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GLUCOKINASE OF PEA SEEDS

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Summary

1. Glucokinase (ATP : D-glucose 6-phosphotransferase, EC 2.7.1.2) was extracted from pea seeds and purified by fractionation with $(NH_4)_2SO_4$ and chromatography on DEAE-cellulose and Sephadex.

2. The relative rates of phosphorylation of glucose, mannose and fructose (final concentration 5 mM) were 100, 64 and 11.

3. The K_m for glucose of pea-seed glucokinase was 70 μM and the K_m for mannose was 0.5 mM. The K_m for fructose was much higher (30 mM).

4. Mg^{2+} ions were essential for activity. Mn^{2+} could partially replace Mg^{2+}.

5. Enzyme activity was not inhibited by glucose 6-phosphate. A number of other metabolites had no effect on glucokinase activity.

6. Pea-seed glucokinase was inhibited by relatively low concentrations of ADP.

Introduction

The reaction catalyzed by hexokinase (ATP : D-hexose 6-phosphotransferase, EC 2.7.1.1) is the principal means of incorporating hexoses into metabolism. The hexokinases from yeast and mammalian tissues have been studied extensively and it is clear that a number of enzyme components are involved, some of which have regulatory properties [1,2]. One isozyme of yeast hexokinase is activated at sub-optimal pH by low concentrations of citrate, malate, P_i, 2-*P*-glycerate, 3-*P*-glycerate, CTP and GTP [3]. Mammalian hexokinases are strongly inhibited by glucose-6-*P*, and it is accepted that in mammalian tissues metabolic control of phosphofructokinase regulates the glucose-6-*P* concentration which in turn regulates hexokinase activity [4,1,2]. In addition to the hexokinases, a glucokinase (ATP : D-glucose 6-phosphotransferase, EC 2.7.1.2) was found in liver [5—8] and glucokinases have been subsequently reported from some other mammalian tissues [9] and yeast [10]. The enzyme from rabbit liver phosphorylated fructose at 8% of the rate given by glucose [8]. Gluco-

kinase from liver is not inhibited by glucose-6-*P* and is believed to be significant in the synthesis of glycogen from glucose in this tissue [7]. A soluble glucokinase has also been found in the slime mould *Dictyostelium discoideum* [11] and a particulate enzyme has been reported from *Euglena gracilis* [12].

Less information is available on the properties of hexokinases from higher plants. Saltman [13] demonstrated both particulate and soluble hexokinase in a number of plant tissues. The enzyme preparation from wheat germ phosphorylated glucose, fructose, mannose and glucosamine at relative rates of 1.00, 1.62, 0.68 and 0.52 [13]. Subsequently, Medina and Sols [14] reported that homogenates from immature pea seeds have, in addition to a particulate hexokinase which phosphorylated glucose and fructose, a soluble fructokinase (ATP : D-fructose 6-phosphotransferase, EC 2.7.1.4). This enzyme phosphorylated glucose at 8% of the rate given by fructose. Nectaries also contain a soluble fructokinase in addition to a particulate hexokinase [15]. Sugar-beet leaf petioles contain both particulate and soluble non-specific hexokinases as well as a soluble fructokinase [16]. Two Michaelis constants for glucose were found with hexokinase from germinating maize scutellum and it was suggested this may indicate the presence of a glucokinase in addition to hexokinase [17].

In the present investigation, the soluble hexokinases from mature pea seeds were fractionated on DEAE-cellulose. Re-chromatography on DEAE-cellulose yielded a glucokinase which had a high affinity for glucose. The enzyme had little activity with fructose. Pea-seed glucokinase was not inhibited by glucose-6-*P* but was inhibited by ADP.

Materials and Methods

Materials

Mature pea seeds (*Pisum sativum* L. var. Progress No. 9) were obtained from F. Cooper Ltd., Wellington, New Zealand. Glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, lactate dehydrogenase, pyruvate kinase, phosphoglucose isomerase, phosphomannose isomerase, NADP, NADH, ATP and other nucleotides, Glc-6-*P*, Fru-6-*P*, Fru-1,6-P_2, 6-*P*-gluconate, 2-*P*-glycerate, 3-*P*-glycerate, 2,3-P_2glycerate, *P-enol*pyruvate, Tris, glucose, mannose, fructose, galactose, sorbose and phenylmethanesulphonyl fluoride were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A., or from Boehringer Mannheim GmbH, Mannheim, (G.F.R.). DEAE-cellulose (DE-32, microgranular) was obtained from Whatman, England, and Sephadex G-200 from Pharmacia (South Seas) Pty. Ltd.

Extraction and fractionation of hexose kinases

Pea seeds were ground and defatted with ether as described by Turner [18]. All subsequent operations were carried out at 2° C. Ether-extracted pea powder (120 g) was suspended in 360 ml of 50 mM $NaHCO_3$ containing 5 mM $MgCl_2$ and 1 mM EDTA (pH 7.8), and rotated on a mechanical roller for 2 h. The mixture was centrifuged at 3000 × *g* for 10 min and the supernatant, after filtration through glass wool and further centrifugation at 23000 × *g* for 30 min was termed the crude enzyme. The crude enzyme was treated with saturated $(NH_4)_2SO_4$ (pH 7.9) and centrifuged at 23000 × *g* for 20 min. The fraction

which precipitated between 30 and 50% saturation was dissolved in 12 ml of 10 mM Tris buffer (pH 7.5) containing 1 mM EDTA, and dialyzed for 2 h against the same buffer.

A volume of the dialyzed ammonium sulphate preparation containing 1000—3000 mU of glucose phosphorylating activity was placed without delay on a DEAE-cellulose column (2.1 × 31 cm) previously equilibrated with 10 mM Tris buffer (pH 7.0) containing 1 mM EDTA (buffer A). The column was eluted with a gradient obtained by introducing 400 ml of 0.4 M KCl in buffer A into 400 ml of buffer A. Fractions of 7.3 ml were collected.

Purification of glucokinase

The fractions (total volume, 50 ml) containing the first peak of glucose phosphorylating activity from the DEAE-cellulose column were pooled, concentrated to approx. 5 ml by ultra-filtration on a Diaflo apparatus (PM-10 membrane, under 280—300 kPa/m^2 nitrogen pressure) and dialyzed against buffer A. The dialyzed material was placed on a DEAE-cellulose column (1.0 × 13 cm) equilibrated with buffer A and eluted with a linear gradient of KCl (0—0.4 M) in buffer A. Fractions of 2.4 ml were collected. Preparations of this type were used for most of the results reported in this communication.

For studies on the effect of concentration of glucose, glucokinase was further purified by treatment with Sephadex G-200. Active fractions from the second DEAE-cellulose column were combined and concentrated to half volume by centrifugation (1000 × *g*) in Amicon semipermeable membrane cones. The concentrate (5 ml) was applied to a Sephadex G-200 column (1.5 × 90 cm) equilibrated with buffer A and eluted with the same buffer. Fractions of 2.1 ml were collected. Fractions containing glucokinase but no detectable 6-phosphogluconate dehydrogenase activity were combined.

Assay of kinase activity

Kinase activity was normally assayed by coupling the production of glucose-6-*P* with the reduction of NADP in the presence of excess glucose-6-phosphate dehydrogenase. Reaction mixtures for the standard assay contained, in a total volume of 1.0 ml, 20 μmol Tris · HCl buffer (pH 8.2), 5 μmol $MgCl_2$, 5 μmol ATP, 0.33 μmol NADP, 0.6 μg glucose-6-phosphate dehydrogenase and an appropriate volume of pea-seed enzyme preparation. When fructose was used as substrate, 3 μg phosphoglucose isomerase was added, and with mannose as substrate 7 μg phosphomannose isomerase and 3 μg phosphoglucose isomerase were added. Correction was made for any reduction of NADP not due to kinase activity or due to hexokinase contained in the supplied glucose 6-phosphate dehydrogenase. The reactions were started by the addition of 5 μmol of hexose and the reaction mixtures were maintained at 39°C. The change in absorbance at 340 nm was followed. Hexose kinase activity was directly proportional to the amount of pea-seed enzyme preparation added. The glucokinase preparation obtained after two DEAE-cellulose fractionations followed by Sephadex G-200 treatment showed no detectable 6-phosphogluconate dehydrogenase activity. With all other preparations, assays were corrected for the presence of 6-phosphogluconate dehydrogenase. Reaction mixtures for the assay of 6-phosphogluconate dehydrogenase contained, in a total volume of 1.0 ml, 20

μmol Tris · HCl buffer (pH 8.2), 5 μmol $MgCl_2$, 0.67 μmol NADP and 0.5 μmol 6-*P*-gluconate. One miliunit (mU) of kinase activity is defined as 1 nmol of glucose-6-*P* produced per min per reaction mixture.

When the phosphorylation of sugars other than glucose, mannose and fructose was being studied, ADP production was coupled with the oxidation of NADH using pyruvate kinase and lactate dehydrogenase. This assay method was also used for investigation of the effects of glucose-6-*P* and fructose-6-*P* on the kinase reaction. Reaction mixtures (total volume, 1.0 ml) contained 20 μmol Tris · HCl buffer (pH 8.2), 5 μmol $MgCl_2$, 20 μmol KCl, 0.5 μmol *P-enol*-pyruvate, 0.14 μmol NADH, 5 μmol ATP, 8 μg pyruvate kinase, 16 μg lactate dehydrogenase and pea-seed enzyme. Reactions were started by addition of hexose. Controls without hexose were used to correct for phosphatase acting on *P-enol*pyruvate and ATP.

Results

DEAE-cellulose chromatography

In preliminary experiments, there were indications that several hexose kinase activities were present in pea-seed extracts. Fig. 1 shows the kinase activities with glucose, fructose and mannose as substrates obtained in fractions after DEAE-cellulose chromatography. For convenience, the eluates were grouped into four main fractions designated I, II, III and IV in order of elution (Fig. 1). Fraction I had higher phosphorylating activity with glucose than with mannose or fructose. There was no activity with galactose as substrate. Fraction II phosphorylated glucose and mannose and, as with Fraction I, the activity with mannose as substrate parallelled that with glucose. Fraction III phosphorylated fructose and has less activity with glucose or mannose as substrate. Fraction IV

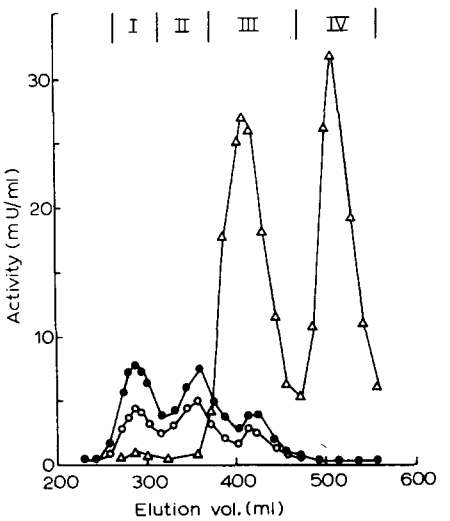


Fig. 1. DEAE-cellulose elution profile of hexose kinases from $(NH_4)_2SO_4$ fraction of pea seeds. The reaction mixtures were of the composition described for the standard assay. ●, glucose, ○, mannose; △, fructose. The eluates were pooled into four main fractions (I, II, III and IV) as indicated.

phosphorylated fructose but had very slight activity with glucose, mannose, galactose or sorbose. The addition of the protease inhibitor phenylmethanesulphonyl fluoride (final concentration 0.2 mM) during the extraction of the pea powder did not produce any change in this pattern of activity.

Purification of pea-seed glucokinase

Re-chromatography of Fraction I on DEAE-cellulose (Fig. 2) produced a single peak of hexose phosphorylating activity. The relative rates of phosphorylation of glucose, mannose and fructose (final concentration 5 mM) were 100, 64 and 11, respectively. This treatment also resulted in an increase in the peak specific activity (mU/mg protein) of glucokinase from 6.3 to 17. The crude pea extract and the 30 to 50% $(NH_4)_2SO_4$ fraction contained high levels of phosphatase acting on *P-enol*pyruvate, ATP and *p*-nitrophenol-*P*. A large part of this phosphatase activity was removed in the first DEAE-cellulose chromatography and there was a further 10-fold reduction in phosphatase in the second DEAE-cellulose fractionation. The glucokinase preparation from the second DEAE-cellulose chromatography contained a substantial amount of 6-phosphogluconate dehydrogenase. Use of a long column of Sephadex G-200 produced a peak specific activity of 50 for glucokinase and effected a partial separation of glucokinase from 6-phosphogluconate dehydrogenase. By selecting fractions a preparation of glucokinase free from 6-phosphogluconate dehydrogenase was obtained.

Effect of substrate concentration

The effect of concentration of glucose, fructose and mannose is shown in

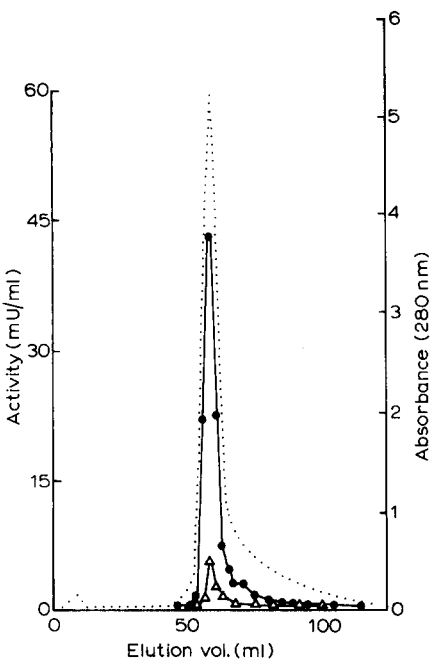


Fig. 2. Re-chromatography of Fraction I on DEAE-cellulose. The reaction mixtures were of the composition described for the standard assay, ●, glucose; △, fructose; · · · · · ·, absorbance (280 nm).

TABLE 1

Substrate	K_m (mM)	V	Relative rates (at 5 mM)
Glucose	0.07	16	100
Mannose	0.5	11	64
Fructose	30	10	11

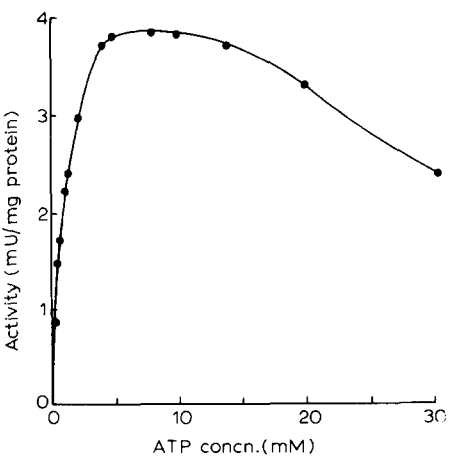
Table I. The K_m for glucose of pea seed glucokinase was 70 μM. Mannose and fructose were phosphorylated by the glucokinase preparation, galactose and sorbose were not phosphorylated. The K_m for fructose (30 mM) was much higher than the value for glucose. The V values for glucose, mannose and fructose were respectively: 16, 11 and 10 mU/min per mg protein.

Effect of concentration of ATP and Mg^{2+}

In the presence of 5 mM $MgCl_2$, increasing the concentration of ATP resulted in an increase in glucokinase activity (Fig. 3). The maximum rate was attained with an ATP concentration of approximately 5 mM. The apparent K_m for ATP in the presence of 5 mM $MgCl_2$ and 5 mM glucose was 1 mM. Pea-seed glucokinase required Mg^{2+} for activity (Fig. 4). Maximum activity was obtained when the concentration of Mg^{2+} was approximately equal to that of ATP (5 mM) and further increase in Mg^{2+} resulted in a decrease in activity. Mn^{2+} could partially replace Mg^{2+}.

Effect of pH

The activity of pea-seed glucokinase in a series of Tris · HCl and imidazole buffers established that the optimum pH was 8.2. Activities of 90% or more



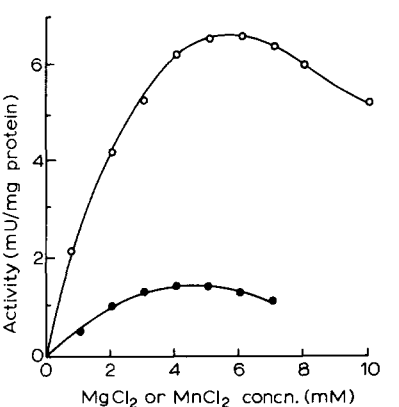


Fig. 3. Effect of ATP concentration on pea-seed glucokinase activity. The reaction mixtures were of the composition described for the standard assay, with the concentration of ATP varied as shown.

Fig. 4. Effect of Mg^{2+} and Mn^{2+} concentration on pea-seed glucokinase activity. The reaction mixtures were of the composition described for the standard assay, with the concentration of $MgCl_2$ or $MnCl_2$ varied as shown. ○, Mg^{2+}; ●, Mn^{2+}.

of the peak pH activity were maintained for pH values from pH 7.85 to pH 8.80.

Inhibition by ADP

Pea-seed glucokinase was inhibited by ADP (Figs. 5 and 6). This inhibition was dependent on the concentration of glucose (Fig. 5) and on the concentration of MgATP (Fig. 6). Under the standard assay conditions (5 mM glucose, 5 mM $MgCl_2$ and 5 mM ATP) 50% inhibition of pea-seed glucokinase was obtained with 1.1—1.4 mM ADP. In separate experiments it was found that UDP was less inhibitory than ADP: under conditions of the standard assay, 1 mM UDP inhibited pea-seed glucokinase by 11%. GDP and IDP (and also AMP) inhibited the reaction to approximately the same extent as UDP.

Effect of other metabolites

Glucose-6-*P* in concentrations ranging from 1—10 mM had no effect on the activity of pea-seed glucokinase. Other compounds which had no significant effect on the reaction rate were as follows: fructose-6-*P* (final concentration 10 mM), fructose-1,6-P_2 (1.0 mM), P_i (10 mM), *P-enol*pyruvate (1.0 mM), 2-*P*-glycerate (1.0 mM), 3-*P*-glycerate (1.0 mM), 2,3-P_2-glycerate (1.0 mM), ethanol (10 mM), lactate (10 mM), ADPglucose (1.0 mM), UDPglucose (1.0 mM), sucrose (10 mM), pyruvate (3.3 mM) and citrate (3.3 mM).

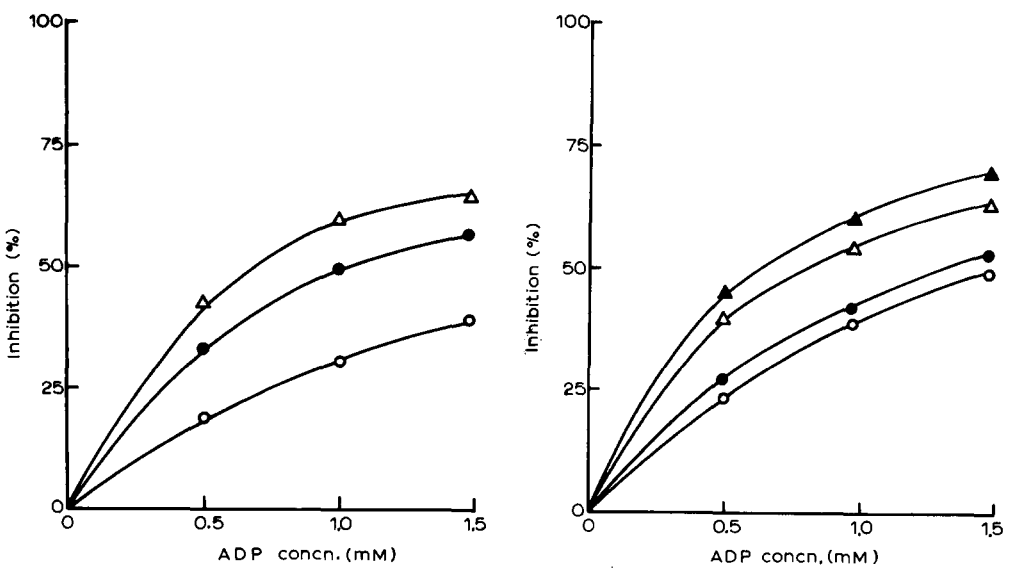


Fig. 5. Effect of ADP and glucose concentration on pea-seed glucokinase activity. The reaction mixtures were of the composition described for the standard assay at different concentrations and with the concentration of ADP varied as shown. △, 1 mM glucose; ●, 5 mM glucose; ○, 30 mM glucose.

Fig. 6. Effect of ADP and MgATP concentration on pea-seed glucokinase activity. The reaction mixtures were of the composition described for the standard assay at different concentrations of $MgCl_2$ and ATP and with the concentration of ADP varied as shown. ▲, 1 mM $MgCl_2$ + 1 mM ATP; △, 2.5 mM $MgCl_2$ + 2.5 mM ATP; ●, 5 mM $MgCl_2$ + 5 mM ATP; ○, 10 mM $MgCl_2$ + 10 mM ATP.

Effect of inhibitors

The addition of $CuSO_4$ (final concentration 1.0 mM), $HgCl_2$ (0.1 mM) and *p*-chloromercuribenzoate (0.1 mM) inhibited pea-seed glucokinase by 82, 73 and 70% respectively. The inhibition by *p*-chloromercuribenzoate was reversed by the addition of dithiothreitol (5 mM). No inhibition was observed with fluoride (10 mM) or arsenate (6 mM).

Discussion

The present investigation has established that a low K_m glucokinase can be obtained from pea seeds. The relative rates of phosphorylation with 5 mM glucose, mannose and fructose were 100, 64 and 11 respectively. The substrate concentration of 5 mM was selected on physiological and kinetic considerations and bears the same relation to the observed K_m glucose (pea-seed glucokinase) as that employed with rabbit liver glucokinase. Thus these figures may be compared with rabbit liver glucokinase where the relative rates with 100 mM glucose, mannose and fructose were 100, 80 and 8 respectively [8]. The K_m for glucose of pea-seed glucokinase was very low at 70 μM whereas that for fructose was 30 mM, i.e. a 400-fold difference. Although the Michaelis constants for rabbit liver glucokinase were much higher than for the pea-seed enzyme, there was again a marked disparity between the K_m values for glucose (12 mM) and fructose (>800 mM) [8]. The low activity of both enzymes with fructose was at least partly due to the low affinity of the enzymes for this hexose. The high affinity for glucose of pea-seed glucokinase may be significant in the metabolism of pea seeds as the concentration of this sugar in the seed is relatively low, e.g. approximately 1.3 mM at 30 days from flowering [19]. It is possible that the concentration of glucose in the cytoplasm is considerably lower than this figure.

Glucose-6-*P* did not inhibit pea-seed glucokinase but the enzyme was inhibited by relatively low concentrations of ADP (50% inhibition with 1.1—1.4 mM ADP under the standard assay conditions). The sensitivity of the enzyme to ADP inhibition was increased by lowering the concentration of glucose or MgATP. Rabbit liver glucokinase was not inhibited by glucose-6-*P* but was inhibited by ADP [8]. The concentration of ADP in developing pea seeds may rise to 1 mM [20] so it is conceivable that the ADP inhibition of pea-seed glucokinase could be of metabolic significance. There is a phase of rapid starch synthesis during the development of the pea seed [21—23] and the inhibition of glucokinase by ADP and the lack of inhibition by glucose-6-*P* may be consistent with the participation of glucokinase in starch formation.

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