Crystalline phosphoglyceric acid mutase*

Although many of the enzymes of glycolysis have been extensively purified or crystallized, phosphoglyceric acid mutase has hitherto resisted attempts at extensive purification[1,2]. This note describes a simple procedure for the crystallization of phosphoglyceric acid mutase from baker's yeast with excellent yields.

Procedure: Crumble two pounds of fresh Fleischmann's baker's yeast into a 3 liter beaker. Warm to 38° in a water bath at 50–60°. Add 100 ml of toluene and mix thoroughly. After about half an hour the yeast liquefies. Stir briefly to ensure mixing. Keep at 38° for 9 to 10 hours. Maximum yields of enzyme are obtained after 9 hours' incubation. Add approximately one volume of cold distilled water and mix. Centrifuge 30 minutes. All centrifugations are at 0°, unless otherwise stated, and are continued until a clear separation is obtained. The times indicated are adequate at approximately 5000 × *g*. The supernatant fluid which contains the enzyme is stirred 10 minutes with 20 g of washed and dried Johns-Manville Super-Cel. The mixture is then filtered through a 6-inch Büchner funnel with a 2–3 mm pad of Super-Cel. The filtrate is the *crude extract*.

To the *crude extract* at 0–5° add 2 *N* acetic acid (50–55 ml) to give a pH of 4.5 to 4.7 (measured at 25°). Centrifuge immediately for 15 minutes. The supernatant fluid is quickly brought to pH 7.1 ± 0.1 with 2 *N* KOH (60–65 ml) to give *fraction* #1. The precipitate which forms on neutralization is not removed. This fraction may be stored overnight at 0° without loss of activity. Although acid treatment provides little purification, it appears to be necessary owing to the instability of the enzyme in the crude extract without acid treatment. To *fraction* #1 add, with stirring, an equal volume of ammonium sulfate solution which has been heated to about 100°. (Throughout this procedure all ammonium sulfate solutions are saturated at 25° and neutralized to pH 7.0 by the addition of concentrated NH_4OH. The pH is determined with a glass electrode for a 1:20 dilution.) The mixture is placed in a hot water bath and maintained with occasional stirring at 75 ± 1° for 20 minutes. It is then cooled on ice to 0–5° and filtered in the cold through a coarse fluted paper. The filtrate is *fraction* #2. To *fraction* #2 add rapidly, with stirring, an equal volume of ammonium sulfate solution at 25°. Place in an ice bath until the solution reaches 0–5°, then centrifuge for 30 minutes. The supernatant fluid is discarded. The precipitate is dissolved in approximately 120 ml of cold deionized water to give *fraction* #3. This fraction may be stored frozen without loss of activity. *Fraction* #3 is diluted to a protein concentration of 10 ± 0.5 mg per ml with water. To the diluted #3 *fraction* at 0° add, during a one- to two-minute interval, 0.9 volumes of acetone measured at — 5°. Let stand 10 minutes at 0°, centrifuge for 10 minutes, and discard the supernatant fluid. The precipitate contains the enzyme and insoluble protein. Take up in about 100 ml of cold deionized water and transfer to a centrifuge tube. Bring the precipitate to a fine suspension and extract 10 minutes at 0°. Centrifuge 15 minutes and keep the supernatant fluid. Reextract the precipitate with about 50 ml cold deionized water.

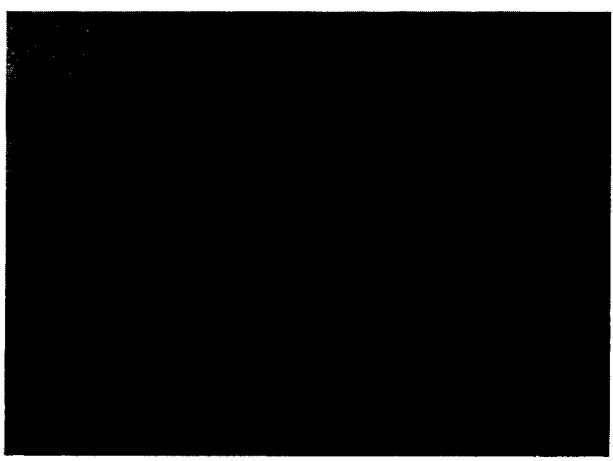

Fig. 1. Photomicrograph of 2 × recrystallized phosphoglyceric acid mutase (× 300). The physical aspect of these crystals is the same before recrystallization. Photomicrograph kindly performed by Dr. H. I. FIRMINGER of the Department of Pathology, University of Kansas Medical Center.

The combined supernatant fluids are filtered through a small pad of glass wool to give *fraction* #4. Bring this fraction to 0°. Add, with stirring, three volumes of ammonium sulfate solution at 25°. Cool to 0° and centrifuge 30 minutes. The

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supernatant fluid is discarded. The precipitate is suspended in the minimum quantity of deionized water at 0° and transferred to a small centrifuge tube. Water is then added dropwise until the solution is just barely turbid. The tube is cooled in an ice-salt bath at —10° to obtain further solubilization. Transfer the unstoppered tube to a refrigerator at 0–5°. Crystallization, as evidenced by the characteristic silky sheen appearing upon swirling the tube, begins within 5–10 hours. The crystals are maximally precipitated after 10 or more hours and settle on prolonged standing. If crystals do not form, reprecipitate with 3 volumes of ammonium sulfate solution and again dissolve in a minimum quantity of cold deionized water at 0–5° and proceed as above. Recrystallization is carried out in the following manner. Centrifuge the crystal suspension and discard the supernatant fluid. Dissolve the crystal pellet in the minimum quantity of deionized water at 0°*. Centrifuge at —10° and allow to crystallize at 0–5° in the refrigerator. Table I shows the summary of the purification procedure.

Fig. 1 shows the appearance of the phosphoglyceric acid mutase crystals after the initial crystallization or two recrystallizations. The crystals contain the enzyme activity, and the protein moves as a single component as evidenced by sedimentation in the ultracentrifuge (Fig. 2). The turnover number under the conditions of assay described under Table I of the 2 × recrystallized enzyme (calculated using the value of 1.73 as the micromolar extinction coefficient of phosphoenolpyruvic acid[3]) is 41,500 moles of substrate transformed per minute per 10^5 g protein. The properties and mechanism of action of crystalline phosphoglyceric acid mutase are under further investigation.

Fig. 2. Sedimentation pattern of phosphoglyceric acid mutase, 2 × recrystallized preparation. Protein concentration was 9.1 mg/ml; 0.1 *M* $(NH_4)_2SO_4$, pH 7.0. Average temperature during centrifugation was 24.1°. Speed 59,780 r.p.m. Bar angle was 70 degrees. Exposures from right to left are at 16, 48, and 80 minutes after speed equilibration. The ultracentrifuge run was kindly performed by Dr. H. EDELHOCH, Department of Pathology, University of Kansas Medical Center.

TABLE I

SUMMARY OF PURIFICATION PROCEDURE

Fraction	*Volume of solution (ml)*	*Total units*	*Total protein (mg)*	*Specific activity*	*Yield %*
Crude extract	1310	2,030,000	34,500	59	100
#1	1395	1,810,000	28,300	64	89
#2	2570	1,690,000	10,665	158	83
#3	185	1,540,000	4,500	342	76
#4	215	1,350,000	810	1670	66
Crystals	16.3	1,040,000	473	2200	51
Mother liquor	10	7,800	76	103	< 1
First recrystallization	3.2	1,020,000	440	2320	50
Second recrystallization	2.7	1,000,000	422	2390	49

The crude extract and fractions #1 and #2 are diluted in deionized water, while fractions #3 and #4 and the crystals are diluted in 0.5 saturated ammonium sulfate solution for assay. The linear rate of formation of phosphoenolpyruvate as followed in the Beckman spectrophotometer at 240 mμ in a coupled assay system containing excess mutase-free enolase is taken as a measure of phosphoglyceric acid mutase activity. An enzyme unit of mutase or enolase is defined as that amount of enzyme which causes an increase in optical density of 0.100 per minute under the conditions of the assay. Each cell contained the following components expressed as μ*M* in 3 ml:

* The unrecrystallized material contains two distinct crystalline forms. The major component (shown in Fig. 1) is the phosphoglyceric acid mutase, which is very soluble in water. In addition there is a minor component (*ca.* 10%, prisms) which is very insoluble in water. This difference in solubility permits the separation of the two crystalline forms. The identity of the prismatic crystals is under investigation.

potassium D-3-phosphoglycerate, 50 (containing 0.12 μM D-2,3-diphosphoglycerate); $MgSO_4$, 10; tris(hydroxymethyl)aminomethane, pH 7.0, 100; and 1.2 enzyme units of mutase-free enolase*. Up to 0.25 enzyme units of mutase may be taken for assay. In the enolase assay DL-2-phosphoglycerate is substituted for the D-3-phosphoglycerate. Protein is estimated by a semimicro adaptation of a standard Biuret method.

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Preparation of paper for quantitative chromatography of corticosteroids

Quantitative chromatography of steroid mixtures is usually performed on columns[1,2,3] despite the existence of paper methods of high resolving power[4,5]; yet paper chromatography offers considerable advantages both in simplicity and reproducibility. The drawback to paper work has been the high blank value of the eluate with many methods of determination, including fluorimetry in sulphuric acid[6] and in potassium *tert*-butoxide[7], ultraviolet spectrophotometry and arsenomolybdate reduction[8]. Washing of papers in the Soxhlet apparatus has not been impressive in reducing blank values, but the following technique has been consistently satisfactory in over a year's use in this laboratory.

Whatman No. 2 "Paper for Chromatography" is cut into 50 × 1.5 cm strips. These are washed chromatographically for 24 hours with 2N ethanolic sodium hydroxide, prepared from freshly redistilled 95% ethanol. The papers are then washed with distilled water until the eluate is free from alkali, and finally with 95% ethanol for three hours. After drying they may be stored indefinitely.

SWEAT's sulphuric acid-induced fluorescence has been employed by us in plasma corticosteroid estimations involving paper chromatography. When 8 cm lengths of the prepared strips are eluted with absolute ethanol and compared fluorimetrically with 1 μg cortisol as standard, blank values never exceed 10% of the cortisol figure, and are usually in the range 3–6%. The eluate from unwashed paper gives a value 1–3 times greater than the standard.

In absorptiometry at 240 mμ, the paperblank reading is 5–10% of that obtained with 10 μg cortisol. Limited experience with the potassium *tert*-butoxide method[9] indicates that blank values are even lower than with sulphuric acid.

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