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A simple and rapid procedure for the preparation of phosphopyruvate hydratase free from phosphoglycerate phosphomutase and pyruvate kinase

Although phosphopyruvate hydratase (2-phospho-D-glycerate hydro-lyase, formerly known as enolase, EC 4.2.1.11) has been crystallized from muscle[1] and from yeast as the Hg^{2+} salt[2], and the purification procedures are fairly simple, they yield preparations which often contain traces of phosphoglycerate phosphomutase (D-phosphoglycerate 2,3-phosphomutase, EC 5.4.2.1) and of pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40). The commonly used assay method for phosphoglycerate phosphomutase requires phosphopyruvate hydratase preparations free from phosphoglycerate phosphomutase[3]. Phosphopyruvate hydratase preparations low in phosphomutase could be obtained, at best, after considerable difficulties and losses. Indeed, the most satisfactory method based on passage of phosphopyruvate hydratase through a Dowex column is often irreproducible[3].

We present here a simple and abridged procedure which we have found to be very reproducible and which yields essentially pure phosphopyruvate hydratase containing neither phosphoglycerate phosphomutase nor pyruvate kinase. Further, the method can be carried out in less than 4 h with minimum equipment and thus is also useful for classroom experiments. The method is based upon the finding that protracted incubation of dry yeast results in labilization of residual phosphoglycerate phosphomutase particularly when alcohol fractionation is carried out in the presence of KCl. As previously shown cations such as potassium also change the kinetic behavior and exchange characteristics of phosphoglycerate phosphomutase[4].

Unless indicated, all centrifugations are carried out for 10 min at 5000 $\times$ g. All steps are carried out at 0–5°. All volumes refer to the original volume for the particular step. The acetone and ethanol are measured at 0° and then cooled to about −50° in a dry ice acetone bath.

Fresh baker's yeast cakes are broken by hand, passed through a wire gauze and then dried slowly by spreading on filter paper. The yeast is occasionally mixed and is well dried after about a week at room temperature. It is then passed through a wire sieve. It can be stored in a bottle for over a year either at room temperature or in the cold room.

Mix 300 g of the dry yeast with 900 ml of distilled water. Incubate in a water bath at 38° and stir occasionally for the first 2 h until the yeast is well suspended. Let it stand for 15±1 h at 38°. Centrifuge at 5000 $\times$ g for 20 min and discard the precipitate. The supernatant fluid is the "crude fraction". To each 100 ml of the crude fraction add 54 ml of acetone. The addition should be made rapidly but maintaining the temperature below 5°. Centrifuge and discard the precipitate. Add acetone to the supernatant fluid (38 ml per each 100 ml of the original crude fraction). Centrifuge, discard the supernatant fluid. Take the precipitate in water to about half the volume of the crude fraction. Centrifuge off and discard any insoluble material. Mix in rapidly sufficient 1 M acetic acid to bring the pH of the preparation to 4.75 (about 7.5 ml) and then 0.1 vol. of 1.2 M KCl. Add 105 ml of ethanol to each 100 ml, centrifuge and discard the precipitate. It is imperative that the ethanol fractionation be carried out rapidly. (It seems that the presence of K^+ during the alcohol fractionation are re-

TABLE I

SUMMARY OF PURIFICATION PROCEDURE

The numbers in the table refer to enzyme units measured as described previously[3] (0.1 absorbance change per min, 1 cm cell, 30°, potassium 2-phospho-DL-glycerate, pH 7, 50 μmoles; $MgSO_4$, 10 μmoles; Tris chloride, pH 7, 100 μmoles). Final volume 3 ml.

Fraction	*Volume (ml)*	*Units/ml*	*Total units*	*Protein (mg/ml)*	*Specific activity*	*Phosphopyruvate hydratase* / *Phosphoglycerate phosphomutase*
Crude	520	1750	910 000	80	22	4.4
Ethanol	50	3500	175 000	5.5	640*	∞

* This fraction may lose activity on freezing and thawing to about specific activity 400; however, it may be precipitated with 4 vol. of ammonium sulfate at pH 7 and the precipitate extracted with about 15 ml of ammonium sulfate[5] and the specific activity is maintained at 600 (at 60% ammonium sulfate saturation) and is then very stable.

sponsible for the complete inactivation of phosphoglycerate phosphomutase. However, on protracted contact they inactivate the phosphopyruvate hydratase; more than 90% is destroyed when the preparation is allowed to stand for several hours at 0° after the ethanol addition.) Add ethanol to the supernatant fluid (65 ml per each 100 ml being fractionated) and centrifuge. This precipitate is the "ethanol fraction". This fraction is taken into about 1/10 the volume of the crude fraction and centrifuged.

The specific activity of the ethanol fraction is comparable to the crystalline muscle enzyme and has about 80% of the activity of the crystalline yeast enzyme; under the conditions reported by WINSTEAD AND WOLD[1] it will catalyze the transfer of approx. 100 μmoles/min per mg protein at 30°. A resume of the purification procedure is presented in Table I.

In 5 h of incubation, twice as much phosphopyruvate hydratase activity can be obtained using 0.2 M sodium phosphate buffer at pH 7. This crude extract can then be fractionated by the method presented here. However, there appears to be a smaller yield at the alcohol step, although this may merit further investigation.

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