DL-Threonine

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Principle

Threonine is oxidized by periodate at neutral pH to acetaldehyde and glyoxylate, the latter undergoing further oxidation within a few minutes to formate and CO_2. After destruction of the excess periodate with a mercaptan, the acetaldehyde is determined with alcohol dehydrogenase (ADH) and reduced diphosphopyridine nucleotide (DPNH)[1]. The decrease in the optical density at 340 mμ due to oxidation of DPNH is a measure of the reaction. The method is more rapid than previous specific methods for the determination of threonine, which involve the diffusion or aeration of acetaldehyde into trapping agents[2-4].

Reagents

1. Potassium dihydrogen phosphate, KH_2PO_4
2. Sodium metaperiodate, $NaIO_4$, A. R.
3. Potassium pyrophosphate, $K_4P_2O_7 \cdot 3H_2O$
4. Potassium hydroxide, 10 N
5. Hydrochloric acid, 1 N
6. Bovine serum albumin
7. Glutathione, GSH
   commercial preparation, see p. 1018.
8. 3-Mercaptopropionic acid *)
9. Reduced diphosphopyridine nucleotide, DPNH
   disodium salt, DPNH-Na_2; commercial preparation, see p. 1011.
10. DL-Threonine
11. Alcohol dehydrogenase, ADH
    crystalline, from yeast. Commercial preparation, see p. 969.

    Purity of the enzyme preparation
    A preparation containing 40000 units[5]/mg. protein satisfies the requirements.

Preparation of Solutions

I. Phosphate buffer (1.0 M; pH 7.5):
   Dissolve 136 g. KH_2PO_4 in 750 ml. distilled water, adjust pH to 7.5 with 10 N KOH and dilute to 1000 ml. with distilled water.

II. Sodium metaperiodate (4% w/v):
   Dissolve 4 g. $NaIO_4$ in distilled water and make up to 100 ml.

III. Threonine standard solution (10^{-3} M):
   Dissolve 11.9 mg. DL-threonine in distilled water and make up to 100 ml.

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*) *e.g.* Eastman Kodak No. 6270.

1) *M. Flavin* and *C. Slaughter*, Analytic. Chem. *31*, 1983 [1959].
2) *A. J. P. Martin* and *R. L. M. Synge*, Biochem. J. *35*, 294 [1941].
3) *B. A. Neidig* and *W. C. Hess*, Analytic. Chem. *24*, 1627 [1952].
4) *G. W. Gaffney*, *W. A. Williams* and *H. McKennis jr.*, Analytic. Chem. *26*, 588 [1954].
5) *E. Racker* in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1955, Vol. I., p. 500.

IV. 3-Mercaptopropionate (10% v/v; p 6):
Dissolve 1 ml. 3-mercaptopropionic acid in a little distilled water, adjust to pH 6 with 10 N KOH and dilute to 10 ml. with distilled water.

V. Reduced diphosphopyridine nucleotide (2.5×10^{-3} M β-DPNH):
Dissolve 7 mg. DPNH-Na_2 in distilled water and make up to 3 ml.

VI. Alcohol dehydrogenase, ADH (0.6 mg. enzyme protein/ml.):
Dilute the suspension in ammonium sulphate solution with a solution containing: 0.1 g. bovine serum albumin, 0.307 g. glutathione and 0.769 g. potassium pyrophosphate ($K_4P_2O_7 \cdot 3H_2O$) per 100 ml. Adjust the pH of this solution to 7.5 with 1 N HCl.

Stability of the solutions

Store solutions III to VI frozen at −16° C. Prepare solutions IV and V freshly each week. Store the suspension of the enzyme in ammonium sulphate solution at −16° C. The diluted enzyme solution keeps for a week at −16° C.

Procedure

Experimental material

The method described here was developed for the assay of threonine synthetase activity[6]. Preliminary experiments showed that it was also suitable for the estimation of threonine in protein hydrolysates[1]. The acid hydrolysate from about 0.1 mg. protein should be neutralized and diluted for the assay.

Spectrophotometric measurements

Preliminary remarks: The pH of the assay mixture is a compromise between the optimal pH values for the periodate oxidation and the ADH reaction. The choice of 3-mercaptopropionate as reducing agent is arbitrary. Other mercaptans are probably equally suitable providing they do not interfere with the ADH reaction.

Method: Wavelength: 340 mμ; light path: 1 cm.; final volume: 1 ml.; room temperature (*ca.* 25° C); measure against water. Three cuvettes can be measured successively.

Pipette into the cuvettes:

sample (containing 0.02 to 0.1 μmoles threonine)
0.10 ml. phosphate buffer (solution I)
distilled water to 0.87 ml.
0.02 ml. periodate solution (II).

Mix well, allow to react for 30 sec., mix in

0.03 ml. mercaptopropionate solution (IV)

and stir for 30 sec. Stir in

0.05 ml. DPNH solution (V)

and read the optical density E_1 twice. If reduction of the periodate is complete the optical density does not decrease. Mix in

0.03 ml. ADH solution*) (VI)

and read the optical density at 30 sec. intervals until no further change occurs (final value: E_2).

*) Use only sufficient enzyme to complete the reaction in 1 to 3 minutes. Larger amounts decrease the specificity of the analysis[5].

[6] *M. Flavin* and *C. Slaughter*, J. biol. Chemistry *235*, 1103 [1960].

Standard: Instead of the sample add 0.02—0.1 ml. DL-threonine standard solution (III), corresponding to 0.02—0.10 μmoles DL-threonine.

Calculations

The decrease in optical density at 340 mμ, after correction for the dilution of the assay mixture on addition of enzyme, is proportional to the amount of threonine between 0.01 and 0.1 μmoles/assay mixture. Nevertheless, for reasons which are not at present known, calculations by the usual formula:

$$\frac{0.97 \times E_1 - E_2}{6.22} = \mu\text{moles threonine/assay mixture}$$

where
0.97 = correction for the dilution of the assay mixture on addition of the enzyme
6.22 = extinction coefficient of DPNH at 340 mμ [cm.^{2}/μmole],

give values which are 33 to 58% too high. Therefore the value found for the sample, $\Delta E_{sam.} = E_1 - E_2$ (uncorrected), is related to the value found for the standard solution, $\Delta E_{stan.}$ So using 0.10 ml. DL-threonine standard solution (III), corresponding to 0.10 μmoles DL-threonine:

$$\frac{\Delta E_{sam.}}{\Delta E_{stan.}} \times 0.1 = \mu\text{moles threonine/assay mixture.}$$

Sources of Error

Protein hydrolysates occasionally contain impurities, which react with periodate without yielding acetaldehyde[1]. In such cases the periodate and mercaptan concentrations in the assay mixture can be doubled.

Specificity

Compounds, such as serine, which react with periodate to yield formaldehyde do not interfere[1]. Some long-chain aldehydes oxidize DPNH in the presence of alcohol dehydrogenase, but the reaction is much slower than with acetaldehyde[5]. Such interference can be minimized by the use of just sufficient enzyme to bring the reaction to completion in 1 to 3 minutes.

Only very few naturally occurring compounds react rapidly with periodate at neutral pH to yield acetaldehyde[7]. Compounds, such as butyleneglycol and acetaldehyde itself are usually removed during the preparation of the sample (heating, dialysis, precipitation). Interference may occur if polymers are present, which on hydrolysis yield sugars (*e.g.* methyl pentoses, 6-deoxyglucose, fucose, rhamnose). The absence of such sugars from the protein hydrolysate must be checked. Alternatively, the method described here should also be suitable for the determination of methyl pentoses.

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[7] *C. G. Huggins* and *O. N. Miller*, J. biol. Chemistry *221*, 377 [1956].