Fructokinase (Fraction III) of Pea Seeds

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ABSTRACT
A second fructokinase (EC 2.7.1.4) was obtained from pea seed (Pisum sativum L. var. Progress No. 9) extracts. The enzyme, termed fructokinase (fraction III), was specific for fructose and had little activity with glucose. With fructose concentrations above 0.25 millimolar, there was strong substrate inhibition at the optimum pH (8.0) and also at pH 6.6. The apparent $K_m$ values at pH 8.0 for fructose and glucose were 0.06 millimolar and 0.14 millimolar, respectively. The apparent $K_m$ for Mg adenosine 5'-triphosphate (MgATP) was 0.06 millimolar and excess MgATP was inhibitory. MgATP was essential for activity but the enzyme was inhibited by excess Mg$^{2+}$ or ATP. Mg adenosine 5'-pyrophosphate was also inhibitory.

Activity was stimulated by the addition of monovalent cations: of those tested K$^+$, Rb$^+$, and NH$_4^+$ were the most effective. The possible role of fructokinase (fraction III) is discussed.

The properties of hexokinase (ATP:d-hexose 6-phosphotransferase, EC 2.7.1.1) from yeast and mammalian tissues have been studied extensively (2, 15). Bakers’ yeast contains two native hexokinases, one of which (form P-II) may be activated by low concentrations of several metabolites (2, 10). Four isozymes of mammalian hexokinase have been identified and the proportions of these vary in different tissues (2, 15, 16). A feature of three of the forms of mammalian hexokinase is strong inhibition by glucose-6-P and this phenomenon is believed to be important in the regulation of hexokinase activity (2). The fourth enzyme form is a glucokinase (ATP:p-glucose 6-phosphotransferase, EC 2.7.1.2) which was first found in liver (7, 18). A ketohexokinase (ATP:d-fructose 1-phosphotransferase, EC 2.7.1.3), which phosphorylates fructose to give fructose-1-P, is also present in liver (3, 8, 14). This enzyme is sometimes referred to as a “fructokinase.”

Saltman (19) demonstrated hexokinase activity in extracts from several plant tissues and later workers have also reported the presence of the enzyme in plants (1, 4–6, 9, 13). Medina and Solis (12) noted the presence of a fructokinase (ATP:d-fructose 6-phosphotransferase, EC 2.7.1.4) in immature pea seeds. Fructokinase activity has been observed also in nectaries (6) and the conducting bundles of sugar beet leaf petioles (11).

The situation in plants with respect to hexose kinases may be comparable in some ways to that in mammalian tissues. In an earlier publication it was shown that mature pea seeds contain several hexose kinases (20). Four fractions, designated I, II, III, and IV in order of elution from a DEAE-cellulose column, were obtained from (NH$_4$)$_2$SO$_4$ fractions of pea seed extracts. Fraction I contained a glucokinase which had $K_m$ values of 0.07 mm and 30 mm for glucose and fructose, respectively (20). The fraction IV enzyme, fructokinase IV, was also characterized (21). This enzyme, a specific fructokinase with low activity toward glucose and mannose, had a requirement for K$^+$ ions. Here, we describe the properties of a different enzyme, fructokinase (fraction III), which was purified from fraction III. This enzyme will be referred to as fructokinase III. Fructokinase III was specific for fructose as substrate and had little activity with glucose. The enzyme was inhibited by fructose (in concentrations greater than 0.25 mm) and by ATP, Mg$^{2+}$, MgATP, and MgADP. Glucose-6-P and fructose-6-P were only slightly inhibitory.

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-P dehydrogenase, P-glucose isomerase, pyruvate kinase, lactate dehydrogenase, NADP, NADH, ATP, ADP, P-enolpyruvate, Tris, p-glucose, d-fructose, d-galactose, d-mannose, l-sorbose, D-mannohexulose, D-tagatose, D-xyllose, d-glucosamine, 2-deoxy-p-glucose, sucrose, and DEAE-cellulose were obtained from Sigma Chemical Co. or Boehringer Mannheim GmbH.

Purification of Fructokinase III. Pea seeds were extracted and the hexose kinases fractionated on a DEAE-cellulose column as described previously (20). The fractions containing the first peak of fructose-phosphorylating activity (fraction III), but none of the fraction IV fructokinase, were pooled and concentrated to approximately 10 ml by ultrafiltration in a Diaflo apparatus (XM-50 membrane, 300 kPa/m$^2$ nitrogen pressure). The concentrate was diazoyled against 10 mm Tris-HCl buffer (pH 8) and placed on a DEAE-cellulose column (1.5 x 20 cm) previously equilibrated with the same buffer. Fructokinase III was eluted with a gradient obtained by introducing 200 ml of 10 mm Tris-HCl (pH 8) containing 0.30 m KCl and 2 mm EDTA, into 200 ml of 10 mm Tris-HCl (pH 8). Fractions of 5 ml were collected. Fructokinase III appeared as a single peak of activity which was eluted with 0.20 m KCl. The fractions containing fructokinase III were pooled and preparations of this type, which contained approximately 0.4 mg of protein/ml, were used to obtain the results presented in this paper. Protein was determined in all fractions (except the crude extract) by measuring the $A$ at 280 nm. An absorption of 1.0 in a 10-mm light path was taken to be equivalent to 1 mg of protein/ml. The enzyme preparation could be stored frozen at -18 C for at least 2 months without loss in activity but if stored at 4 C only 50% of activity remained after 24 hr. Freezing and thawing after elution from the first DEAE-cellulose column resulted in 30% loss of fructose-phosphorylating activity.

Assay of Fructokinase Activity. 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 3 ml, 75 mmol of Tris-HCl buffer (pH 8), 0.6 mmol of fructose, 3 mmol of ATP, 4.5 mmol of MgCl$_2$, 1 mmol of NADP, 3 mmol of P-glucose isomerase, 0.6 mmol of glucose-6-P dehydrogenase, and a volume of the fructokinase III preparation containing 30 to 50 mm of protein. When glucose was used as substrate P-glucose isomerase was omitted. Reaction mixtures were maintained at 30 C and the increase in $A$ at 340 nm was

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TABLE I  

Purification of Fructokinase III

<table>
<thead>
<tr>
<th>Stage</th>
<th>Protein Content</th>
<th>Fructokinase Activity</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>30-50% (NH₄)₂SO₄</td>
<td>2640</td>
<td>9600</td>
<td>3.9</td>
</tr>
<tr>
<td>1st DEAE-cellulose</td>
<td>61</td>
<td>2200</td>
<td>36</td>
</tr>
<tr>
<td>Ultrafiltration</td>
<td>42</td>
<td>2000</td>
<td>48</td>
</tr>
<tr>
<td>2nd DEAE-cellulose</td>
<td>12</td>
<td>1125</td>
<td>94</td>
</tr>
</tbody>
</table>

1Includes fructokinases III and IV

followed. One mU² of fructokinase activity is defined as 1 nmol of glucose-6-P produced/min. Fructokinase activity was directly proportional to the amount of pea seed enzyme used in the assay. The enzyme preparation was free of 6-P-gluconate dehydrogenase and no phosphatase activity with p-nitrophenyl-P at pH 8 was detected.

When the phosphorylation of sugars other than fructose and glucose was 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 III activity. Reaction mixtures contained in a total volume of 1 ml, 25 μmol of Tris-HCl buffer (pH 8), 20 μmol of KC1, 1 μmol of ATP, 1.5 μmol of MgCl₂, 1 μmol of P-enolpyruvate, 0.14 μmol of NADH, 8 μg of pyruvate kinase, 16 μg of lactate dehydrogenase, a volume of the fructokinase III preparation containing 30 to 50 μg protein, and the substrate sugar. No phosphatase activity with P-enolpyruvate or ATP was detected.

RESULTS

When the DEAE-cellulose column fractions were assayed under the standard conditions, fraction III contained approximately three times as much fructose-phosphorylating activity as fraction IV. After elution from the second DEAE-cellulose column, the fructokinase III preparations had specific activities of approximately 90 mU/mg of protein. The fructose-phosphorylating activities at different stages of a typical purification are shown in Table I.

Effect of Hexose Concentration. The activity of fructokinase III increased until the fructose concentration reached 0.20 mM but with concentrations higher than 0.25 mM the enzyme showed strong substrate inhibition (Fig. 1). With a fructose concentration of 1.5 mM, the activity was only 50% of that obtained with 0.20 mM fructose. The apparent Kₘ value of fructokinase III for fructose was 0.06 mM at pH 8. In contrast to fructokinase IV (21), substrate inhibition with fructose was also observed when the enzyme was assayed in imidazole-HCl buffer at pH 6.6 (Fig. 1). The optimum concentration of fructose at pH 6.6 was 0.25 mM. Fructokinase III also phosphorylated glucose but the maximum rate was only 9% of that obtained with fructose (Fig. 2). No substrate inhibition was observed with glucose and the apparent Kₘ was 0.14 mM. A comparison of the relative activities of fructokinase III with fructose and glucose is given in Table II. D-Mannose, 2-deoxy-D-glucose, and D-glucosamine were phosphorylated to a small extent and there was slight activity with the ketoses D-tagatose and D-xyllose (Table III).

Effect of Concentration of ATP and Mg²⁺. Fructokinase III showed no activity in the absence of Mg²⁺ and phosphorylation of fructose was maximal when ATP and MgCl₂ were present in approximately equal concentrations. The enzyme was inhibited by an excess of either MgCl₂ or ATP. Figure 3 shows the effect on fructokinase III activity of increasing MgCl₂ concentration. When the concentration of MgATP was varied (in the presence of a fixed excess of 0.5 mM MgCl₂), the activity of fructokinase III increased until the MgATP concentration was 1.0 mM (Fig. 4). With higher MgATP concentrations the enzyme showed substrate inhibition. The apparent Kₘ of fructokinase III for MgATP was 0.06 mM under the standard assay conditions. Inhibition by excess MgATP was also observed in assays in imidazole-HCl buffer at pH 6.6 (Fig. 4).

Effect of pH. Fructokinase III showed a broad pH optimum when assayed under standard conditions in a series of imidazole-HCl and Tris-HCl buffers. The optimum pH was 8.0 and 90% or

![FIG. 1. Effect of fructose concentration on pea seed fructokinase III activity. Reaction mixtures at pH 8 were of the composition described for the standard assay with the concentration of fructose varied as shown. Reaction mixtures at pH 6.6 contained 75 μmol of imidazole-HCl buffer. ○: pH 8; ●: pH 6.6.](image)

![FIG. 2. Phosphorylation of glucose by pea seed fructokinase III. Reaction mixtures were of the composition described for the standard assay with fructose replaced by glucose in the concentrations shown.](image)

TABLE II  

Kinetic Constants of Fructokinase III

<table>
<thead>
<tr>
<th>Hexose</th>
<th>V</th>
<th>kₘ</th>
<th>V/kₘ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>98</td>
<td>0.060</td>
<td>1633</td>
</tr>
<tr>
<td>D-Fructose</td>
<td>0.6</td>
<td>0.14</td>
<td>61</td>
</tr>
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</table>
TABLE III
Substrate Specificity of Fructokinase III

<table>
<thead>
<tr>
<th>Hexose</th>
<th>Phosphorylating Activity (mU/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Fructose (0.2 mM)</td>
<td>129</td>
</tr>
<tr>
<td>D-Fructose</td>
<td>42</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>9.1</td>
</tr>
<tr>
<td>D-Mannose</td>
<td>5.1</td>
</tr>
<tr>
<td>D-Tagatose</td>
<td>1.1</td>
</tr>
<tr>
<td>D-Galactose</td>
<td>0.9</td>
</tr>
<tr>
<td>D-Mannohexulose</td>
<td>0.0</td>
</tr>
<tr>
<td>L-Sorbose</td>
<td>0.0</td>
</tr>
<tr>
<td>D-Cellulose</td>
<td>0.0</td>
</tr>
<tr>
<td>2-Deoxy-D-glucose</td>
<td>6.9</td>
</tr>
<tr>
<td>D-Glucosamine</td>
<td>7.4</td>
</tr>
</tbody>
</table>

Reaction mixtures were of the composition described for the standard assay with pyruvate kinase and lactate dehydrogenase as coupling enzymes. Hexose concentrations were 5 mM unless otherwise shown.

FIG. 4. Effect of ADP concentration on pea seed fructokinase III activity. Reaction mixtures were of the composition described for the standard assay with the concentration of MgCl₂ varied as shown.

FIG. 5. Effect of MgADP and MgATP concentration on pea seed fructokinase III activity. Reaction mixtures were of the composition described for the standard assay at different concentrations of ATP and MgCl₂ (plus 0.5 mM excess MgCl₂) and with MgADP added as shown. ●: 1.0 mM MgATP; □: 2.0 mM MgATP; ■: 5.0 mM MgATP.

Table IV

<table>
<thead>
<tr>
<th>Addition</th>
<th>Fructokinase III Activity (μU/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K⁺</td>
<td>72</td>
</tr>
<tr>
<td>Rb⁺</td>
<td>72</td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>75</td>
</tr>
<tr>
<td>Na⁺</td>
<td>49</td>
</tr>
<tr>
<td>Li⁺</td>
<td>52</td>
</tr>
<tr>
<td>Cs⁺</td>
<td>46</td>
</tr>
</tbody>
</table>

Effect of Monovalent Cations. Fructokinase III as prepared contained approximately 200 mM KCl. In experiments where the effect of added monovalent cations was studied, the enzyme preparation was dialyzed against 10 mM Tris-HCl buffer (pH 8) for 60 min and the coupling enzymes dialyzed in the same way. Reaction mixtures prepared from the dialyzed fructokinase III and coupling enzymes contained 0.2 mM K⁺, 3.0 mM Na⁺, and 0.7 mM NH₄⁺.

TABLE IV
Effect of Monovalent Cations on Fructokinase III Activity

More of the maximum activity was obtained between pH 7.4 and pH 8.4.

Effect of ADP. Fructokinase III was inhibited by ADP. Under the standard assay conditions (0.2 mM fructose, 1 mM ATP, and 1.5 mM Mg²⁺), 50% inhibition of fructokinase III activity was obtained with 1.5 mM MgADP. Increasing the concentration of fructose increased the extent of inhibition by MgADP, e.g. when the fructose concentration was 1 mM, 1.5 mM MgADP inhibited fructokinase III activity by approximately 70%. In contrast, increase in concentration of the other substrate, MgATP, decreased the inhibition given by MgADP (Fig. 5). The sigmoid curve in the presence of 5 mM MgATP suggests that MgADP may function as a cooperative inhibitor. A sigmoid curve was also observed in some assays when the MgATP concentration was 2 mM. In separate experiments it was found that UDP was less inhibitory than ADP: under conditions of the standard assay, 1.5 mM MgUDP inhibited pea seed fructokinase III by 10%.

Effect of Metabolites. Both glucose-6-P (final concentration 6 mM) and fructose-6-P (6 mM) inhibited fructokinase III in standard assay reaction mixtures by 25%. The inhibition given by glucose-6-P and fructose-6-P was less than 5% when the concentration of either of these metabolites was 1.5 mM.

Effect of Monovalent Cations. Fructokinase III as prepared contained approximately 200 mM KCl. In experiments where the effect of added monovalent cations was studied, the enzyme preparation was dialyzed against 10 mM Tris-HCl buffer (pH 8) for 60 min and the coupling enzymes dialyzed in the same way. Reaction mixtures prepared from the dialyzed fructokinase III and coupling enzymes contained 0.2 mM K⁺, 3.0 mM Na⁺, and 0.7 mM NH₄⁺. Table IV shows the effect of the addition of chlorides of monovalent cations on fructokinase III activity. With no addition the activity was 47 mU/mg of protein and the addition of K⁺, Rb⁺, or NH₄⁺ (final concentration 60 mM) stimulated fructokinase III activity by 53 to 60%. Na⁺, Li⁺, and Cs⁺ were much less effective stimulators of fructokinase III and at a final concentration of 60...
mm showed little effect; when the final concentration was increased to 120 mm there was a 17 to 28% stimulation of enzyme activity.

**DISCUSSION**

This investigation has shown that pea seeds contain a second specific fructokinase, fructokinase III, which is different from the fructokinase IV described previously (21). The maximum rate of phosphorylation of fructose was 11 times the maximum rate obtained with glucose and the value of the expression \( V/K_m \) with fructose as substrate was 27 times the figure with glucose. Glucose was an even poorer substrate for fructokinase IV; with this enzyme the maximum rate with fructose was 38 times that given by glucose and the value of \( V/K_m \) with fructose was 263 times the value obtained with glucose (21). The apparent \( K_m \) values for fructose of fructokinase III (0.06 mm) and fructokinase IV (0.057 mm) were very similar.

Pea seed fructokinase III was inhibited by the substrate fructose in concentrations above 0.25 mm at the standard assay pH (8.0) and also at pH 6.6. On the other hand, fructokinase IV was subject to fructose inhibition at the optimum pH (8.2) but not at pH 6.6 (21). Fructokinase III was inhibited at pH 8.0 by excess of the other substrate, MgATP: in these experiments an excess of 0.5 mm \( Mg^{2+} \) was maintained over the total ATP concentration to reduce the proportion of free ATP. Fructokinase III was also inhibited by \( Mg^{2+} \) in excess of the ATP concentration and by ATP in excess of the \( Mg^{2+} \) concentration. With fructokinase IV there was little inhibition by MgATP at pH 8.2 (21). Fructokinase III had considerable activity in the absence of added monovalent cations but K+, which could be partially replaced by Na+, stimulated the enzyme by more than 50%. In contrast, pea seed fructokinase IV was almost inactive without added monovalent cations in the reaction mixture and showed a specific requirement for K+ ions (21).

The total fructose-phosphorylating capacity in pea seed extracts is greater than the total glucose-phosphorylating capacity (20). During the development of the pea seed there is a substantial conversion of sucrose to starch (22) and the initial step in this process is believed to be catalyzed by sucrose synthase yielding fructose and UDP-glucose (23). The fructose formed in this reaction must be converted to fructose-6-P for entry into glycolysis, polysaccharide synthesis, etc. This points to a requirement for considerable fructose-phosphorylating activity. There may be a smaller demand for glucose phosphorylation during most of the period of growth of the pea seed.

There seems to be potential for regulation of pea seed fructokinase III since the enzyme was inhibited by \( Mg^{2+} \), free ATP and MgATP, and was stimulated by monovalent cations. Pea seed fructokinase III was sensitive to inhibition by MgADP and this inhibition became cooperative with increase in MgATP concentration. The concentration of ADP in developing pea seeds may reach 1 mm (17), a potentially inhibitory level. ADP is a product of the ADP-glucose-starch synthase reaction and regulation of fructokinase III by ADP could possibly be of metabolic significance. The inhibitory effect of concentrations of fructose higher than 0.25 mm raises the possibility of inhibition of fructokinase III by levels of fructose which could be expected in peas; in developing pea seeds the concentration of fructose ranges from 1.3 to 21 mm (24).

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