Pyrophosphate

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All inorganic pyrophosphatases so far described require Mg^{2+} for their action. According to[1] the actual substrate of the enzyme is $[MgP_2O_7]^{2-}$. For further details of the mechanism of the reaction, see[2]. Inorganic pyrophosphatases have been found in yeast[3,4], rat brain[5], erythrocytes[1,6,7], insect muscle[8] and potatoes[9].

Principle

Inorganic pyrophosphatase catalyses the reaction:

(1) $$P_2O_7^{4-} + H_2O \xrightarrow[Mg^{2+}]{\text{pyrophosphatase}} 2\,HPO_4^{2-}$$

The orthophosphate formed is determined colorimetrically by the method of *Fiske* and *Subbarow*. Inorganic pyrophosphatase from yeast[3] has a very high affinity for its substrate (Michaelis constant *ca.* 3×10^{-5} M) and the pH optimum is 7.

Reagents

1. Potassium dihydrogen phosphate, KH_2PO_4
2. Imidazole
3. Magnesium chloride, $MgCl_2 \cdot 6\,H_2O$
4. Hydrochloric acid, A. R., 1 N
5. Sodium pyrophosphate, $Na_4P_2O_7 \cdot 10\,H_2O$
6. Ammonium molybdate, $(NH_4)_6Mo_7O_{24} \cdot 4\,H_2O$
7. Sulphuric acid, A. R., 10 N and 1 N
8. Ferrous sulphate, $FeSO_4 \cdot 7\,H_2O$
9. Inorganic pyrophosphatase

   prepared from yeast according to[3]. For a short description, see Appendix, p. 647.

   Purity of the enzyme preparation

   The enzyme preparation obtained according to[3] (p. 647) satisfies the requirements. It still contains many of the glycolytic enzymes (certainly triosephosphate dehydrogenase), but these contaminants do not interfere. The preparation is free from ATPase.

Preparation of Solutions

I. Imidazole buffer (0.05 M; pH 7.3):
Dissolve 0.27 g. imidazole in distilled water, add 1.6 ml. 1 N HCl and make up to 80 ml.

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1) *L. Block-Frankenthal*, Biochem. J. *57*, 87 [1954].
2) *M. Cohn*, J. biol. Chemistry *230*, 369 [1958].
3) *K. Bailey* and *E. C. Webb*, Biochem. J. *38*, 394 [1944].
4) *M. Kunitz*, J. gen. Physiol. *35*, 423 [1952].
5) *B. Naganna* and *V. K. N. Menon*, J. biol. Chemistry *174*, 501 [1948].
6) *A. E. Robbins*, *M. P. Stulberg* and *P. D. Boyer*, Arch. Biochem. Biophysics *54*, 215 [1955].
7) *A. Malkin* and *O. F. Denstedt*, Canad. J. Biochem. Biophysics *34*, 121 [1956].
8) *D. Gilmour* and *J. H. Calaby*, Enzymologia *16*, 34 [1953].
9) *B. Naganna*, *A. Raman*, *B. Venugopal* and *C. E. Sripathi*, Biochem. J. *60*, 215 [1955].

II. Magnesium chloride (*ca.* 0.02 M):
Dissolve 0.41 g. $MgCl_2 \cdot 6\ H_2O$ in distilled water and make up to 100 ml.

III. Pyrophosphate standard solution (0.01 M; pH 7.3):
Dissolve 2.23 g. $Na_4P_2O_7 \cdot 10\ H_2O$ in 400 ml. distilled water, adjust to pH 7.3 with 4.7 ml. 1 N HCl and dilute to 500 ml. with distilled water.

IV. Phosphate standard solution (1.161×10^{-3} M or 50 μg. P/ml.):
Dissolve 109.5 mg. KH_2PO_4 in distilled water and make up to 500 ml.

V. Molybdate (*ca.* 0.02 M):
Dissolve 25 g. $(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$ in 200 ml. distilled water, carefully add 500 ml. 10 N H_2SO_4 and dilute to 1000 ml. with distilled water.

VI. Ferrous sulphate (*ca.* 0.36 M):
Dissolve 5 g. $FeSO_4 \cdot 7\ H_2O$ in 30 ml. distilled water, add 1 ml. 1 N H_2SO_4 and dilute to 50 ml. with distilled water. Prepare the solution freshly each day.

VII. Pyrophosphatase (25—75 mg. protein/100 ml.):
Dissolve the requisite amount of enzyme in 25 ml. imidazole buffer (solution I) and dilute to 100 ml. with distilled water.

Stability of the solutions

All the solutions, except VI and VII, keep for several weeks, if well stoppered. Prepare the ferrous sulphate solution (VI) and the enzyme solution (VII) freshly each day. Solutions of the enzyme in imidazole buffer (*i.e.* not diluted with distilled water) keep for several weeks at 0° C.

Procedure

Experimental material and deproteinization

In the analysis of pure pyrophosphate solutions it is sufficient to inactivate the enzyme by the addition of the acid molybdate solution. The denatured protein is left in the mixture, since it does not interfere with the colour reaction. If the sample contains much protein, it must be deproteinized with trichloroacetic acid and the protein precipitate removed; molybdate forms a protein-molybdate-phosphate complex which interferes. If the sample contains acid-labile phosphate, another colour reaction for phosphate must be chosen (see "Phosphate determination").

Enzymatic reaction

For each series of measurements prepare a blank containing buffer instead of enzyme solution.

Pipette into test tubes (1.5 cm. diameter, 12 cm. long):

2.00 ml. buffer (solution I)
0.20 ml. sample (containing *ca.* 2 μmoles pyrophosphate)
0.02 ml. $MgCl_2$ solution (II).

Mix and place in a water bath at 25° C. Mix in

0.20 ml. enzyme solution (VII) or 0.20 ml. buffer (solution I) for the blank

and incubate for 15 min. at 25° C*). Stop the reaction by the addition of

0.10 ml. molybdate solution (V).

*) Check whether this time is sufficient for complete hydrolysis. The curve (pyrophosphate hydrolysed/time) is linear up to 92% hydrolysis.

Phosphate determination

Transfer the contents of the tubes after the enzymatic reaction to 25 ml. volumetric flasks with three 2 ml. portions of distilled water. Add to each flask:

2.5 ml. molybdate solution (V)
2.0 ml. $FeSO_4$ solution (VI)
distilled water to 25 ml.

Mix thoroughly and after 5 min. read the optical density at 600 mμ or an adjacent wavelength against the blank.

If pure pyrophosphate solutions are analysed, the enzymatically liberated phosphate should agree exactly with that found on hydrolysis (10 min.) with 1 N HCl. Biological material may contain acid-labile organic phosphate or organic pyrophosphate, which is not hydrolysed by the enzyme, but from which phosphate is liberated by the action of the acid molybdate solution. In this case, determine the enzymatically hydrolysed phosphate according to *Lowry* and *Lopez*[10]:

After 15 min. incubation with the enzyme adjust the pH of the mixture to 4.0 with
4 volumes 0.1 M acetate buffer (pH 4.0)
and dilute with
0.125 M acetate buffer (pH 4.0)
so that the PO_4^{3-} concentration is 1.5×10^{-5} to 10^{-4} M (bring all the mixtures to the same volume). Then add:

0.1 volumes ascorbic acid solution (1% w/v)
0.1 volumes of a solution of 1 g. $(NH_4)_6Mo_7O_{24} \cdot 4\,H_2O$ in 100 ml. 0.05 N H_2SO_4.

Read the optical densities at 700 mμ against the blank (buffer instead of enzyme) 5 and 10 min. after the addition of molybdate. In addition prepare standards (see "Standard curve", but do not add molybdate solution (V)!). If the optical densities read at 5 and 10 min. are different, extrapolate to zero time (= molybdate addition).
Very crude extracts may retard the colour formation and in such cases consult the original paper[10].

Standard curve

Pipette into 25 ml. volumetric flasks 1, 2, 4, 6 and 8 ml. phosphate standard solution (IV) (50 to 400 μg. P), add 2.6 ml. molybdate solution (V) and proceed as described under "Phosphate determination". Plot the optical densities against μg. P/flask or μmoles PO_4^{3-}/flask.

Calculations

Obtain from the standard curve the phosphate content of the flask corresponding to the measured optical densities. If the abscissa scale of the standard curve is in μg. P/flask then:

$$5 \times (\mu g.\ P/flask) = \mu g./ml.\ sample$$

$$\frac{5 \times (\mu g.\ P/flask)}{62} = \mu moles\ P_2O_7^{4-}/ml.\ sample$$

where

5 = conversion from 0.2 ml. sample to 1 ml.
62 = 2× atomic weight of phosphorus

[10] *O. H. Lowry* and *J. A. Lopez*, J. biol. Chemistry *162*, 421 [1946].

Sources of Error

None of the glycolytic enzymes contained in the sample interfere with the determination. According to[3] Ca^{2+} inhibits the reaction by being antagonistic to Mg^{2+}. The extent of the inhibition depends on the ratio of Ca^{2+}/Mg^{2+}. Substrate in excess of the Mg^{2+} concentration also inhibits.

Specificity

Inorganic pyrophosphatase is absolutely specific for inorganic pyrophosphate.

Appendix

Preparation of inorganic pyrophosphatase*) from yeast[3]

Extraction: Crumble 3.2 kg. baker's yeast, warm to 38° C and mix with 200 ml. toluene. After 30 min., add 2500 ml. water at 38° C, incubate for 18 hours at 38° C and then centrifuge. Dialyse the supernatant overnight against running tap water. The contents of the dialysis sac should be virtually free from inorganic phosphate. Volume *ca.* 4600 ml.

1st Ammonium sulphate precipitation: To every 1000 ml. solution at room temperature add 283 g. $(NH_4)_2SO_4$ (46% saturation) and centrifuge. The protein salted out of solution floats. Filter the solution through muslin and discard the residue. To every 1000 ml. of the filtrate add 109 g. $(NH_4)_2SO_4$ (63% saturation). Filter off the precipitate on a gravity filter overnight at room temperature, and discard the filtrate.

2nd Ammonium sulphate precipitation: Dissolve the protein on the filter paper in 700 ml. distilled water. Centrifuge off insoluble material and filter the still turbid supernatant with slight suction through a 2.5 cm. layer of paper pulp. To the filtrate add 0.747 volumes of $(NH_4)_2SO_4$ solution saturated at 20° C (43% saturation). Centrifuge off the precipitate and discard**). To every 1000 ml. supernatant add 133 g. $(NH_4)_2SO_4$ (65% saturation). Filter off the precipitate with suction.
The preparation keeps for several years at 0° C. It has an activity at 25° C of about 0.05 units+) or a Q_P value+) of 800–2400.

*) The method described here yields a pyrophosphatase preparation which contains no ATPase. The procedure of *Heppel* and *Hilmoe*[11] gives the same result, but is rather more complicated.

**) If the supernatant is allowed to stand for 24 hours at 4° C, the triosephosphate dehydrogenase crystallizes out, but this step can be omitted.

+) According to[3] a pyrophosphatase unit is the amount of enzyme which liberates 1 mg. P (*i.e.* 32.2 μmoles PO_4^{3-}) from pyrophosphate in 30 min. The specific activity (expressed as units/mg. protein nitrogen) can be converted to a Q_P value, which is defined as the μl. of a hypothetical gas/mg. protein/hour. It is assumed that 1 mole PO_4^{3-} corresponds to 1 mole of the gas. The relation between the units/mg. protein nitrogen and the Q_P is as follows:

$$Q_P = \frac{22.4 \times 6 \times 10^3}{31 \times 2} \times (\text{units/mg. protein nitrogen})$$

where
22.4 = volume of 1 μmole gas [μl.]
6 = approximate conversion of mg. protein nitrogen to mg. protein
10^3 = conversion of mg. P to μg. P
31 = atomic weight of P (conversion of μg. to μmoles)
2 = conversion from 30 min. to 1 hour.

11) *L. A. Heppel* and *R. J. Hilmoe*, J. biol. Chemistry *192*, 87 [1951].