Plant Physiol. (1977) 60, 666-669

# Fructokinase (Fraction IV) of Pea Seeds[1]

Received for publication June 1, 1977 and in revised form July 19, 1977

JOHN F. TURNER, DOROTHY D. HARRISON, AND LES COPELAND
*Department of Agricultural Chemistry, University of Sydney, N.S.W. 2006, Australia*

## ABSTRACT

**A fructokinase (EC 2.7.1.4) was obtained from pea (*Pisum sativum* L.) seeds. This enzyme, termed fructokinase (fraction IV), was specific for fructose as substrate and had little activity with glucose or mannose. Excess fructose inhibited the enzyme at the optimum pH (8.2) but not at pH 6.6. MgATP was inhibitory at pH 6.6. The apparent Michaelis-Menten constants at pH 8.2 were 0.057 mM for fructose and 0.10 mM for MgATP. $Mg^{2+}$ ions were essential for activity; $Mn^{2+}$ could partially replace $Mg^{2+}$. Fructokinase (fraction IV) had a requirement for $K^+$ ions which could be substantially replaced by $Rb^+$ or $NH_4^+$ but not by $Na^+$. The enzyme was inhibited by MgADP. The possible significance of fructokinase (fraction IV) in plant carbohydrate metabolism is discussed.**

Phosphorylation of hexoses by ATP is usually the initial reaction in the metabolism of these sugars. There are two native isoenzymes of yeast hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) and these differ with respect to glucose phosphorylation rates and regulatory properties (1, 11). Mammalian hexokinases have been separated into four fractions three of which are inhibited by glucose-6-P (1, 11). The other fraction is a glucokinase (ATP:D-glucose 6-phosphotransferase, EC 2.7.1.2) originally found in liver (3, 13, 20). Liver glucokinase has a high *km* (12 mM) for glucose, a very high *km* (>800 mM) for fructose, and is not inhibited by glucose-6-P (1). Liver also contains a ketohexokinase (ATP:D-fructose 1-phosphotransferase, EC 2.7.1.3) which phosphorylates fructose to yield fructose-1-P (2, 4, 7, 10). Ketohexokinase, sometimes referred to as "fructokinase," also acts on D-tagatose and, to a lesser extent, on L-sorbose (12).

Plant hexokinases have been known for a considerable period but less definitive information is available. Saltman (14) extracted hexokinase from several plant tissues and studied some properties of a preparation from wheat germ. This enzyme preparation phosphorylated glucose, fructose, mannose, and glucosamine. Wheat germ hexokinases have been purified and some physicochemical characteristics studied (5, 9). The presence of a fructokinase (ATP:D-fructose 6-phosphotransferase, EC 2.7.1.4) in immature pea seeds was the subject of a brief report by Medina and Sols (8). This enzyme phosphorylated glucose at 8% of the rate given by fructose with hexose concentrations of 50 mM. Kursanov *et al.* (6) noted that hexokinase in the soluble fraction from the conducting bundles of sugar beet leaf petioles phosphorylated fructose more rapidly than glucose. $K^+$ ions activated the phosphorylation of fructose but depressed that of glucose and this led the authors to suggest the presence in the cytoplasm of two different hexose kinases: one a specific fructokinase, and the other a nonspecific hexokinase.

A previous communication from this laboratory described the separation of hexose kinases from pea seed extracts into four main fractions (17). These fractions were numbered I to IV in order of elution from a DEAE-cellulose column. Fraction I, a glucokinase, had *km* values for glucose and fructose of 70 μM and 30 mM, respectively (17). In the present investigation the properties of fraction IV (a fructokinase) have been studied. This enzyme will be referred to as fructokinase IV in this communication. Fructokinase IV had a high affinity and high activity with fructose but had very low activity with glucose or mannose. No phosphorylation was observed with D-tagatose. The enzyme had a requirement for $K^+$ ions.

## MATERIALS AND METHODS

Pea seeds (*Pisum sativum* L. var. Progress No. 9) were obtained from F. Cooper Ltd., Wellington, New Zealand. Glucose-6-P dehydrogenase, pyruvate kinase, lactate dehydrogenase, P-glucose isomerase, P-mannose isomerase, NADP, NADH, ATP and other nucleotides, glucose-6-P, fructose-6-P, 6-P-gluconate, 2-P-glycerate, 3-P-glycerate, 2,3-$P_2$-glycerate, P-enolpyruvate, tris, MES, D-fructose, D-glucose, D-mannose, D-galactose, L-sorbose, 2-deoxy-D-glucose, D-glucosamine, D-tagatose, and D-mannoheptulose were obtained from Sigma Chemical Co., or Boehringer Mannheim GmbH.

**Preparation of Fructokinase IV.** Pea seeds were finely ground and defatted with ether (16). Extraction of the defatted pea powder and fractionation with $(NH_4)_2SO_4$ (30-50% saturation) were carried out as described previously (17). A volume of the dialyzed $(NH_4)_2SO_4$ preparation containing 3,000 to 6,000 mU[2] fructose-phosphorylating activity was placed without delay on a DEAE-cellulose column (2.6 × 40 cm) previously equilibrated with 0.025 M KCl in 10 mM tris-HCl (pH 7) containing 1 mM EDTA (buffer A). The column was washed with 0.025 M KCl in buffer A until the *A* at 280 nm was less than 0.300. Hexose kinases were then eluted with a gradient obtained by introducing 400 ml 0.275 M KCl in buffer A into 400 ml 0.025 M KCl in buffer A. Fractions of 120 drops (approximately 7.3 ml) were collected. The fractions containing fructokinase IV but none of the fraction III fructokinase (17) were pooled and concentrated to approximately 10 ml by ultrafiltration on a Diaflo apparatus (PM-10 membrane, under 280-300 kPa/$m^2$ $N_2$ pressure). Preparations of this type contained approximately 2 mg protein/ml and 220 mM KCl and were used to obtain the results presented in this communication. The preparation was free from glucose-6-P dehydrogenase and 6-P-gluconate dehydrogenase. The enzyme could be stored at 2 C with 10% loss of activity per day or at −18 C with no loss of activity in 6 months.

**Assay of Fructokinase Activity.** The enzyme activity was assayed by coupling the production of glucose-6-P (derived from fructose-6-P) with the reduction of NADP in the presence of excess glucose-6-P dehydrogenase. Reaction mixtures for the standard assay contained, in a total volume of 1 ml, 20 μmol tris-HCl buffer (pH 8.2), 0.5 μmol fructose, 5 μmol ATP, 7

[1] This investigation was supported by the University of Sydney Research Grant.

[2] Abbreviations: mU: milliunit; *V*: maximum velocity.

μmol $MgCl_2$, 0.33 μmol NADP, 3 μg P-glucose isomerase, 0.6 μg glucose-6-P dehydrogenase, and an appropriate volume of the pea seed enzyme preparation. When glucose was used as substrate, P-glucose isomerase was omitted; and with mannose as substrate, 7 μg P-mannose isomerase was added. Correction was made for any reduction of NADP not due to hexose kinase activity or due to hexokinase contained in the coupling enzymes. The enzyme reactions were started by the addition of hexose. Reaction mixtures were maintained at 30 C and the change in *A* at 340 nm was followed. One mU fructokinase activity is defined as 1 nmol glucose-6-P produced/min.

When the phosphorylation of sugars other than fructose, glucose, and mannose was being examined, the production of ADP was coupled with the oxidation of NADH using pyruvate kinase and lactate dehydrogenase. This method of assay was also used for investigation of the effects of glucose-6-P and fructose-6-P on fructokinase activity. Reaction mixtures (total volume 1 ml) contained 20 μmol tris-HCl buffer (pH 8.2), 5 μmol ATP, 7 μmol $MgCl_2$, 20 μmol KCl, 0.5 μmol P-enolpyruvate, 0.14 μmol NADH, 8 μg pyruvate kinase, 16 μg lactate dehydrogenase and the pea seed enzyme. Reactions were started by the addition of hexose. Controls without hexose were used to correct for any phosphatase acting on P-enolpyruvate and ATP.

## RESULTS

**Effect of Hexose Concentration.** Under the standard assay conditions (pH 8.2) the activity of fructokinase IV increased until the fructose concentration was 0.75 mM (Fig. 1). Further increase in fructose led to a decrease in fructokinase IV activity, thus with a concentration of 5 mM the rate was only 52% of that given with 0.75 mM fructose. Fructokinase IV had little activity with glucose or mannose as substrates (Fig. 2). The *V* with glucose was less than 3% of that with fructose and the value with mannose was even lower (Table I). The apparent *km* values for fructokinase IV under the standard assay conditions with fructose, glucose, and mannose were 0.057 mM, 0.40 mM, and 1 mM, respectively (Table I).

The effect of fructose concentration on fructokinase IV activity at pH 6.6 differed from that obtained at pH 8.2 (Fig. 1). At the lower pH, increase in fructose was not inhibitory. The *km* for fructose at pH 6.6 was 0.069 mM, *i.e.* close to the value obtained at pH 8.2. The lack of fructose inhibition at pH 6.6 was not due to the lower concentration (0.5 mM) of MgATP in these experiments. Use of 0.5 mM MgATP at pH 8.2 did not reduce the extent of inhibition by fructose.

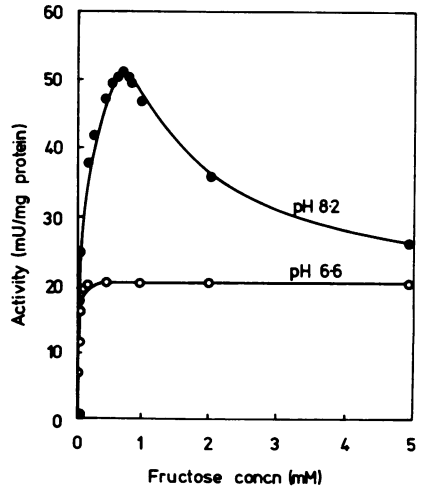


FIG. 1. Effect of fructose concentration on pea seed fructokinase IV activity. Reaction mixtures at pH 8.2 were of the composition described for the standard assay, with the concentration of fructose varied as shown. Reaction mixtures at pH 6.6 contained 20 μmol MES buffer, 0.5 μmol ATP, and 2.5 μmol $MgCl_2$.

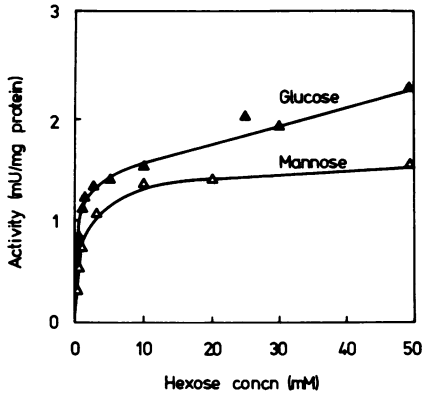


FIG. 2. Phosphorylation of glucose and mannose by fructokinase IV. Reaction mixtures were of the composition described for the standard assay with fructose replaced by glucose or mannose in the concentrations shown.

TABLE I

Kinetic Constants of Fructokinase IV

Reaction mixtures were of the composition described for the standard assay with the hexose varied.

| Hexose | *km* | V | V/*km* |
|---|---|---|---|
| | mM | mU/mg protein | Ratio |
| D-Fructose | 0.057 | 60 | 1053 |
| D-Glucose | 0.40 | 1.6 | 4 |
| D-Mannose | 1.0 | 1.4 | 1.4 |

The activity of fructokinase IV was tested with other sugars. No significant phosphorylation was observed with the following: D-tagatose, L-sorbose, 2-deoxy-D-glucose, D-glucosamine, D-galactose, and D-mannoheptulose.

**Effect of Concentration of ATP and $Mg^{2+}$.** In the presence of 5 mM ATP, increasing the concentration of $MgCl_2$ resulted in an increase in fructokinase IV activity (Fig. 3). The maximum rate was attained when the $MgCl_2$ concentration was approximately 1 to 2 mM in excess of that of ATP. At low concentrations $MnCl_2$ could effectively replace $MgCl_2$. Figure 4 shows the effect of increasing MgATP concentrations (with a fixed excess of 2 mM $MgCl_2$) on the rate of phosphorylation of fructose by fructokinase IV. With excess MgATP there was very slight inhibition of activity at pH 8.2 and a more pronounced inhibition at pH 6.6. The *km* for MgATP (in the presence of 2 mM excess $MgCl_2$) was 0.10 mM at pH 8.2 and 0.035 mM at pH 6.6.

**Effect of Concentration of Monovalent Cations.** Fructokinase IV as prepared contained 220 mM KCl which gave a concentration of KCl in the standard assay mixtures of approximately 30 mM. In experiments involving the effect of addition of salts of monovalent metals, the KCl concentration in the fructokinase IV preparations was reduced by dialysis for 2 hr against 10 mM tris-HCl buffer (pH 8.2). The coupling enzymes were similarly treated to remove $(NH_4)_2SO_4$. The ATP concentration was reduced from 5 mM (in the standard assay) to 1 mM to decrease the $Na^+$ present. Table II shows the effect of the addition of chlorides of monovalent cations on the phosphorylation of fructose by fructokinase IV. There was little activity in the absence of added monovalent cation. The reaction mixtures prepared with dialyzed fructokinase IV and coupling enzymes contained 0.5 mM $K^+$, 0.2 mM $NH_4^+$, and 4.3 mM $Na^+$ and the small activity with no addition was probably due to the $K^+$ and $NH_4^+$. $K^+$ was the most effective stimulator of fructokinase IV: addition of 30 mM KCl increased activity 6.4-fold. $Rb^+$ and

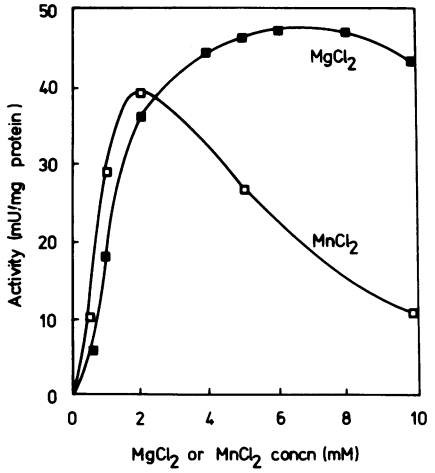


FIG. 3. Effect of $Mg^{2+}$ and $Mn^{2+}$ concentration on pea seed fructokinase IV activity. Reaction mixtures were of the composition described for the standard assay, with the concentration of $MgCl_2$ or $MnCl_2$ varied as shown.

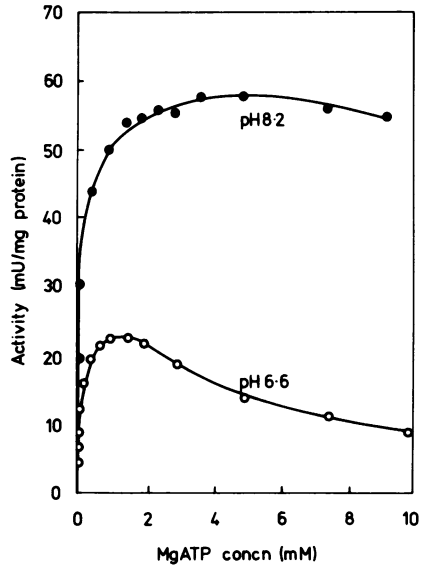


FIG. 4. Effect of MgATP concentration on pea seed fructokinase IV activity. Reaction mixtures were of the composition described for the standard assay with 0.75 μmol fructose and with ATP and $MgCl_2$ (plus 2 mM excess $MgCl_2$) varied as shown. Reaction mixtures at pH 6.6 contained 0.5 μmol fructose and 20 μmol MES buffer.

$NH_4^+$ were also stimulators but were not as effective as $K^+$. $Na^+$, $Cs^+$, and $Li^+$ produced little effect.

**Effect of pH.** The activity of pea seed fructokinase IV in a series of tris-HCl and imidazole-HCl buffers is shown in Figure 5. The optimum pH was approximately 8.2 and activities of 90% or more of the peak activity were maintained for pH values from pH 7.5 to pH 9.3. There was no difference in enzyme activity at pH 7.4 in tris-HCl and imidazole-HCl buffers.

**Effect of ADP.** Fructokinase IV was inhibited by ADP, the extent of inhibition being dependent on the concentration of fructose and MgATP. With the standard reaction mixtures, the addition of 2 mM MgADP gave 9% inhibition of enzyme activity. In reaction mixtures containing 5 mM fructose and 0.1 mM MgATP (plus 2 mM $MgCl_2$), 2 mM MgADP inhibited fructokinase IV by 62%.

**Effect of Metabolites.** Glucose-6-P (final concentration 5 mM) and fructose-6-P (5 mM) inhibited fructokinase IV in standard assay reaction mixtures by 22 and 13%, respectively. There was no significant inhibition by 5 mM glucose-6-P or fructose-6-P when the tris-HCl buffer (pH 8.2) was replaced by MES buffer at pH 6.6. The following compounds had no effect on the reaction rate at pH 8.2 (tris-HCl buffer) or pH 6.6 (MES buffer): 2-P-glycerate (final concentration 1 mM), 3-P-glycerate (1 mM), 2,3-$P_2$-glycerate (1 mM), P-enolpyruvate (1 mM), lactate (10 mM), ethanol (10 mM), citrate (1–10 mM). Pi (final concentration 10 mM) inhibited fructokinase IV by 13%.

TABLE II

Effect of Monovalent Cations on Fructokinase IV Activity

Reaction mixtures were of the composition described for the standard assay except that the ATP concentration was 1 mM and the fructokinase IV preparation and the coupling enzymes were dialyzed as described in the text. Chlorides of monovalent cations were added as shown to give a final concentration of 30 mM.

| Addition | Fructokinase IV Activity |
|---|---|
| | mU/mg protein |
| None | 2.4 |
| $K^+$ | 15.3 |
| $Rb^+$ | 12.4 |
| $NH_4^+$ | 9.5 |
| $Na^+$ | 2.8 |
| $Cs^+$ | 2.6 |
| $Li^+$ | 2.4 |

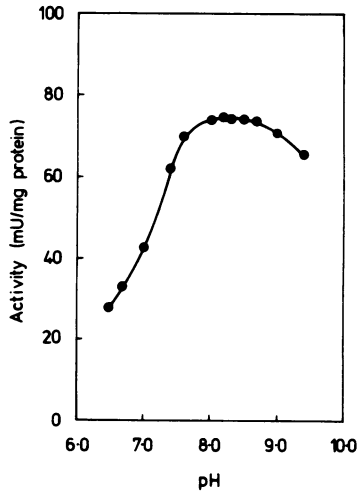


FIG. 5. Effect of pH on pea seed fructokinase IV activity. Reaction mixtures were of the composition described for the standard assay, except that 20 μmol tris-HCl (pH 7.4–9.4) and 20 μmol imidazole-HCl (pH 6.5–7.4) buffers were employed.

## DISCUSSION

The present investigation has established that a specific fructokinase, fructokinase IV, can be obtained from pea seeds. Fructose was the preferred substrate; glucose and mannose were poorly utilized. The *V*/*km* for fructose was 263 times the figure obtained for glucose. It may be concluded that fructokinase IV is unlikely to be of significance in the phosphorylation of glucose. Unlike the ketohexokinase of beef liver, pea seed fructokinase IV did not phosphorylate D-tagatose or L-sorbose. Fructokinase IV had a high affinity at pH 8.2 and at pH 6.6 for fructose. In the developing pea seed the over-all fructose concentrations range from 1.3 mM to 21 mM (19).

Increasing fructose beyond the optimun concentration at pH 8.2 led to inhibition of fructokinase IV activity. At pH 6.6 there was no indication of fructose inhibition. Conversely, when MgATP was varied, there was little inhibition of pea seed

fructokinase IV by excess MgATP at pH 8.2 but there was definite inhibition at pH 6.6. Fructokinase IV may have a role in glycolysis and the reaction would be a logical point of regulation (18). Fructokinase IV was less sensitive to ADP inhibition than fraction I glucokinase (17). The enzyme was not sufficiently sensitive to inhibition by glucose-6-P and fructose-6-P to indicate a regulatory role for these compounds. Glucose-6-P is an important effector of mammalian hexokinases (1, 11).

Fructokinase IV required $K^+$ ions for activity and $Rb^+$ and $NH_4^+$ could partially substitute for $K^+$. $Na^+$, $Cs^+$, and $Li^+$ could not replace $K^+$, suggesting a specific requirement for the latter. It is of interest that the pyruvate kinase of higher plants has a requirement for $K^+$ which can be partly fulfilled by $Rb^+$ and $NH_4^+$ but not by $Na^+$ (15). Pyruvate kinase is probably involved in the regulation of glycolysis (18).

Pea seeds possess considerable fructose-phosphorylating potential in fractions III and IV (17) and this is consistent with available information on the carbohydrate metabolism of the seed. It is believed that sucrose transported into the pea seed is degraded by a reversal of sucrose synthase yielding UDP-glucose and fructose (18). This points to a requirement by the tissue for fructose phosphorylation to facilitate incorporation of the sugar into starch or metabolism through glycolysis.

*Acknowledgment* – The authors wish to thank K. R. Curtis for technical assistance.

## LITERATURE CITED

1. Colowick SP 1973 The hexokinases. Enzymes 9: 1-48
2. Cori GT, S Ochoa, MW Slein, CF Cori 1951 The metabolism of fructose in liver. Isolation of fructose-1-phosphate and inorganic pyrophosphate. Biochim Biophys Acta 7: 304-317
3. Dipietro DL, C Sharma, S Weinhouse 1962 Studies on glucose phosphorylation in rat liver. Biochemistry 1: 455-462
4. Hers HG 1952 La fructokinase du foie. Biochim Biophys Acta 8: 416-423
5. Higgin TJC, JS Easterby 1974 Wheatgerm hexokinase: physical and active-site properties. Eur J Biochem 45: 147-160
6. Kursanov AL, SV Sokolova, MV Turkina 1970 Hexokinase in conducting tissues of sugar-beet and its possible connection with transport of sugars through cell membranes. J Exp Bot 21: 30-39
7. Leuthardt F, E Testa 1951 Die Phosphorylierung der Fructose in der Leber. Helv Chim Acta 34: 931-938
8. Medina A, A Sols 1956 A specific fructokinase in peas. Biochim Biophys Acta 19: 378-379
9. Meunier JC, J Buc, J Ricard 1971 Isolation, purification and characterization of wheat germ hexokinases. FEBS Lett 14: 25-28
10. Parks RE, E Ben-Gershom, HA Lardy 1957 Liver fructokinase. J Biol Chem 227: 231-242
11. Purich DL, HJ Fromm, FB Rudolf 1973 The hexokinases: kinetic, physical and regulatory properties. Adv Enzymol 39: 249-326
12. Raushel FM, WW Cleland 1973 The substrate and anomeric specificity of fructokinase. J Biol Chem 248: 8174-8177
13. Salas J, M Salas, E Vinuela, A Sols 1965 Glucokinase of rabbit liver. Purification and properties. J Biol Chem 240: 1014-1018
14. Saltman P 1953 Hexokinase in higher plants. J Biol Chem 200: 145-154
15. Tomlinson JD, JF Turner 1973 Pyruvate kinase of higher plants. Biochim Biophys Acta 329: 128-139
16. Turner JF 1957 The biosynthesis of sucrose. Biochem J 67: 450-456
17. Turner JF, QJ Chensee, DD Harrison 1977 Glucokinase of pea seeds. Biochim Biophys Acta 480: 367-375
18. Turner JF, DH Turner 1975 The regulation of carbohydrate metabolism. Annu Rev Plant Physiol 26: 159-186
19. Turner JF, DH Turner, JB Lee 1957 Physiology of pea fruits. IV. Changes in sugars in the developing seed. Aust J Biol Sci 10: 407-413
20. Vinuela E, M Salas, A Sols 1963 Glucokinase and hexokinase in liver in relation to glycogen synthesis. J Biol Chem 238: PC1175-PC1177