PARTIAL PURIFICATION AND EFFECT OF ADENINE NUCLEOTIDES ON THE ACTIVITY OF PHOSPHOGLYCERATE KINASE OF GERMINATING MUNG BEANS (*PHASEOLUS AUREUS*)

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ABSTRACT

In mung beans phosphoglycerate kinase activity is maximum at 36 hr of germination, after which it decreases sharply. The enzyme has been partially purified from mung beans. The enzyme activity assay solution contains 3-phosphoglycerate, ATP, NADH, glyceraldehyde 3-phosphate dehydrogenase (excess) and phosphoglycerate kinase (rate limiting). At 10 mM ATP, there is an initial lag period (20 sec) during which the rate of disappearance of NADH increases gradually before a steady state rate is reached. No lag is observed at lower ATP concentrations (up to 5 mM) or if ADP (1–100 μM) is present in the assay solution.

INTRODUCTION

PHOSPHOGLYCERATE kinase (ATP: D-3-phosphoglycerate 1-phosphotransferase; EC. 2.7.2.3) (PGK), an enzyme of glycolytic pathway has been purified from a large number of sources such as skeletal muscle[1], erythrocytes[2], kidney[3], halibut muscle[4], yeast[5], *Fasciola hepatica*[6], *E. coli*[7] and thermophilus bacteria[8]. There are very few reports on phosphoglycerate kinase of higher plants[9,10], where it is involved in glycolysis as well as carbon fixation in some tissues[11]. In the present paper, we report the partial purification of phosphoglycerate kinase from germinating mung beans and modulation of its activity by adenine nucleotides.

MATERIALS AND METHODS

Tris-(hydroxymethyl) aminomethane (Tris), glyceraldehyde-3-phosphate dehydrogenase (GPDH), 3-phosphoglyceric acid, NADH, CM-cellulose, ATP, bovine serum albumin, ADP and ammonium persulphate, were purchased from Sigma Chemical Co., St. Louis, Missouri, USA; acrylamide, N,N′-methylene bisacrylamide, riboflavin, N,N,N′,N′-tetramethyl-ethylenediamine from T. Schuchardt Munich, Germany; amidoblack from SDS Lab Chem. Industry. A commercial sample of acetone was refluxed over potassium permanganate for 4–5 days (till the pink colour persisted) and distilled twice before use. EDTA, sucrose and acetic acid were guaranteed reagents from Sarabhai M. Chemicals, India. Other chemicals were of analytical reagent grade. All solutions were prepared in double distilled water from an all-glass assembly.

Enzyme Assay: The enzyme (PGK) is assayed by combining its reaction with that of GPDH of a different source (skeletal muscle or yeast). Assay buffer contained 30 mM Tris, 5 mM magnesium chloride and 10 mM 3-phosphoglyceric acid. Its pH was adjusted to 7.5 with 1 M hydrochloric acid. A 5 mM solution of ATP was prepared in the assay buffer. The test solution contained 0.9 ml ATP solutions in assay buffer 0.1 ml freshly prepared NADH (0.2 mM) and 0.01 ml GPDH (1000 units/ml). The reaction was started by adding 0.01 ml of appropriately diluted PGK solution. Disappearance of NADH was monitored by noting the absorbance at 366 nm at 15 sec intervals on an Eppendorf photometer. ε_{NADH} at 366 nm is 3.11 $\times 10^3$ M^{-1} cm^{-1}. An enzyme unit has been defined as the amount of enzyme which brings about the oxidation of 1 μ mole of NADH in one minute under our test condition.

Protein was estimated by the method of Lowry *et al*[12]. The specific activity is given in terms of enzyme units/mg protein.

Germination of mung beans: The seeds (*Phaseolus aureus*) were soaked for 5–6 hr and then spread over moist filter paper on a moist sand bed for the required period of time.

Isolation of enzyme: The following steps were carried out at 0–4°C, unless stated otherwise.

1. *Extraction of enzyme*: Sixty five grams of 36 hr germinated seeds were homogenized in 100 ml of 10 mM Tris buffer containing 1 mM EDTA, pH 6.7 (extraction buffer) and centrifuged to get a clear extract.

2. *Acid treatment*: The crude extract was brought to pH 5.0 with 1 M HCl added dropwise with vigorous stirring. The suspension was centrifuged and pH of supernatant adjusted to 6.7 with 1 M Tris solution.

3. *Ammonium sulphate fractionation*: The above solution was brought to 65% $(NH_4)_2SO_4$ saturation by adding solid ammonium sulphate with continuous stirring for 12–15 min. The contents were then centrifuged. The pellet was discarded and the supernatant brought to 82% $(NH_4)_2SO_4$ saturation. After 1 hr stirring the precipitate was collected by centrifugation and suspended in 10 ml extraction buffer.

4. *Acetone fractionation*: The above solution was cooled to semifrozen state and treated with precooled acetone (−15°C) with vigorous shaking (5 ml acetone/10 ml solution), centrifuged and the precipitate discarded. To the supernatant, more acetone was added at −15°C (6 ml acetone/10 ml supernatant). The precipitate was collected and suspended in 4 ml extraction buffer.

5. CM-*cellulose treatment*: The above enzyme solution was added to CM-cellulose which had been previously washed according to the method of Peterson and Sober[13] and equilibrated with the extraction buffer (1 g CM-cellulose/100 mg protein). The suspension was stirred for 1 hr and centrifuged and the supernatant was collected.

A sample protocol of this procedure is summarised in table 1.

Polyacrylamide gel electrophoresis

Gel electrophoresis of enzyme solution was performed at pH 8.3 as described by Reisfield *et al*[14]. Protein bands were stained as usual with amidoblack. The partially purified enzyme showed three protein bands.

RESULTS AND DISCUSSION

Effect of germination on PGK *activity*: In mung beans, there is a gradual increase in PGK activity from 0 to 36 hr of germination. At longer periods the activity declines sharply (figure 1). The specific activity is maximum in the 30–42 hr period. The enzyme was partially purified with 36 hr germinated seeds (table 1).

Effect of adenine nucleotides on PGK *activity*: The conditions for enzyme assay described earlier are optimum for the PGK activity. Absorbance versus time plot is linear for 60–90 sec and facilitates determination of the initial steady state rate of reaction. At higher ATP concentrations (10 mM and above), the

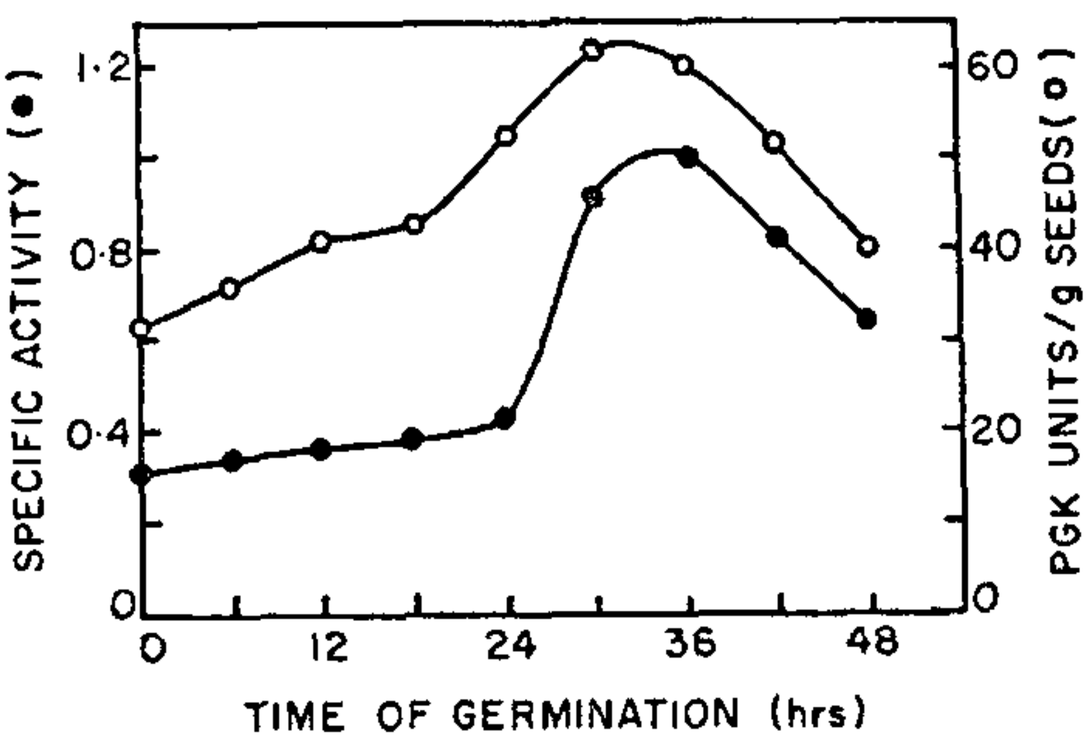

Figure 1. Effect of time of germination on the PGK activity of mung beans. Seeds (2 g) were thoroughly washed, soaked in tap water for 5–6 hours and spread over mosit filter paper on a moist sand bed. After the desired time intervals, the seeds were homogenised in 10 ml of extraction buffer. The suspension was centrifuged and the clear extract assayed for PGK activity and protein. PGK activity is expressed as units/g dry seeds and specific activity as units/mg protein.

Table 1 *Summary of purification of phosphoglycerate kinase from 65 g germinated green gram seed.*

Step	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification	Recovery (%)
Crude extract	98	770.3	842.8	0.90	—	—
Acid treatment	95	679.2	568.1	1.19	1.3	88.2
Ammonium sulfate fractionation	10	650.0	57.9	11.81	13.1	84.4
Acetone fractionation	4	442.0	23.2	19 27	21.4	57.4
CM-cellulose treatment	4	416.0	15.5	26.71	29.7	53.7

reaction shows a lag period (figure 2, curve 1), which varies with ATP concentration (table 2). No lag is observed at 1–5 mM ATP. The lag period increases from 10 to 20 mM ATP. At still higher concentrations, ATP inhibits the reaction completely.

It appears that at 10 mM or higher ATP concentrations, the reaction is *initially* inhibited and that this inhibition is overcome when the reaction has progressed for a short period. Since the concentrations of the reactants do not change significantly during this period, it may be inferred that the reversal of inhibition is due to some product of the reaction, which must be effective at very small concentrations. Products of the combined action of PGK and GPDH are ADP, NAD^+ and glyceraldehyde 3-phosphate. Addition of even small concentrations of ADP (1–10 μM) brought about complete disappearance of the lag (figure 2 curve 2). Higher concentrations of ADP (1 mM) are inhibitory (data not shown). Addition of NAD^+ or glyceraldehyde 3-phosphate had no effect on the reaction rate or the lag period.

PGK isolated from other sources has not been reported to be sensitive to the adenine nucleotides in the manner described above. From the present data, it appears that the activity of PGK in mung beans (and consequently the rate of glycolysis) may be under a fine control through the ATP/ADP ratio. Details of this phenomenon are being worked out.

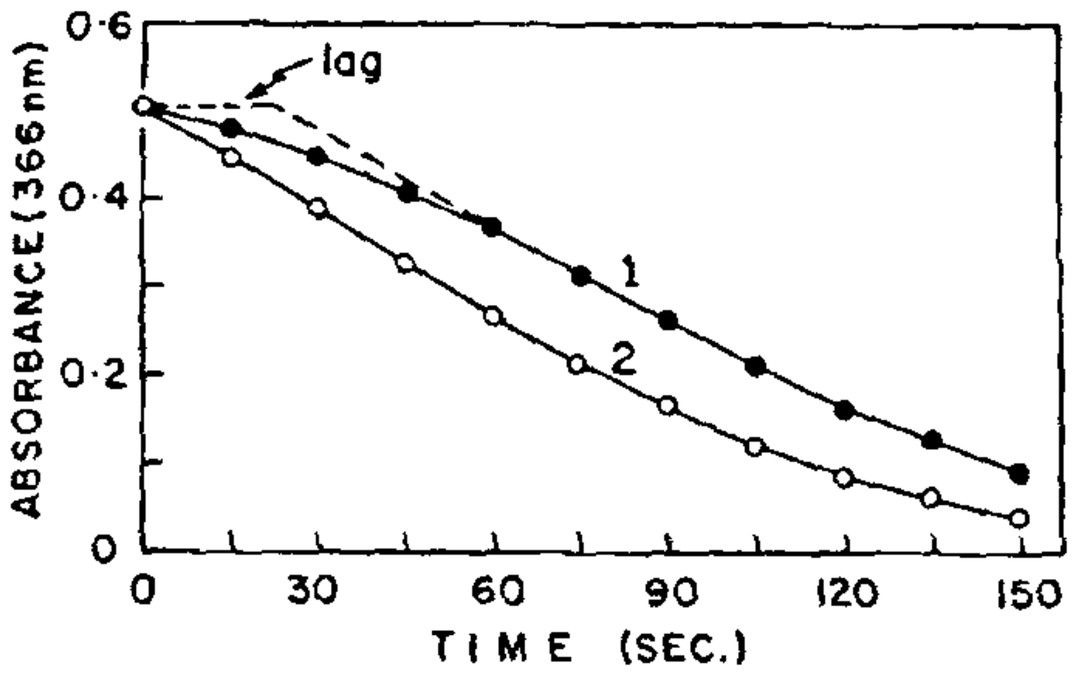

Figure 2. Effect of adenine nucleotides on the activity of phospho-glycerate kinase of mung beans. Concentrations of reactants (other than ATP and ADP) and pH and procedure are described under methods. ATP concentration was 10 mM. In curve 2, 10 μM ADP was also added. PGK (3.8 μg protein/ml) was added at zero time and absorbance at 366 nm was monitored. Duration of lag period has been calculated by extrapolation as indicated.

Table 2 *Lag period observed in assay of PGK activity at different adenine nucleotide concentrations.*

ATP (mM)	Lag period* (sec)
1	Nil
2.5	Nil
5	Nil
10	20
15	24
20	27
30	Complete inhibition
10 + ADP (1–100 μM)	Nil

*lag period was determined as shown in figure 2 (curve 1).

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