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ISOLATION OF RABBIT MUSCLE GLUCOSEPHOSPHATE ISOMERASE BY A SINGLE-STEP SUBSTRATE ELUTION

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Summary

A simple method has been developed for the rapid isolation of crystalline glucosephosphate isomerase (EC 5.3.1.9) from rabbit muscle. The enzyme is first bound to cellulose phosphate by adding the ion exchanger to a solution of the crude tissue extract. After filtering and washing the cellulose with buffer, the isomerase is specifically eluted in a batch process by its substrate, glucose 6-phosphate. The entire procedure is very rapid and results in a good recovery (at least 50%) of the enzyme with specific activity of approximately 900 units per mg. The enzyme is homogeneous by polyacrylamide gel electrophoresis in the presence or absence of sodium dodecyl sulfate and by analytical ultracentrifugation.

Introduction

Within the past five years glucosephosphate isomerase (EC 5.3.1.9) has increasingly become the center of a variety of structural-functional studies in several laboratories [1—4], and currently, primary sequence and crystallographic studies are underway. The enzyme was first isolated in crystalline form by Noltmann [5,6] over ten years ago from rabbit muscle, which has, in general, served as the most common source of enzyme. However, the existing procedure for the isolation of rabbit muscle glucosephosphate isomerase, while quite reproducible, is rather long and tedious (requiring eight fractionation steps and repeated recrystallizations) *. In addition, the method makes use of somewhat nonspecific fractionation methods (e.g. precipitation by zinc acetate, organic solvents and salts, and adsorption on bentonite) and results in recoveries of only 20—25% [5,6].

* The enzyme can also be isolated by a combination of the original procedure and successive chromatographic steps on carboxymethyl cellulose. However, specific information regarding recovery is not available [7].

Our laboratory has recently shown that glucosephosphate isomerase from human erythrocytes and cardiac muscle can be specifically eluted from cellulose phosphate by its substrates [2,8]. We thus felt that a substrate elution method might be developed for the rapid isolation of rabbit muscle glucosephosphate isomerase.

Materials and Methods

Cellulose phosphate (0.91 mequiv/g) was obtained from Sigma and prewashed sequentially with 1 M HCl and NaOH. Substrates, coenzymes, and coupling enzymes were also from Sigma. Glucosephosphate isomerase was assayed spectrophotometrically as previously described [8] using fructose 6-phosphate as the substrate and measuring the rate of conversion to glucose 6-phosphate by coupling with excess glucose 6-phosphate dehydrogenase. Protein concentrations were determined at 280 nm using an extinction coefficient ($E^{1\%}_{1\,cm}$=) of 13.2 [6]. One unit of enzyme is defined as the amount catalyzing the conversion of one micromole of fructose 6-phosphate to glucose 6-phosphate per min at 30° C, and specific activity is expressed as units per mg of protein.

Results

Isolation procedure

The following procedure has been developed for the isolation of rabbit muscle glucosephosphate isomerase. All steps are performed in an ice bath or in a cold room at 0—4° C. The entire isolation procedure can easily be completed in less than two working days. Although the following procedure is described for starting with 200 g of tissue, it has also been carried out on larger (500—1000 g) and smaller (50 g) scales.

Tissue extraction. Fresh or frozen rabbit skeletal muscle (200 g) is passed through a meat grinder and mixed with 400 ml of 50 mM triethanolamine, 1 mM EDTA, 0.1% v/v 2-mercaptoethanol, pH 8.2. The tissue is then homogenized for 1 min at high speed on a Waring blendor, pH promptly adjusted to 6.6, and then stirred for 30 min in the cold. After centrifugation (15 min at 20 000 × *g*), the supernatant solution is collected, filtered through glass wool, and dialyzed overnight * against Buffer A (10 mM imidazole, 1 mM EDTA, 0.1% v/v 2-mercaptoethanol, pH 6.6).

Adsorption to cellulose phosphate: The dialyzed enzyme is stirred in a large beaker and cellulose phosphate, which has been previously cleaned and equilibrated in Buffer A, is added to the extract. For an initial extract of 200 g of tissue (i.e. approximately 107 000 units of enzyme) 150 g of packed-wet weight cellulose (20 g dry weight) are needed to bind 70—80% of the isomerase. The mixture is stirred for 20 min, then poured into a large (19 × 8 cm) porcelain Buchner funnel containing Whatman No. 541 filter paper. The cellulose is filtered under mild vacuum and then washed with additional Buffer A (approxi-

* Alternatively, this desalting and buffer exchange can be more rapidly accomplished by ultrafiltration.

mately 5 liters) until the effluent exhibits an absorbance at 280 nm of less than 0.01. The cellulose is then resuspended in 1-liter of Buffer B (10 mM imidazole, 1 mM EDTA, 0.1% v/v 2-mercaptoethanol, pH 7.2), and the pH of the suspension carefully adjusted to pH 7.2 with NaOH *. The cellulose is then washed with Buffer B until the effluent is protein-free (approximately 7 liters). This entire process requires approximately three hours.

Elution of the enzyme. The cellulose is removed from the Buchner funnel, resuspended in approximately 700 ml of Buffer B and poured into a 7.5 cm diameter glass column and packed under 5 lb/inch^2 of nitrogen (the height of the resulting cellulose pad is approximately 8 cm). The enzyme is then specifically eluted from the cellulose phosphate with 350 ml of 10 mM imidazole, 0.1% v/v 2-mercaptoethanol, 3 mM glucose 6-phosphate **, pH 7.2. Under these conditions the enzyme is eluted with the front of the substrate and can be collected in a volume of 100 to 150 ml.

The enzyme eluted in this manner exhibits a specific activity of approximately 850 to 950 units per mg and is homogeneous as judged by several criteria (vide infra). The total recovery of pure enzyme from the substrate elution is at least 50%. The remainder of the enzyme activity can be fully accounted for as the amount not initially bound to the cellulose (approximately 20%) or material lost during the two washing steps prior to substrate elution. All of the steps following the initial dialysis of the tissue extract can be completed in one working day.

Crystallization. Although crystallization is not essential to achieve purity, the enzyme isolated in this manner can be easily crystallized as follows. The effluent from the substrate elution is concentrated by dialysis overnight against a saturated solution of ammonium sulfate (buffered to pH 8.2 with 25 mM triethanolamine, 1 mM EDTA, 0.1% v/v 2-mercaptoethanol). The precipitated protein is collected by centrifugation (20 000 $\times g$, 30 min) and resuspended in the above buffer such that the protein concentration is approximately 10 mg per ml. A solution of saturated ammonium sulfate is slowly added until reaching 0.48 saturation (4°C), and any amorphous material which precipitates at this point is removed by centrifugation. The solution is then transferred to room temperature and left overnight during which crystallization occurs (Fig. 1). Table I summarizes the results of the isolation procedure.

Homogeneity

The specific activity of the enzyme isolated by this procedure (850 to 950 units per mg) is essentially identical with that of the enzyme isolated by the procedures of Noltmann [5—7]. The purified enzyme migrates as a single band in both standard and sodium dodecyl sulfate polyacrylamide gel electrophoresis. Analytical ultracentrifugation studies (both sedimentation velocity and meniscus-depletion sedimentation equilibrium) are also indicative of a monodisperse protein (M_r = 132 000 and $s_{20,w}$ (5.0 mg/ml) = 7.0 S).

* The entire filtration process and resuspension can be carried out in the Buchner funnel after releasing the vacuum. Stirring of the slurry is best achieved by a slow (15—20 rev./min) overhead blade stirrer during the wash period.

** The other substrate, fructose 6-phosphate, can also be used to elute the enzyme under identical conditions with comparable results.

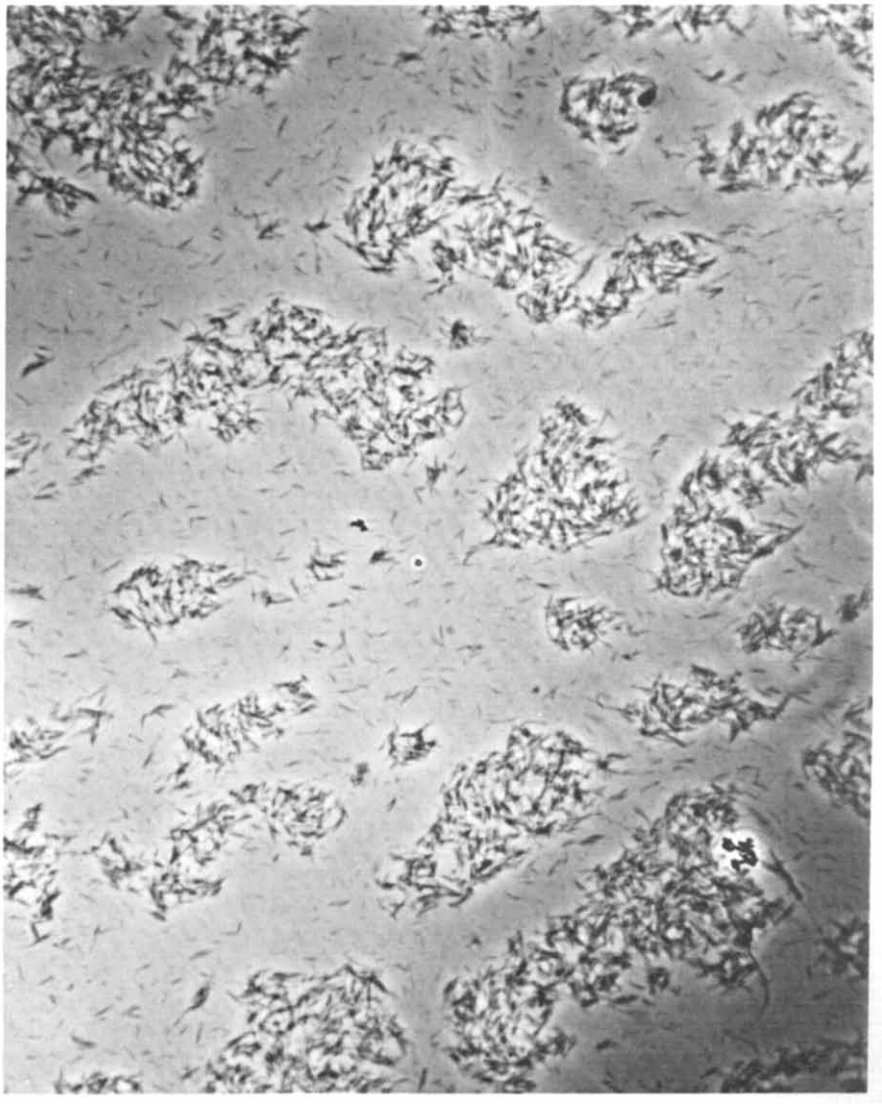

Fig. 1. Crystals of rabbit muscle glucosephosphate isomerase. The enzyme was crystallized from ammonium sulfate as described in the text. Photographs were taken under oil immersion using phase contrast optics. Total magnification is 420 X.

TABLE I

ISOLATION OF GLUCOSEPHOSPHATE ISOMERASE FROM RABBIT MUSCLE

200 g of initial tissue.

Fraction	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification	% Recovery
Tissue extract	107 000	13 400	8.0	(1)	(100)
Cellulose phosphate					
a. pH 6.6 wash	31 000	—	—	—	29
b. pH 7.2 wash	12 400	—	—	—	12
c. Substrate elution	55 000	59	920	115	51
Crystallization	50 400	54	933	116	47

Discussion

The new isolation procedure described in this study has a number of distinct advantages over the previous method for the purification of this enzyme. The new procedure is much faster and requires only the single substrate induced elution from the ion exchanger as compared to the previous eight-step fractionation process and repeated recrystallizations requiring several weeks. The new method is very mild and avoids the rather harsh fractionating agents (e.g. zinc acetate, acetone, ethanol, and bentonite), used in the previous method which could alter the conformational or catalytic properties of the enzyme. The inclusion of 2-mercaptoethanol in all buffers and the short time required to isolate the pure enzyme permits the enzyme to be isolated without generating the multiple electrophoretic forms which can arise from sulfhydryl oxidation [8]. Therefore, the new isolation procedure provides a far better method for obtaining the pure enzyme in better yields, in minimal time, with minimal labor and expense.

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References

1 James, G.T. and Noltmann, E.A. (1973) J. Biol. Chem. 248, 730—737
2 Tilley, B.E., Gracy, R.W. and Welch, S.G. (1974) J. Biol. Chem. 249, 4571—4579
3 Schray, K.J., Benkovic, S.J., Venkovic, P.A. and Rose, I.A. (1973) J. Biol. Chem. 248, 2219—2224
4 Tsuboi, K.K., Fukunaga, K. and Chervenka, C.H. (1971) J. Biol. Chem. 246, 7586—7594
5 Noltmann, E.A. (1964) J. Biol. Chem. 239, 1545—1550
6 Noltmann, E.A. (1966) Methods Enzymol. 9, 557—568
7 Blackburn, M.N., Chirgwin, J.M., James, G.T., Kempe, T.D., Parsons, T.F., Register, A.M., Schnackerz, K.D. and Noltmann, E.A. (1972) J. Biol. Chem. 247, 1170—1179
8 Gracy, R.W. and Tilley, B.E. (1975) in Methods in Enzymology 41, 392—400