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An Improved Procedure for the Isolation of 3-Phosphoglycerate Kinase from Yeast

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3-Phosphoglycerate kinase has been isolated from yeast by a new procedure. Over 1g was obtained from 450g of granulated baker's yeast; it had a specific activity of up to 940units/mg at 30°C. Six distinct crystalline forms have been grown, at least one of these being suitable for X-ray diffraction studies. The crystalline preparation is pure, judged by starch-gel or sodium dodecyl sulphate–polyacrylamide-gel electrophoresis; the latter method indicating that the enzyme is monomeric, with a molecular weight near to 50000.

Bücher (1947) described a procedure for the isolation of the enzyme 3-phosphoglycerate kinase (ATP–3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3) from yeast. [It should be noted that in the previous publication on the isolation of the muscle enzyme (Scopes, 1969) the footnote gave an incorrect identification of the enzyme, confusing it with EC 2.7.1.31.] This method, though complex, has not been superseded, and is used for making the enzyme commercially. The preparation (C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany) is claimed to be crystalline: nevertheless impurities amounting to about half of the protein have been removed from it (Larsson-Raźnikiewicz, 1970). Recently a preparation of higher specific activity has been marketed.

Detailed studies of this enzyme are under way, in particular X-ray diffraction work on crystalline preparations (P. L. Wendell, personal communication). For this reason an improved method for preparing the enzyme is highly desirable. The present paper describes a relatively simple method of obtaining more than 1g of pure enzyme from a 450g tin of granulated baker's yeast. The best preparations have had specific activities of over 900units/mg at 30°C in near-saturating conditions of substrate concentration, comparable with the specific activity of the muscle enzyme (Scopes, 1969). Several different crystalline forms have been obtained, and at least one of these is suitable for X-ray diffraction studies.

MATERIALS AND METHODS

D-3-Phosphoglycerate (barium salt), ATP (sodium salt), and NADH were obtained from C. F. Boehringer und Soehne G.m.b.H. Other chemicals were A.R. grade. 'Active Dried Yeast' was obtained in 450g vacuum packed tins from Distillers Co. (Yeast) Ltd., Morden, Surrey, U.K., and could be stored at 1°C for many months before use.

Column chromatography was carried out on polymethylmethacrylate columns (Wright Scientific Co. Ltd., Kenley, Surrey, U.K.), and effluents were monitored at 254nm. CM-cellulose was the microgranular form (Whatman CM 32).

Phosphoglycerate kinase was assayed as described by Scopes (1969) and protein was determined by the Lowry method, by using crystalline bovine serum albumin (Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.) as a standard (Lowry, Rosebrough, Farr & Randall, 1951). All samples for protein determination were first dialysed to remove interfering compounds such as NH_4^+.

PROCEDURE

All stages of this isolation procedure were carried out at room temperature.

Cytolysis of the yeast was achieved with ammonia, by a procedure based on that described by Morena, Santos & Grisolia (1968). A litre of 0.5M-ammonia containing 1g of EDTA (disodium salt) was stirred vigorously while 450g of granulated yeast was added slowly over a period of 30min. Any lumps forming were dispersed, and stirring was continued for several hours; the thick sludge was then left overnight. The stirring was recommenced, 20–24h after adding the yeast and 800ml of 0.5M-lactic acid was mixed in. The pH was adjusted to 7.0 ± 0.5 with cold 5M-lactic acid adjusted to pH3.5 with ammonia. The mixture was then centrifuged at 20000*g* for 15min and the residue discarded. This extract contained many of the soluble enzymes of yeast in excellent yield; it was suitable for the preparation of

glucose 6-phosphate dehydrogenase, alcohol dehydrogenase, hexokinase and several other enzymes as well as 3-phosphoglycerate kinase.

Ammonium sulphate (100g/litre of extract) was dissolved in the extract, then the pH was further lowered to 4.3 (measured on a 10×diluted sample) by using cold 5M-ammonium lactate, pH3.5, as before. About 50ml were required, and the mixture was stirred vigorously during the addition to minimize denaturation of proteins. After 10min at pH4.3, the mixture was centrifuged (20000*g* for 15min) and the precipitate discarded. A further 250g of ammonium sulphate/litre of supernatant was dissolved in the supernatant over a period of 15min and the mixture was left to equilibrate for 30min before centrifugation as before. The supernatant was discarded, and the precipitate taken up in 800ml of 40mM-tris base–1mM-EDTA; M-tris was added if necessary to raise the pH to 7.5±0.2.

Ammonium sulphate (340g/litre) was stirred into this solution over a period of 15min; after 30min equilibration it was centrifuged and the precipitate discarded. A further 150g of ammonium sulphate/litre of supernatant was added and the resulting precipitate was collected by centrifugation as before. This precipitate was dissolved in a small amount of 1mM-EDTA, pH7.0.

At this point it was necessary to remove most of the salt from the fraction before application to a CM-cellulose column. This could be done either by dialysis, or more rapidly by some other technique, such as use of a large Sephadex G-25 column. Dialysis was carried out against running tap water for at least 3h, then overnight against 5litres of 1mM-EDTA, pH7.0. In the morning the buffer was changed, and dialysis continued for several hours more. Then a solution of 1g of protamine sulphate in 50ml of water, adjusted to pH7.0 with tris, was added to the protein, and the resulting precipitate was removed by centrifugation. (The protamine treatment was not essential, but it minimized turbidity developing in the fraction during application to the CM-cellulose column.) Then, 0.01vol. of M-tris was added to the supernatant, and the pH lowered to 6.0 with M-cacodylic acid. This solution, after dilution with buffer (10mM-tris–1mM-EDTA, adjusted to pH6.0 with cacodylic acid) to 800ml, was pumped on to a CM-cellulose column at about 200ml/h. The column dimensions were 16cm²×30 cm, and the CM-cellulose was equilibrated with the tris–EDTA–cacodylate buffer.

When all the protein solution had been applied to the column, a linear gradient of potassium chloride was commenced. A mixer consisting of two beakers, one with 1litre of buffer, the other with 1 litre of buffer containing 0.2M-potassium chloride was used to form the gradient, and the flow rate was adjusted to about 120ml/h. The elution pattern is illustrated in Fig. 1. Tubes containing more than 300units of the kinase/ml were combined, and the protein salted out, by using 600g of ammonium sulphate/litre of eluate. The precipitated protein was collected by centrifugation at 20000*g* for 30 min and dissolved in a small amount of water. The solution was diluted to between 40 and 50ml, and clarified by centrifugation. One-half was taken and applied to a Sephadex G-100 column (8cm²×110cm) by using 0.15M-sodium chloride–1mM-EDTA–10mM-imidazole, pH7.0, as column buffer. The dense protein and ammonium sulphate solution was applied to the base of the column to minimize density instabilities, but when most of the solution had run in, the column was inverted, so that a sharp trailing edge was formed. Elution of the proteins was carried out overnight with a flow rate of 30–40 ml/h. The last peak was the kinase nearly pure at this stage (Fig. 2). The other half of the fraction was treated identically.

Any remaining impurities could be removed by crystallization. The enzyme peak was again salted out by using 600g of ammonium sulphate/l, and the precipitate was dissolved in 20mM-potassium

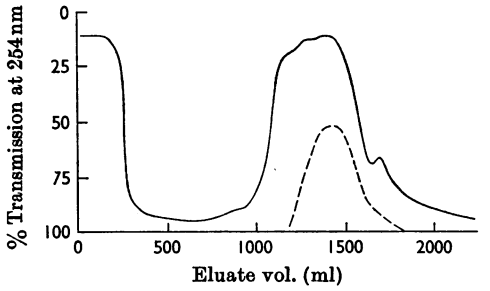


Fig. 1. Elution of phosphoglycerate kinase from CM-cellulose column in pH6.0 buffer. ——, Protein; ----, 3-phosphoglycerate kinase activity.

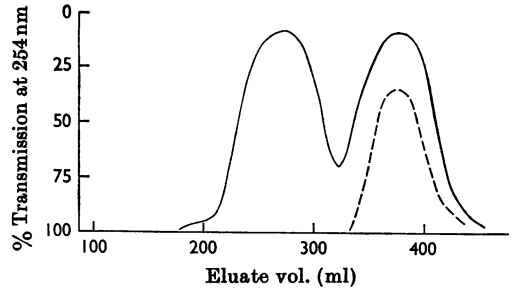
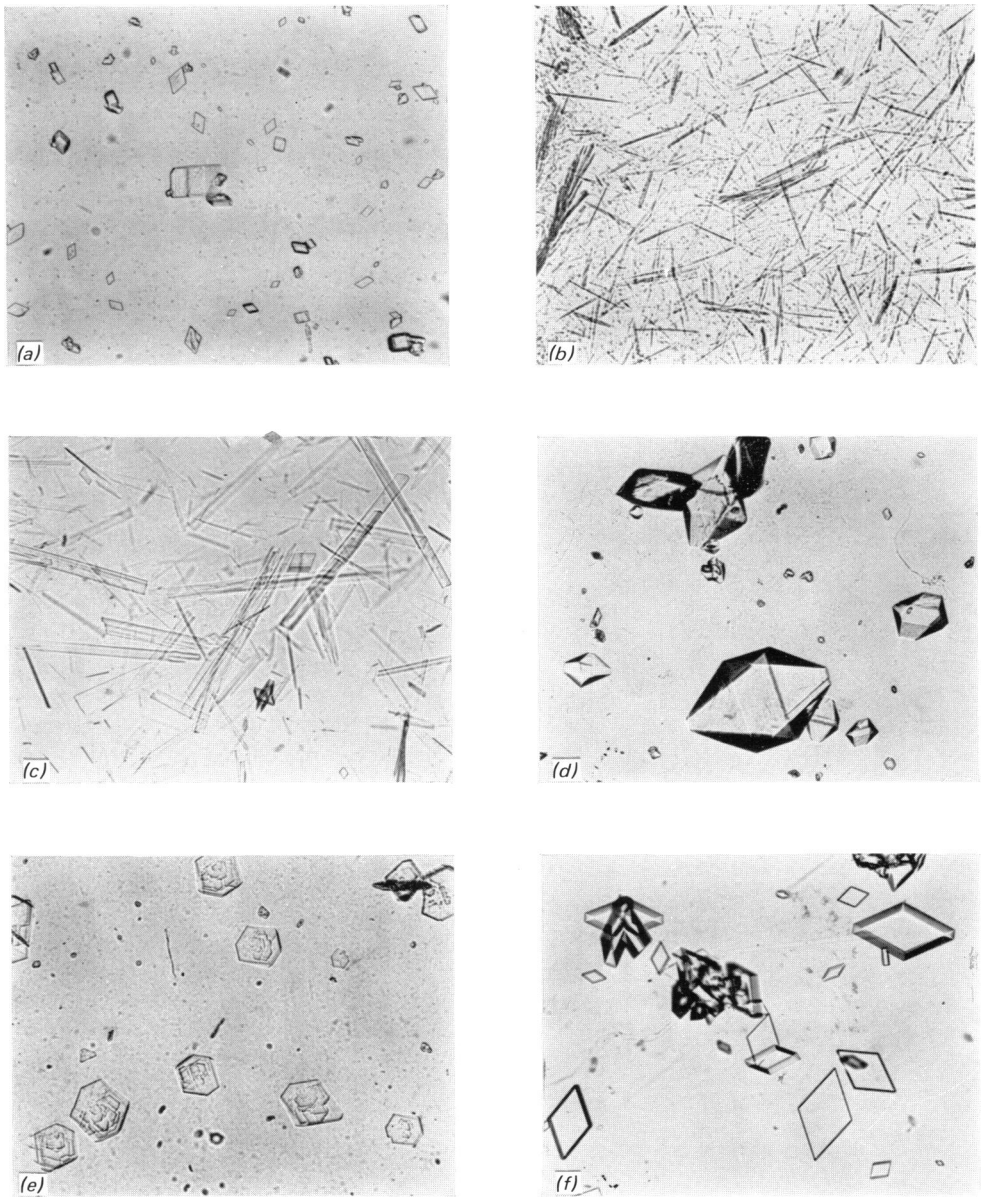


Fig. 2. Elution of phosphoglycerate kinase from Sephadex G-100 column. ——, Protein; ----, 3-phosphoglycerate kinase activity.



EXPLANATION OF PLATE 1

Some crystalline forms of yeast phosphoglycerate kinase: grown (*a*) at pH6.0; (*b*) at pH6.5; (*c*), (*d*), (*e*) and (*f*) at pH values between 7.0 and 8.3. 1,4-Dioxan was present in all except (*b*). Magnification ×75.

Table 1. *Summary of isolation procedure, from 450g of granulated baker's yeast*

	Volume (ml)	Total protein (g)	Total activity (k-units)	Specific activity (units/mg)	Recovery (%)
Day 1: Start, ammonia extract					
Day 2: Collect extract	1300	49	2300	47	100
0.8–2.3M-$(NH_4)_2SO_4$, pH4.3	880	26.5	1800	68	78
2.3–3.3M-$(NH_4)_2SO_4$, pH7.5	150	17.5	1620	92.5	70
Dialysis overnight					
Day 3: Dialysis, protamine, on to CM-cellulose	800	17.2	1620	94	70
Off CM-cellulose overnight	390	2.40	1220	510	53
Day 4: Collect peak, salt out, one-half on to Sephadex G-100					
Off Sephadex G-100 overnight					
Day 5: Second half on to Sephadex G-100	160	1.42	1160	820	50
Off Sephadex G-100 overnight					
Day 6: Collect peaks, salt out for crystals					
Crystals		1.18	1000	850	43

phosphate buffer, pH 6.5. It was made turbid with saturated ammonium sulphate: the protein concentration was about 20mg/ml at this point. The turbid solution was left at room temperature to crystallize, which usually took several days. Seeding with previously obtained crystals hastened the process.

A summary of the isolation procedure is given in Table 1. On a smaller scale (e.g. from 250g of granulated yeast) the procedure can be shortened as follows: day 2, ammonium sulphate fractionations, de-salting on Sephadex G-25, and application to CM-cellulose, then elution from CM-cellulose overnight; day 3, whole sample on to Sephadex G-100; day 4, salt out for crystallization. Recovery of the enzyme before crystallization was 50%, and the recovery at each individual step has been repeated several times with very little variation. Rechromatography on Sephadex G-100 has produced enzyme with specific activity as high as 940units/mg, but on crystallization some loss in activity occurs.

The crystals obtained in phosphate buffer, pH 6.5, were needles, sometimes up to 1mm long, but quite unsuitable for X-ray crystallography. Other forms of crystals have been obtained, and are illustrated in Plate 1. The exact conditions for producing a particular form of crystal are not easily definable, and the shapes often seem to be a matter of chance. However, most of the chunky types of crystal have only been grown in the presence of 1,4-dioxan at a concentration of 1–4%. The polyhedral form (Plate 1*d*) grows over a period of weeks at pH 7.0–8.5, with about 2% dioxan and a protein concentration of the enzyme solution between 10 and 15mg/ml. Seeding with the appropriate form of crystal can shorten this time considerably. This form has been most used in the X-ray studies, the results of which are presented by Watson, Wendell & Scopes (1971).

Some studies on twice-crystallized enzyme indicated that E_{278} is 0.535 for a 1mg/ml solution, based on a dry weight determination. Alkaline tryptophan/tyrosine ratio determination (Beavan & Holiday, 1952) gave a value of 0.27, in good agreement with the amino acid composition ratio 2:7 found by Larsson-Raźnikiewicz (1970). Colorimetric determination of tryptophan (Spies & Chambers, 1949) on the present preparation gave a value of 2 residues in a molecule of 47000 daltons. However, these contents of tryptophan and tyrosine should theoretically give E_{278} of only 0.44, and the difference is large enough to suggest the presence of some other substance, contributing to at least 10% of the extinction at 278nm.

DISCUSSION

The procedure described produced a substantial amount of pure 3-phosphoglycerate kinase from yeast, with overall recovery from the extract of about 50%. The preparation had a higher specific activity than that of Bücher (1947), and crystallized quite easily. The procedure was similar to that used for isolation of the enzyme from skeletal muscle (Scopes, 1969), except that the simple ammonium sulphate fractionation and heat treatment for the muscle enzyme were replaced by two ammonium sulphate fractionations at different pH values. It is significant that the yeast enzyme precipitates in the range 0.8–2.3M-ammonium sulphate at pH 4.3, but not before 2.3M at pH 7.5. It is also clear that

this applies to many other proteins in the extract. The CM-cellulose column separation used for the muscle enzyme was varied only by lowering the buffer pH by 0.5units to ensure that the yeast enzyme was completely adsorbed, and by using a larger size of column, as the quantity of enzyme involved was considerably greater. A longer Sephadex G-100 column was used for better separation; but the enzyme was not quite pure after this step, and removal of final impurities was achieved by crystallization.

The crystallized enzyme ran as a single component on starch-gel electrophoresis, though as with other phosphoglycerate kinases, with considerable streaking towards the anode (Scopes, 1968). There was no evidence of the minor components found by Larsson-Raźnikiewicz (1970). The enzyme also gave only a single band on polyacrylamide–sodium dodecyl sulphate-gel electrophoresis, at a position corresponding to 50000daltons. This compares with a molecular weight of 45000 given by Larsson-Raźnikiewicz (1970), and 47000 as determined by tryptophan content.

The preparation has been used successfully for the enzymic determination of ATP (Adam, 1963), phosphocreatine and 3-phosphoglycerate. It contains no myokinase, and is stable in the crystalline form at room temperature for many months.

I am grateful to Dr Mārtha Larsson-Raźnikiewicz for providing me with a pre-print of her manuscript.

Note added in proof. Since submission of this manuscript a paper describing the properties, but not the preparation, of yeast 3-phosphoglycerate kinase has appeared (Krietsch & Bücher, 1970). There is excellent agreement between their results and those properties described here.

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