L-Alanine

Determination with Glutamate-Pyruvate Transaminase and Lactic Dehydrogenase

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Principle

L-Alanine is converted to pyruvate by glutamate-pyruvate transaminase (GPT) and α-oxoglutarate:

(1) α-Oxoglutarate + L-alanine $\overset{GPT}{\rightleftharpoons}$ L-glutamate + pyruvate

Lactic dehydrogenase (LDH) reduces pyruvate in the presence of reduced diphosphopyridine nucleotide (DPNH) to lactic acid:

(2) Pyruvate + DPNH + H^+ $\overset{LDH}{\rightleftharpoons}$ L-lactate + DPN^+

The disappearance of DPNH can be followed spectrophotometrically at 340 or 366 mμ. The equilibrium of the indicator reaction (2) is far to the right ($K = 7 \times 10^4$ l./mole at pH 7 and 25° C). However, a quantitative conversion of alanine to pyruvate is not possible because the Michaelis constant of the transaminase is too high. With excess of both enzymes and DPNH the rate of the coupled reaction with limited alanine concentrations is strictly proportional to the amount of alanine added. Measurement of the reaction rate permits the determination of alanine by use of a standard curve prepared with known alanine concentrations[1] (see "Kinetic methods", p. 6).

Reagents

1. Potassium dihydrogen phosphate, KH_2PO_4, A. R.
2. Disodium hydrogen phosphate, $Na_2HPO_4 \cdot 2H_2O$, A. R.
3. Sodium hydroxide, A. R., 2 N
4. α-Oxoglutarate
5. L-Alanine
6. Reduced diphosphopyridine nucleotide, DPNH
   disodium salt, DPNH-Na_2; commercial preparation, see p. 1011.
7. Glutamate-pyruvate transaminase, GPT
   from pig heart, suspension in 1.6 M ammonium sulphate. Commercial preparation, see p. 977.
8. Lactic dehydrogenase, LDH
   crystalline, from skeletal muscle, suspension in 2.2 M ammonium sulphate solution. Commercial preparation, see p. 986.

Purity of the enzyme preparations

Both enzymes must be as free as possible from glutamic dehydrogenase, otherwise DPNH will be oxidized by the high concentrations of α-oxoglutarate used. Commercially available LDH *) is sufficiently pure. GPT can be obtained in a few steps from pig heart[2] and is sufficiently pure for this method.

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*) From C. F. Boehringer & Soehne GmbH, Mannheim (Germany).

1) *G. Pfleiderer, L. Grein* and *Th. Wieland*, Ann. Acad. Sci. fennicae, Ser. A II, *60*, 381 [1955].

2) *L. Grein* and *G. Pfleiderer*, Biochem. Z. *330*, 433, [1955].

Preparation of Solutions

I. Phosphate buffer (M/15; pH 7.2):
a) Dissolve 11.876 g. $Na_2HPO_4 \cdot 2H_2O$ in doubly distilled water and make up to 1000 ml.
b) Dissolve 9.078 g. KH_2PO_4 in doubly distilled water and make up to 1000 ml.
Mix solutions a) and b) in the ratio of 72 : 28 volumes.

II. α-Oxoglutarate (0.1 M):
Dissolve 1.46 g. α-oxoglutaric acid in *ca.* 50 ml. doubly distilled water, neutralize with 2 N NaOH and dilute with doubly distilled water to 100 ml.

III. Reduced diphosphopyridine nucleotide (*ca.* 1.2×10^{-2} M β-DPNH):
Dissolve 50 mg. DPNH-Na_2 in 5 ml. doubly distilled water.

IV. Alanine standard solution (2 mg./ml.):
Dissolve 20 mg. L-alanine in doubly distilled water and make up to 10 ml.

V. Lactic dehydrogenase, LDH (*ca.* 1 mg. protein/ml.):
Dilute the crystalline suspension with 2.2 M ammonium sulphate solution.

VI. Glutamate-pyruvate transaminase, GPT (*ca.* 10 mg. protein/ml.):
Use the preparation obtained according to[2] without dilution. Dilute the commercial preparation with 1.6 M ammonium sulphate solution.

Stability of the solutions

Solutions I–IV may be stored, well stoppered, for *ca.* 14 days in a refrigerator or deep-freeze, but it is advisable to prepare a fresh alanine standard solution before starting a large series of measurements. At 0°C the LDH suspension is stable for several months with virtually no loss of activity; the GPT suspension is stable for 6–8 weeks at 0–4°C. Higher ammonium sulphate concentrations are to be avoided with GPT, since on standing, its prosthetic group, pyridoxal phosphate, is slowly split off and the enzyme is irreversibly inactivated.

Procedure

Preliminary treatment of the experimental material

Tissue extracts: Deproteinize tissue extracts by heating for 3 min. in a boiling water bath and centrifuge off coagulated protein. If the alanine content of the sample is very low, freeze-dry the supernatant and re-dissolve the residue.

Protein analysis: Hydrolyse protein by heating for 15 to 72 hours with 5 N HCl at 110°C. Free from excess HCl on a water bath or in a vacuum desiccator over conc. H_2SO_4 and KOH. Take up the residue in a little water and repeat evaporation process. Dissolve the residue in water, neutralize with 2 N NaOH and dilute to a known volume.

Standard curve

A standard curve should be prepared for each series of measurements. Take portions of the L-alanine standard solution (IV) (0.04 to 0.20 ml., corresponding to 80 to 400 μg. L-alanine) and measure the ΔE/min. under the test conditions described below. The rates, corrected if necessary, are plotted graphically, ΔE/min. (ordinate) versus μg. L-alanine (abscissa). The standard curve should pass through the origin (see under "Sources of error" p. 380).

Spectrophotometric measurements

Wavelength: 340 or 366 mμ; light path: 1 cm.; final volume: 4.0 ml. Room temperature must be constant for a series of measurements. Prepare two determinations with different amounts of sample.

Pipette successively into the cuvettes:

0.20 ml. α-oxoglutarate solution (II)
0.10 to 0.20 ml. alanine standard solution (IV) or pre-treated sample
0.06 ml. DPNH solution (III)
0.01 ml. LDH suspension (V)
buffer (solution I) to 3.96 ml.

Mix, observe for several minutes any small change in optical density (ΔE_1/min.) which may occur. By mixing in

0.04 ml. GPT suspension (VI)

start the transaminase reaction. Take readings of the decrease in optical density at 60 second intervals for about 5 min. (ΔE_2/min.).

Calculations

Both the transaminase reaction and any reaction before addition of transaminase are linear with time. Therefore the values for the rates can be averaged. The corrected rate of the transaminase reaction is ΔE_2/min. $-\Delta E_1$/min. $= \Delta E$/min. These values are used to prepare the standard curve if known amounts of alanine have been added, or to obtain the alanine concentration of unknown samples from the standard curve.

Sources of Error

If a blank value occurs due to oxidation of DPNH by impurities the standard curve will not pass through the origin. In such cases, to obtain the correct standard curve, a parallel line is drawn through the zero point.

With protein hydrolysates containing very small amounts of alanine it is possible that the large excess of other amino acids may competitively inhibit the transaminase reaction. This inhibition can be corrected for by measuring alanine standards in the presence of a constant amount of the sample solution and relating the increase in reaction rate obtained to the amount of alanine added[3]. Also refer to the chapter on "Pyridoxal Phosphate", p. 606.

Specificity

Only L-alanine, not the D isomer, reacts under the conditions described here. In moderate excess, other amino acids neither react nor inhibit. α-Aminobutyric acid which can also react with GPT causes no additional oxidation of DPNH even in large excess.

[3] *L. Grein*, Ph. D.-Thesis, Universität Frankfurt/Main 1955.