 0.4 g. $(NH_4)_2SO_4$/ml. The lyophilized preparation is stable for 3—5 months at −30° C. Its specific activity is 40—50 units (for definition, see footnote on p. 263). The enzyme is highly sensitive to the inhibitory action of metal chelating agents[8]. For further properties see[9].

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[7] *E. Kun* and *H. G. Williams-Ashman*, Experientia *18*, 261 [1962].

[8] *E. Kun* and *D. W. Fanshier*, Biochim. Biophysica Acta *48*, 187 [1961].

[9] *D. W. Fanshier* and *E. Kun*, Biochim. Biophysica Acta *58*, 266 [1962].