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PURIFICATION OF PHOSPHOFRUCTOKINASE USING TRANSITION-STATE ANALOGUE AFFINITY CHROMATOGRAPHY

S. P. J. BROOKS and K. B. STOREY*
Institute of Biochemistry and Department of Biology, Carleton University, Ottawa, Ontario K1S 5B6 (Canada)
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SUMMARY

A novel purification of phosphofructokinase has been achieved in a two step process using ion-exchange affinity chromatography and a transition-state analogue affinity column matrix. The procedure can be performed in one day, and gives a 25% yield of the starting material. The transition-state analogue chromatography is carried out using an ADP-agarose column in the presence of fructose 6-phosphate, magnesium ions and nitrate ions. In the presence of nitrate ion plus substrate, phosphofructokinase binds immobilized ADP while other proteins pass through the column. Previous studies with creatine kinase have shown that the nitrate ion mimics the planar phosphate in the transition state resulting in a complex which is stable under the relatively high ionic strength of the column buffer. This permits the elution of phosphofructokinase in a single peak of high specific activity. This column typically results in a 20–30 fold increase in specific activity with only a small loss of activity.

INTRODUCTION

Phosphofructokinase (PFK) can exist in several different states *in vivo* depending on the degree of enzyme polymerization and subunit phosphorylation. Both these factors regulate the enzyme's kinetic activity with the polymerized enzyme having a higher activity[1–3], and the phosphorylated enzyme having a lower activity, although this has only been unequivocally demonstrated in lower vertebrates and invertebrates[4–7]. In order to investigate the importance of these effects on PFK catalytic activity it is necessary to analyze the purified enzyme under various *in vitro* conditions. Because of the known instability of the enzyme during purification it is important to devise purification schemes which incorporate the least number of steps, and which can be performed in the shortest amount of time. The present paper presents a rapid purification of PFK which requires only two chromatography steps and two polyethylene glycol precipitations. The procedure can be completed in a single day minimizing losses due to inherent enzyme instability. The protocol represents an extension of previous work on kinase transition states which indicated that nitrates could mimic planar phosphate groups in the transition state allowing a stable complex to form between substrate, ADP and nitrate ion[8,9].

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MATERIALS AND METHODS

Animals, tissues and chemicals

Turtles (*Pseudymes scripta*) were obtained from Carolina Biological Supply Company (Burlington, NC, U.S.A.) and kept in dechlorinated water at 10–14°C. Tissues were removed within one minute after decapitation, frozen in liquid nitrogen, and stored at −80°C until use. All chemicals were obtained from Sigma (St. Louis, MO, U.S.A.) or Boehringer-Mannheim (Montreal, Canada) and were of the highest quality available.

Enzyme and protein assays

All enzyme activities were performed at pH 7.0 in 50 m*M* imidazole buffer containing 50 m*M* potassium chloride, 5 m*M* magnesium chloride and 2 m*M* potassium phosphate at 20°C. PFK was assayed in the presence of 0.5 m*M* MgATP, 4 m*M* fructose 6-phosphate, 15 m*M* ammonium chloride, 0.15 m*M* NADH, and 1 IU each of aldolase, triose phosphate isomerase, and α-glycerol phosphate dehydrogenase. Adenylate kinase activity was assayed using 1 m*M* MgATP, 2 m*M* AMP, 5 m*M* phospho(enol)pyruvate, 0.15 m*M* NADH, and 1 IU of lactate dehydrogenase and pyruvate kinase. Phosphoglucose isomerase activity was assayed using 10 m*M* fructose 6-phosphate, 0.15 m*M* NADH, and 1 IU of glucose 6-phosphate dehydrogenase. Reactions were monitored by following NADH disappearance at 340 nm using a Gilford Model 240 spectrophotometer.

Protein concentration was determined by the change in absorbance at 280 nm (DEAE-Sephadex G-25 column chromatography), by the Bio-Rad protein assay kit (Coomassie Blue G-250 dye-binding method[10]), or by measuring the reaction with *o*-phthalaldehyde[11]. Bovine serum albumin was used as the standard for all protein measurements.

Purification of phosphofructokinase

All steps of the purification were performed at 22°C.

Crude homogenate. Approximately 0.4 g of tissue was homogenized with 4 volumes of homogenization buffer [50 m*M* imidazole, pH 7.0, 30 m*M* β-mercaptoethanol, 5 m*M* EDTA, 5 m*M* EGTA, 100 m*M* sodium fluoride, 1 m*M* phenylmethylsulfonyl fluoride (PMSF)], and centrifuged at 16 000 *g* for 10 min.

First polyethylene glycol (PEG) precipitation. The supernatant from the previous step was made up to 11% (w/v) PEG 8000 and mixed at room temperature for 20 min. The suspension was centrifuged at 12 000 *g* and the pellet was resuspended by adding 0.5 ml of buffer A (20 m*M* potassium phosphate, pH 7.2, 1 m*M* EDTA, 5 m*M* β-mercaptoethanol, 1 m*M* fructose 6-phosphate, 30 m*M* sodium fluoride) to the pellet and gently homogenizing.

DEAE Sephadex G-50 chromatography. The resuspended pellet from step 2 was loaded on a 1.5-ml DEAE-Sephadex G-50 column pre-equilibrated with buffer A. The column was washed with 20 ml buffer A, and developed with a 15-ml 0–0.6 *M* phosphate gradient in buffer A (see Fig. 1). Fractions of 1 ml were collected, and the tubes with specific activity greater than 2 IU/mg were pooled.

Second PEG precipitation. The pooled fractions from step 3 were made up to 20% (w/v) PEG, mixed 20 min, and centrifuged for 20 min at 16 000 *g*. The supernatant

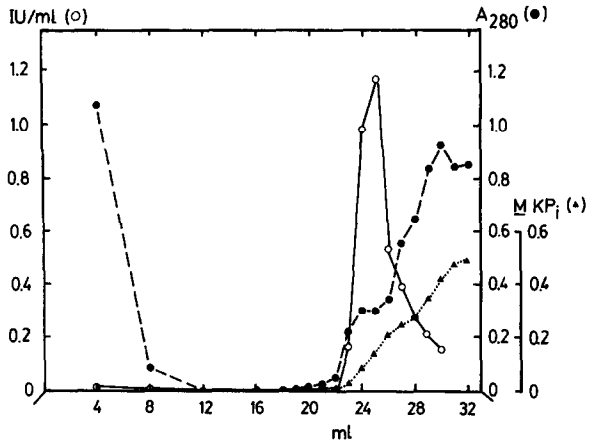

Fig. 1. DEAE Sephadex G-50 column chromatography of turtle white muscle PFK. The resuspended pellet from step 2 was loaded on a 1.5-ml DEAE-Sephadex G-50 column previously equilibrated with buffer A. The column was washed with 20 ml of buffer A to elute non-bound protein. After the column eluent was free of contaminating protein ($A_{280} = 0$), the column was developed with a 15-ml gradient of 0–0.6 *M* potassium phosphate at pH 7.2 in buffer A (▲). Fractions of 1 ml were collected and analysed for protein (A_{280}, ●), and PFK activity (○). Analysis of adenylate kinase and phosphoglucomutase activity indicated that these enzyme activities did not bind to the column matrix (data not shown).

was discarded and the pellet resuspended in 0.5 ml of buffer B [20 m*M* 4-(2-hydroxyethyl)-1-piperazimethanesulphonic acid (HEPES), pH 8, 25 m*M* β-mercaptoethanol, 30 m*M* potassium nitrate, 2 m*M* magnesium chloride, 10 m*M* fructose 6-phosphate].

ADP-agarose affinity chromatography. The enzyme was then loaded on a 1 ml ADP-agarose column (adenosine 5′-diphosphate agarose, Sigma No. 4398, attached through the N^6-amino group) which was pre-equilibrated in buffer B. The column was washed with 15 ml of buffer B and developed with a 15 ml of 0–5 m*M* MgATP gradient. Fractions of 0.5 ml were collected and those with a specific activity greater than 100 IU/mg were pooled.

Concentration and storage of the enzyme. The final enzyme was placed in a dialysis bag, concentrated against solid sucrose and dialyzed against 500 ml of stabilization buffer (SB) (40 m*M* potassium phosphate, pH 7.0, 10 m*M* β-mercaptoethanol, 0.1 m*M* EDTA, 2% (v/v) glcerol). The final enzyme is stored in SB plus 6 m*M* fructose 6-phosphate. Note that the final enzyme is unstable and looses activity quickly, even when stored at 4°C. Consequently, it should not be stored for periods greater than one week.

RESULTS AND DISCUSSION

Table I shows the results of a typical purification of PFK from turtle white muscle. The enzyme was purified approximately 240 fold and the final specific activity is similar to that reported for other PFK enzymes isolated from other animal tissues[12–15].

The procedure presented in this paper can also be used to purify PFK isozymes from other turtle tissues. For example, both turtle heart and turtle brain PFK were

TABLE I

PURIFICATION OF PHOSPHOFRUCTOKINASE FROM TURTLE WHITE MUSCLE

Enzyme was isolated from approximately 0.43 mg white muscle tissue.

Stage	*Total protein (mg)*	*Total activity (IU)*	*Specific activity (IU/mg)*	*Yield (%)*	*Purification (fold)*
Crude	17.5	7.72	0.44	100	1
PEG pellet No. 1	14.7	6.65	0.45	86	1
DEAE-Sepharose G-25 peak fractions	0.85	5.99	7.05	78	16
PEG pellet No. 2	0.40	3.57	8.93	46	21
ADP-Agarose peak fractions	0.012	1.352	112.67	18	256
Concentated enzyme	0.010	1.063	106.30	14	242

purified to similar specific activities (150 and 95 IU/mg respectively) by using the protocol outlined in this paper. It was important to increase the fructose 6-phosphate concentration to 30 m*M* and decrease the potassium nitrate concentration to 25 m*M* when purifying these latter isozymes because a large proportion of these enzymes did not bind to the column when a lower concentration of fructose 6-phosphate was used. The requirement for increased fructose 6-phosphate concentrations reflects the different kinetic constants for these two isozymes; both turtle brain and turtle heart PFK have K_m values which are 5–10 fold higher than that measured for the muscle isozyme. Since the ADP-agarose column functions by binding PFK through the active site, the concentration of fructose 6-phosphate in buffer A must be changed to compensate for this fact.

It was important to include sodium fluoride and EDTA in both the homogenization buffer and buffer A to prevent any changes in the phosphorylation state of the enzyme during the purification procedure. Sodium fluoride is a potent competitive inhibitor of alkaline phosphatase and EDTA removes Mg^{2+}, a necessary component of protein kinase reactions. The rapidity of the procedure helps to minimize the modifications which may occur during isolation, as does the addition of PMSF to the initial homogenization medium.

The graph of Fig. 1 shows the elution profile of PFK from the DEAE-Sephadex G-50 column. This step resulted in an approximately 16-fold purification of the enzyme, with 75% of the enzyme recovered in a single peak (see Table I). Before applying the peak fractions from the DEAE column to the ADP-agarose column, it is necessary to precipitate the enzyme with PEG. This step serves to concentrate the enzyme so that it may be resuspended in a small amount of buffer B at a relatively low phosphate concentration. Thus, if the DEAE peak fractions are simply brought up to 10 m*M* fructose 6-phosphate and 30 m*M* potassium nitrate in buffer A, the enzyme does not bind to the ADP-agarose column.

Elution of PFK from the ADP-agarose column is accomplished with a 0–5 m*M* MgATP gradient (Fig. 2). The enzyme elutes at a MgATP concentration of 0.1 m*M* (a value which reflects the K_m value for MgATP of 30 μM). The specific activity of the enzyme is closely monitored at this point so that only pure enzyme is collected. Note that the first fraction containing activity has contaminating protein and is usually discarded.

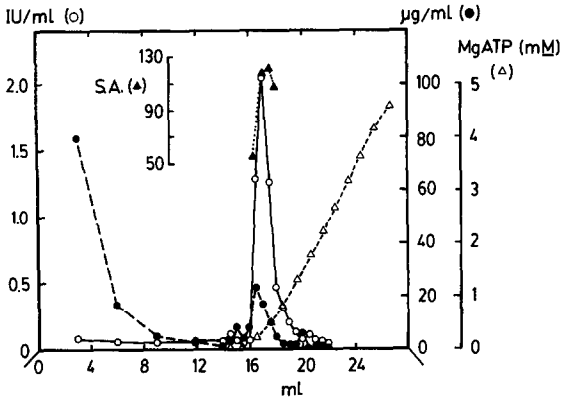

Fig. 2. ADP–agarose column chromatography of turtle white muscle PFK. The second PEG precipitate was resuspended in 0.5 ml buffer B and loaded on a 1-ml ADP-agarose column previously equilibrated in buffer B. The column is washed with buffer B until no further protein is washed from the column (approximately 15 ml). The column is then developed with a 15-ml 0–5 m*M* MgATP gradient in buffer B. Fractions of 0.5 ml are collected and monitored for protein concentration (*o*-phthalaldehyde method) and activity. Only fractions with a high specific activity are collected.

The protocol for the transition state analogue affinity chromatography step can be modified to enable one to purify several different enzymes which have ATP as one of their substrates. The principal of the column procedure was initially developed for creatine kinase[9] and is based on initial work by Milner-White and Watts[8] who demonstrated that a transition state analogue could be formed if ADP, substrate, and potassium nitrate are incubated together at an appropriate pH value. The nitate anion mimics a planar phosphate in the transition state and serves to bridge the ADP and substrate, forming a relatively stable (and highly specific) complex. The specificity of the column allows one to selectively purify different enzymes by adding excess of specific substrates and, as such, can be considered a general tool for purifying a number of different kinase molecules.

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