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Note

Improved methods for purification and assay of glycerol kinase from *Escherichia coli*

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Glycerol kinase is an essential enzyme for the dissimilation of glycerol by *Escherichia coli* [1]. It was first purified over a decade ago [2,3], and its biochemical and physical properties have been extensively characterized [2–6]. For example, the enzyme shows an important feedback inhibition by fructose-1,6-bisphosphate. The purification procedures described have yielded recoveries of homogeneous glycerol kinase of 20–60% [2–6]. These procedures, which involve repeated salt precipitation, are time-consuming and give relatively low yields.

A number of methods for assaying glycerol kinase have been reported [1–10]. These methods, however, tend to be laborious and time-consuming. They involve coupling the glycerol kinase reaction to other enzymes, such as L-α-glycerol phosphate dehydrogenase [7,8] or pyruvate kinase and lactate dehydrogenase [5], which are assayed spectrophotometrically. Other assays involve radiochemical determinations of the product [^{14}C]L-α-glycerol phosphate, which can be precipitated as the lead salt [9] or adsorbed on DEAE-cellulose filter paper [6,10].

Recently, while attempting to isolate inhibitors of *E. coli* proteases, we have found fortuitously that glycerol kinase has affinity for heparin. In the course of that work, we noted that an unidentified protein bound to a heparin column and consumed ATP, provided that the medium contained glycerol. This protein was glycerol kinase. On this basis, we have developed an improved protocol for rapid

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purification of this enzyme that uses affinity chromatography on heparin, and also a sensitive and rapid protocol for assaying its activity.

EXPERIMENTAL

Materials

[r^{32}P]ATP was purchased from ICN Radiochemicals (Irvine, CA, U.S.A.), heparin–agarose from Bethesda Research Labs. (Gaithersburg, MD, U.S.A.) and polyethyleneimine (PEI) cellulose plates from Brinkman (Westbury, NY, U.S.A.). All other chemicals were of analytical grade and were obtained from Sigma (St. Louis, MO, U.S.A.).

E. coli K12-strain, RGC121 (*CapR*$^+$, *Leu*$^-$, *Pro*$^-$, *Pur*$^-$, *Trp*$^-$, *Thi*$^-$) was provided by Dr. A. Markovitz (University of Chicago). This cell was grown in Luria broth supplemented with 1% glycerol, harvested at stationary phase and kept frozen at −70°C.

Purification

All operations for the purification of glycerol kinase were carried out at 4°C. The frozen cells (40 g) were resuspended in 80 ml of 10 m*M* Tris–HCl (pH 7.5), 5 m*M* magnesium chloride, 1 m*M* ATP and 10% (v/v) glycerol, and were disrupted by a French press at 96.54 MPa. After ultracentrifugation at 100 000 *g* for 3 h, the extract was diluted with the same buffer to give a final protein concentration of ca. 15 mg/ml. The resulting solution was heated at 70°C for 8 min and centrifuged to remove the precipitated materials. The supernatant was diluted with an equal volume of the same buffer, but lacking glycerol and ADP. Solid ammonium sulphate was added to give 50% saturation. After centrifuging at 30 000 *g* for 1 h, ammonium sulphate was added to the supernatant to give 70% saturation. The pellet obtained by centrifuging again at 30 000 *g* for 1 h was resuspended in 25 ml of 10 m*M* Tris–HCl (pH 7.5), 10 m*M* magnesium chloride and 5 m*M* glycerol. It was dialysed against this buffer for 9 h with changes of the buffer every 3 h. The dialysed protein solution was loaded onto a heparin–agarose column (15 cm×1.5 cm I.D.) equilibrated with the same buffer. After washing the unbound proteins with 150 ml of the equilibration buffer, the bound proteins were eluted with a linear gradient of 0 to 100 m*M* sodium chloride (total volume of 200 ml) by collecting 3-ml fractions at a flow-rate of 20 ml/h.

Assays

During the purification of glycerol kinase, the activity of the enzyme was measured as described by Thorner and Paulus [3], except that the reaction buffer contained 50 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) (pH 7.0) in a final volume of 200 μl.

The purified glycerol kinase was assayed by incubating the reaction mixtures (30 μl) containing 25 m*M* Tris–HCl (pH 8.5), 2 m*M* magnesium chloride, 1 m*M* [r^{32}P]ATP (50 000 cpm), 10 μg of bovine serum albumin and various amounts of the purified glycerol kinase. After incubating at 37°C for 10–60 min, the reaction was stopped by adding 5 μl of a solution containing 5% (w/v) sodium

dodecyl sulphate (SDS) and 70 m*M* ammonium molybdate. A 2-μl sample of the reaction mixture was spotted on a PEI plate, and [^{32}P]glycerol phosphate formed during the incubation period was separated from the remaining [r^{32}P]ATP by ascending thin-layer chromatography in 1 *M* lithium chloride solution. This chromatographic step usually took less than 20 min. The plates were then dried with hot air (from a hair dryer) and exposed to Kodak X-OMAT R film overnight.

Therefore, the routine assay of glycerol kinase was performed by simply cutting out the glycerol phosphate spots from the PEI plate and measuring their radioactivity with a liquid scintillation spectrometer. The radioactivity in the remaining portion of the plate was also determined for estimating the total radioactivity spotted on the plate.

Polyacrylamide gel electrophoresis (PAGE) in the presence of SDS was carried out as described by Laemmli [11]. The protein concentration was determined by the dye-binding assay of Bradford [12] or by the method of Lowry et al. [13].

RESULTS AND DISCUSSION

Purification

As shown in Fig. 1A, the elution profiles of protein and the activity of glycerol kinase from the heparin–agarose column precisely overlapped. Because the specific activity of the enzyme appears constant over the area of this peak, the enzyme eluted from the column is likely to be homogeneous. To confirm the purity of the enzyme, aliquots from the fractions were analyzed by SDS-PAGE. Fig. 1B shows that glycerol kinase in all fractions appears as a single polypeptide. The purified enzyme was dialysed against 50 m*M* Tris–HCl (pH 7.5) containing 2 m*M* glycerol, 1 m*M* EDTA and 1 m*M* 2-mercaptoethanol, and concentrated to 10 mg/ml by ultrafiltration on a PM10 membrane (Amicon). It was kept at $-70\,^{\circ}$C until use.

The results of these purification steps are summarized in Table I. A total purification of 41-fold was achieved, with a final recovery of 75%. Thus, under these growth conditions, glycerol kinase was a major gene product constituting 1–2% of cell protein in a wild-type cell.

Hayashi and Lin [2] initially purified the enzyme by a procedure entailing heat treatment at 52°C. This enzyme has also been isolated by Thorner and Paulus [6], who used a more gentle method that does not involve this heating step. The latter investigators argued that the heating and low pH treatment might have depressed the yield of the enzyme. Subsequently, they extended their studies on the heat stability of the enzyme, and showed that glycerol and ADP protect the enzyme from heat inactivation [6]. Thus, the condition was adopted in the heating step used in our purification. However, the purification protocols used by these investigators and others involve eight or nine steps, including repeated salt precipitation [2,3,6]. Therefore, by the introduction of a single ammonium sulphate precipitation step and chromatography on a heparin–agarose column, we have obtained a highly purified enzyme in less time and with higher yields than were reported earlier [2,3,6].

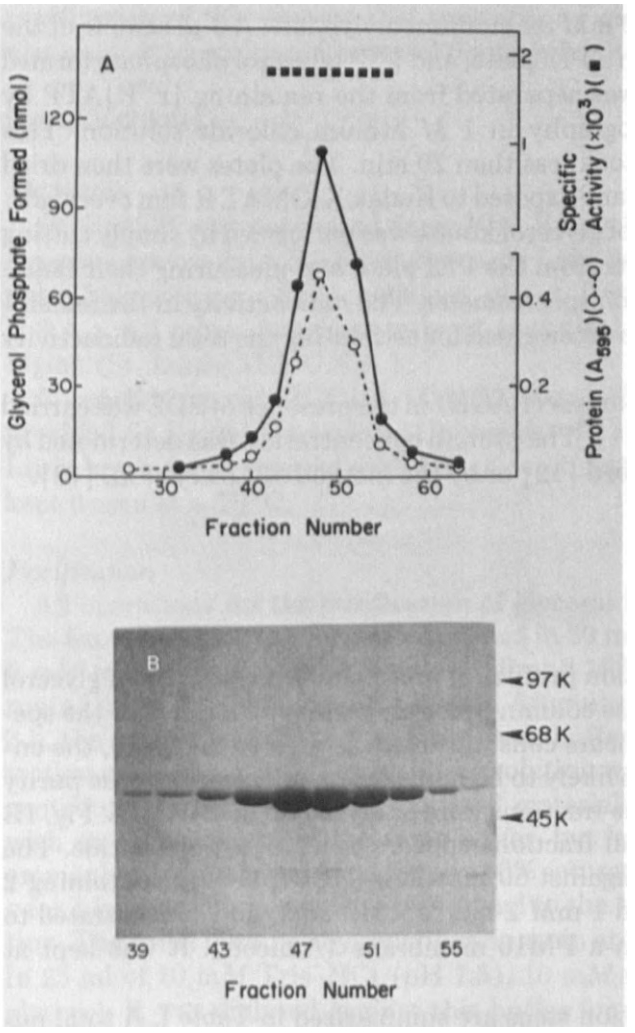

Fig. 1. (A) Heparin–agarose column chromatography of glycerol kinase. The glycerol kinase preparation obtained from the ammonium sulphate precipitation step was loaded on a heparin–agarose column, and fractions of 3 ml were collected as described in the text. The activity of the enzyme was measured by incubating the reaction mixtures (0.2 ml) containing 50 m*M* HEPES (pH 7.0), [r^{32}P]ATP (50 000 cpm), 1 m*M* ATP, 1 m*M* glycerol, 0 1 mg of bovine serum albumin and 5 μl of 100-fold diluted each fraction. After incubation at 37°C for 15 min, the [^{32}P]L-α-glycerol phosphate produced was measured as described by Thorner and Paulus [3,6]. The specific activity is expressed relative to the amount of protein in each fraction. (B) SDS-PAGE: an aliquot of each fraction (from 39 to 57) was analysed on the 10% gel to determine the purity of glycerol kinase; the activity of the enzyme in each fraction was proportional to the intensity of the Coomassie-stained protein band shown.

Assay

A variety of protocols for assaying the activity of glycerol kinase has been reported [1–10]. For example, Thorner and Paulus [3,6] used [r^{32}P]ATP for radiochemical determination of the product [^{32}P]L-α-glycerol phosphate. In their

TABLE I

SUMMARY OF PURIFICATION

Determination of glycerol kinase activity was carried out under linear assay conditions as described in ref. 3. One unit (U) is defined as 1 nmol [^{32}P]L-α-glycerol phosphate produced per hour.

Step	Total protein (mg)	Total activity (U×10^3)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Crude extract	4100	189	46	100	1
Heat treatment	572	184	322	97	7
Ammonium sulphate precipitation	202	163	805	86	18
Heparin–agarose	75.5	142	1882	75	41

assay, inorganic orthophosphates (Pi) were separated from the products by precipitating Pi with molybdate after the hydrolysis of remaining [r^{32}P]ATP to Pi. However, this assay and others seem to be rather time-consuming and laborious. Therefore, to improve the assay method, we used the PEI cellulose plates to separate the [^{32}P]glycerol phosphate produced from [r^{32}P]ATP and glycerol (Fig. 2). As shown in this autoradiogram, a clear separation of glycerol phosphate from Pi and ATP was achieved by ascending chromatography in 1 *M* lithium chloride. The relative mobility (R_F value) for glycerol phosphate was found to be 0.73. The remaining ATP and Pi migrated as a single spot with an R_F of 0.02 (lane B). Therefore, for routine assays, the radioactivity in the top half of the plate, which contains only glycerol phosphate, was measured. With this protocol, the production of glycerol phosphate increased linearly with increasing amount of the enzyme up to 30 ng (Fig. 3).

PEI cellulose plates have been widely used for the separation of phosphorylated compounds, such as in ATPase assays [14,15]. In our assay, ammonium molybdate was added when the reaction was terminated. This agent formed a complex with free Pi that contaminate the ATP and thus arrested this migration during the ascending chromatography. Normally, the mobility of the free Pi on the PEI plate is very close to that of glycerol phosphate. However, these compounds were well separated by the addition of ammonium molybdate (Fig. 2). Thus, the activity of glycerol kinase can be accurately assayed even in samples contaminated with certain ATPase or phosphatase activities.

The assay protocol described above has a number of advantages over the procedures reported previously: (1) it is rapid because the only time-consuming steps are sample spotting and the ascending chromatography of PEI plates, which together take less than 15 min for a plate carrying 20 assay samples; (2) it is quite reproducible because it is not necessary to remove other labelled species (e.g., ATP or Pi) by precipitation with salt or by washing off filter papers; (3) it is sensitive because, unlike other protocols, the concentration of glycerol in samples containing as little as 4 nmol of glycerol, can be determined (Fig. 3).

Rapid and accurate determination of glycerol or of triglyceride concentration

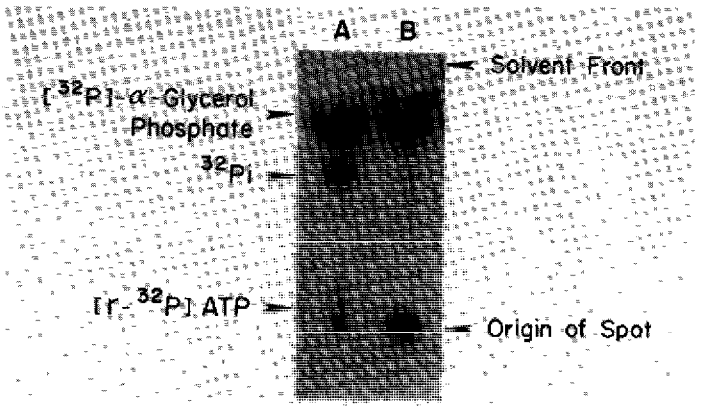

Fig. 2 Separation of glycerol phosphate from ATP and Pi. The reaction mixtures containing 30 ng of the purified glycerol kinase were prepared as described in the text. After incubation at 37°C for 30 min, the reaction was terminated by adding 5 μl of either a solution containing 5% (w/v) SDS (lane A) or a solution containing 70 m*M* ammonium molybdate in addition to 5% SDS (lane B). The reaction products were then separated by ascending chromatography on a PEI cellulose plate (2×6 cm) and exposed to X-ray film.

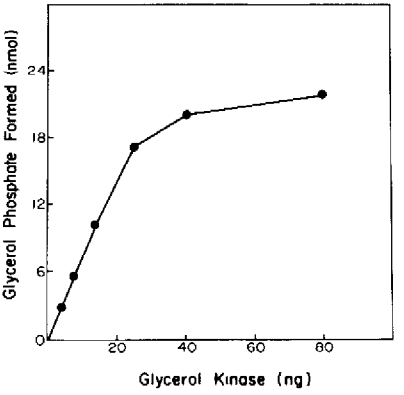

Fig. 3. Determination of glycerol kinase activity with the new assay protocol. Reaction mixtures containing various amounts of the enzyme (from 4 to 80 ng) were incubated at 37°C for 15 min. The reaction was then terminated by adding 5 μl of the solution containing SDS and ammonium molybdate. These data were reproduced with less than 5% error in three different trials of this experiment.

in blood or in tissue is of importance in medical diagnosis and clinical investigations. We have also found that this protocol can be used for assaying the glycerol concentration in human serum. The concentration of glycerol in the serum samples was estimated to range from 0.05 to 0.1 m*M* (data not shown), which is in good agreement with earlier reports [16–19]. Thus, these improvements in the methods for purification of the enzyme and in its assay might have applications not only in research but also in medical diagnosis and industrial biotechnology.

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