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ISOLATION AND PROPERTIES OF HEXOKINASE FROM LORANTHUS LEAVES

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Key Word Index—*Dendrophthoe falcata*; Loranthaceae; mistletoe; angiospermic parasite; hexokinase; phosphotransferase.

Abstract—Hexokinase was partially purified from the leaves of *Dendrophthoe falcata*. The optimum pH for the enzyme was 8.5. The enzyme was sensitive to *p*-CMB and the inhibition could be reversed by 2-mercaptoethanol. The optimum temperature was 40° and energy of activation 6900 cal/mol. The enzyme had an absolute requirement for a divalent metal ion. Although Mg^{2+} was the preferred metal, it could be partially replaced by Mn^{2+} and Ca^{2+}. ATP was the most effective phosphoryl donor. Glucose was the best substrate, the K_m values of 0.14 and 0.26 mM were obtained at saturated and sub-saturated ATP concentration. Phosphorylation coefficients show the following order of reactivity of sugars: glucose > mannose > 2-deoxy D-glucose > fructose > glucosamine > galactose > ribose. The K_m value for ATP was 0.16 mM, which increased to 0.35 mM in the presence of 0.5 mM ADP. ADP and 5'-AMP were competitive inhibitors with respect to ATP, and K_i values were 0.4 and 1.2 mM respectively.

INTRODUCTION

This laboratory is engaged in studies on starch metabolism in loranthus leaves [1,2]. Sucrose synthesized photosynthetically in leaves or derived from the host plant is metabolized in the parasitic plant. A key step in the utilization of sugars is their phosphorylation catalyzed by hexokinase, which can be considered as the first step in the sequence leading to the synthesis of reserve substances. Hexokinase activity has been demonstrated in a number of plant tissues [3–5], but detailed studies are lacking. The following is a report on hexokinase in the leaves of *D. falcata*.

RESULTS

Demonstration of hexokinase activity in loranthus leaves

Hexokinase activity could not be demonstrated in the homogenate of leaves prepared in Tris-medium, PVP-supplemented medium or Triton-supplemented media, employing a one step grinding procedure. Grinding the leaves with "elimination medium" yielded dispersion which on centrifugation at 1600 *g* had a light yellow colored supernatant. It contained low protein and no enzyme activity. This was discarded and the residue ground with the "extraction medium". On centrifugation, a deep green extract was obtained with considerable enzymic activity and protein; about 31% protein of the homogenate was solubilized by grinding with the extraction medium.

Purification of hexokinase

Sephadex G-25 treatment and $(NH_4)_2SO_4$ fractionation resulted in enhanced recovery of the enzyme from the crude extract (Table 1). This can easily be understood since the tissue contained large amounts of endogenous inhibitory phenolics [1] which could have been removed by these treatments. The enzyme could not be adsorbed on DEAE-cellulose but negative adsorption led to about 1.4-fold enrichment of the enzyme. The enzyme was adsorbed on calcium phosphate gel, but large amounts of the gel was required (gel:protein ratio = 15). These steps resulted in 20-fold purification of the enzyme from the crude extract but this was an apparent value, since the crude extract had inhibited activity. The DEAE-cellulose and calcium phosphate gel treatment resulted in about 6-fold purification of the enzyme.

Table 1. Purification of hexokinase from *D. falcata* leaves

Fraction	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg protein)	Recovery of activity (%)
Crude extract	160	11.2	80.0	0.14	100
Sephadex G-25 filtration	200	20.0	68.0	0.29	179
$(NH_4)_2SO_4$, 20–75%	11	26.4	54.1	0.49	236
DEAE-cellulose	110	25.3	37.4	0.68	226
Calcium phosphate gel	330	23.1	8.3	2.78	206

Freedom from contaminating enzyme activities

The enzyme fraction appeared to be free of the following enzymes: glucose-6-phosphate dehydrogenase, phosphoglucomutase, glucosephosphate isomerase, glucose dehydrogenase, glucose-6-phosphatase and ATPase. No utilization of NADP was observed in absence of ATP in the hexokinase assay system. No phosphogluconate dehydrogenase activity which would complicate the coupled assay for hexokinase was detected.

Enzyme stability

The 20–75% $(NH_4)_2SO_4$ fraction was stable for several weeks when stored at 2–4° and only 20% loss in activity occurred in 5 weeks. In contrast, the partially purified enzyme was fairly unstable and 50–60% loss in activity occurred after 24 hr. It was essential that 50 mM sucrose and 20–50 mM 2-mercaptoethanol were present throughout the purification procedure and also during storage, since rapid loss in activity resulted in their absence.

Enzyme linearity

The hexokinase activity was linear up to 15 μg of enzyme protein when tested for 10 min. Keeping the enzyme protein constant at 10 μg level, the activity was linear up to 20 min. The following experiments were carried out in the linearity range of enzyme protein and time.

Spectrophotometric assays

The amount of glucose phosphorylated by hexokinase reaction was the same whether the product glucose-6-phosphate (G-6-P) formed is measured by linking it to reduction of NADP in the presence of added G-6-P dehydrogenase, either by direct or indirect spectrophotometry or the product ADP is measured by coupling it with pyruvate kinase and lactate dehydrogenase in the presence of phosphoenolpyruvate and NADH. It would be seen further that ADP inhibited loranthus hexokinase activity. Since no stimulation of hexokinase activity occurred when ADP-trapping enzymes were added, it could be concluded that inhibitory amounts of ADP were not produced during the spectrophotometric assays.

Effect of pH on enzyme activity

Employing indirect spectrophotometry, the effect of pH was studied in the range 6–10. Tris–maleate buffer was used in the range 6–6.5, Tris–HCl buffer in the range 7–9, and glycine–NaOH buffer in the range 9–10. The sugar phosphate formed was determined in a 1 ml aliquot using excess of glucose-6-phosphate dehydrogenase at pH 8.5 in the presence of 20 μmol of Tris–HCl buffer (pH 8.5). The enzyme had a sharp pH optimum at 8.5. The enzyme activity with Tris–HCl buffer at pH 9 was higher than with glycine–NaOH buffer at the same pH.

Effect of temperature on enzyme activity

Using an indirect spectrophotometric method, the enzyme activity at various temperatures (15 to 50°) was assayed after preincubation of the assay system (excepting ATP) for 5 min at the desired temperature. The enzyme activity increased with temperature till a maximum was reached at 40°; thereafter there was an abrupt decrease in activity at 45°. The Arrhenius plot of the data indicated an energy of activation of 6900 cal/mol.

Requirement for metal ions

The loranthus enzyme showed an absolute requirement for divalent metal ions. Mg^{2+} was preferred but Mn^{2+} and Ca^{2+} could partially replace Mg^{2+}. Mn^{2+} was only 30% effective as Mg^{2+} when tested in 0.1 and 0.5 mM concentration. Ca^{2+} was 25% effective as Mg^{2+} when present in 0.1 mM concentration, 33% in 0.5 mM and was ineffective in 2 mM concentration.

Ratio of ATP to Mg^{2+}

Keeping the glucose concentration fixed at 2 mM and ATP at 0.66 mM, the concentration of Mg^{2+} was varied in the range 0.16 to 3.3 mM. Maximum activity was observed at ATP:Mg^{2+} ratio of 1:1 and 1:2 (Table 2). The hexokinase activity was inhibited if the ratio is increased over 1:2 indicating that excess of free Mg^{2+} was inhibitory.

Effect of metal ions

The effect of metal ions was tested in the presence of 0.5 mM Mg^{2+} and 0.5 mM ATP. Monovalent ions, Na^+ and K^+, tested in 15 and 20 mM respectively and divalent ions, Mn^{2+} and Ca^{2+} in 0.5 mM concentration, did not activate or inhibit the reaction. In a separate experiment, it was also observed that Na^+ and K^+ did not have any effect on hexokinase activity, when glucose was replaced by fructose as a substrate for the enzyme.

Effect of thiol group blocking agent and protectants

The enzyme was sensitive to -SH blocking agent, and was inhibited 25 and 60% respectively when incubated with 0.03 and 0.3 mM *p*-CMB. The inhibition by 30 μM *p*-CMB was completely reversed when the preparation was incubated further with 0.3 mM 2-mercaptoethanol. Cysteine, dithiothreitol and 2-mercaptoethanol, tested in 0.1 and 0.5 mM concentration did not produce any effect on hexokinase activity.

Sugar specificity and phosphorylation coefficient

The sugar specificity was tested at a Mg–ATP ratio of 1:1 and measured by the method based on ADP estimation. The enzyme was unspecific and acted on a number of sugars. Glucose was found to be the best substrate. Plots of enzyme activity vs glucose concentration (25 to 2000 μM) at two fixed ATP concentrations, 0.22 mM (subsaturated) 0.66 mM (saturated) were hyperbolic in nature. Lineweaver–Burk plots of the data indicated K_m values of 0.14 and 0.26 mM respectively in the pres-

Table 2. Effect of variable concentration of Mg^{2+} at a fixed concentration of ATP

Mg^{2+} concentration (mM)	Mg^{2+}/ATP	Enzyme activity (unit)
Nil		Nil
0.16	0.25	0.005
0.33	0.5	0.012
0.49	0.75	0.014
0.66	1	0.019
1.32	2	0.019
1.98	3	0.016
3.30	5	0.009

Glucose and ATP concentrations were 2 and 0.66 mM respectively. 8 μg of loranthus enzyme protein was employed.

Table 3. Affinity of sugars with reference to glucose

Sugars	V_{max} (unit)	K_m (mM)	Phosphorylation coefficient
Glucose	0.028	0.14	1.0
Mannose	0.026	0.19	0.68
2-Deoxy D-glucose	0.034	0.44	0.38
Fructose	0.014	0.58	0.12
Glucosamine	0.018	0.76	0.11
Galactose	0.020	1.10	0.09
Ribose	0.020	1.25	0.08
Xylose	0.011	2.7	0.02

The enzyme assay was based on ADP estimation, and 8 μg of enzyme protein was used for each assay.

ence of saturated and subsaturated ATP. Reciprocal plots for other sugars tested in the presence of saturated concentration of ATP were also straight lines indicating normal Michaelis kinetics. The K_m's for hexokinase towards several sugars are given in Table 3. Included are also the values for the phosphorylation coefficient defined as: K_m (glucose)/K_m (substrate) × V_{max} (substrate)/ V_{max} (glucose). The phosphorylation coefficient is a useful indicator of the relative susceptibility of the substrate to phosphorylation [6]. Phosphorylation coefficients show the following order of reactivity of sugars: glucose > mannose > 2-deoxy D-glucose > fructose > glucosamine > galactose > ribose. The relative maximal rate of fructose and glucose utilization (fructose V_{max}/ glucose V_{max}) for loranthus enzyme was 0.5, as compared to 1.5–2.0 for maize endosperm [3], 1.3 for sugar beet [7], and 3.0 and 1.4 for soluble and particulate enzymes from castor bean [4].

Nucleotide specificity

ATP was the most effective donor of phosphoryl group. ITP, an analogue of ATP, was only 30% as active as ATP at equimolar concentration. GTP and UTP could be utilized as the phosphoryl donor but only at 20 and 13% rate respectively compared to ATP.

Effect of metabolites

A number of metabolites were tested in a search for effectors of hexokinase. All the solutions were neutralized and pH adjusted to 7. L-Malate and succinate had no effect on the reaction, tested in the concentration range of 2–10 mM. Oxaloacetate in 2 mM concentration had no effect, but produced 25% inhibition in 5 mM concentration. 2-Oxoglutarate produced 20% inhibition in 2 mM concentration and 25% in 5 mM concentration. Whereas, 3-phosphoglycerate had no effect on the reaction, another glycolytic intermediate, phosphoenolpyruvate inhibited the hexokinase activity; 25% inhibition occurred at 5 mM concentration and 40% in 15 mM concentration. Inorganic phosphate, tested in 5 and 10 mM concentration did not produce any effect on phosphate-free enzyme preparation. Assay based on ADP estimation revealed no product inhibition by glucose-6-phosphate tested at 10, 20 and 50 mM concentrations with D-mannose as the substrate. D-Xylose also did not produce any effect when used in 2, 5 and 10 mM concentrations with D-glucose as the substrate.

Influence of ATP concentration on hexokinase reaction

To determine the effect of nucleotide concentration, ATP was varied in the range 25 to 800 μM, keeping ATP/Mg^{2+} ratio fixed at 1 : 1 and glucose at 2 mM. The substrate saturation curve was hyperbolic, with no homotropic cooperative effects. The optimum activity was found at 0.66 mM ATP, after which a fall in activity was observed. The K_m arrived at by Lineweaver–Burk plot was 0.16 mM. ADP inhibited the reaction competitively and 0.5 mM ADP increased the apparent K_m value for ATP to 0.35 mM.

Inhibitor constant for ADP and AMP

Employing two fixed concentrations of ATP, 0.22 and 0.66 mM and with glucose at 2 mM, ADP concentration was varied in the range of 0.16–5 mM. Dixon plot of the data indicated a K_i value of 0.4 mM for ADP. 5'-AMP also inhibited the hexokinase reaction with a K_i value of 1.2 mM. The inhibition was competitive with respect to ATP.

DISCUSSION

Hexokinase activity could not be demonstrated in loranthus leaves unless special measures were taken to remove endogenous phenolics. The use of phenol fixing agent, PVP or the detergent, Triton X-100, which is known to prevent phenol protein interaction [8–10] proved ineffective in eliciting hexokinase activity. Success was, however, achieved by the use of the two step grinding procedure of Khanna *et al.* [1]. This consisted of first extracting major endogenous phenolics by grinding the leaves in alkaline medium in the presence of 2-mercaptoethanol and then subsequently extracting hexokinase by the use of Triton X-100. In addition, the incorporation of sucrose was essential to stabilize the hexokinase activity.

The loranthus enzyme had a pH optimum at 8.5, similar to the *Euglena* enzyme which has an optimum pH between 8.0–8.5 [11]. The enzyme from wheat germ seems to show two peaks of activity at pH 7.9 and 10.7 [12]. The failure to demonstrate hexokinase activity in loranthus leaves without Mg^{2+} establishes the essentiality of Mg^{2+}, as observed for other plant hexokinases [5,12]. Mg^{2+}, however, could partially be replaced by Mn^{2+} and Ca^{2+}. No requirement for K^+ could be established for loranthus hexokinase which is reported to stimulate sugar beet fructokinase [7]. While sharing a number of properties with hexokinase from higher plants, the enzyme from the angiospermic parasite possesses some unique features. In contrast to various plant hexokinases [3,4,7], the parasite hexokinase had low activity for fructose. The energy of activation of the enzyme from the parasitic tissue was lower than for wheat germ hexokinase [12]. Unlike wheat germ hexokinase [12], the loranthus enzyme was sensitive to -SH blocking agents, and required a thiol group protectant for its activity. The enzyme activity and, therefore, sugar metabolism in loranthus may be regulated by change in sulfhydryl content.

The sugar substrate specificity for loranthus enzyme was quite broad. The activity with 2-deoxy-glucose and glucosamine indicates that modifications on position 2 of glucose is non-critical. Ribose and xylose, both five carbon sugars, can also be phosphorylated by the enzyme, although poorly compared to glucose. This together with the fact that G-6-P is not inhibitory could be interpreted that the C-6 of glucose is not necessary for hexokinase activity of loranthus. Roustan *et al.* [13]

reported that xylose competitively inhibited the yeast hexokinase reaction against glucose. However, xylose in the present case produced no inhibition when tested up to 20 mM concentration with glucose or mannose as a substrate.

The K_m value of loranthus hexokinase for glucose was similar to the value reported for pea stem [14] and maize scutellum hexokinase [3], and was somewhat lower than reported for wheat germ hexokinase [12]. The loranthus enzyme had very low affinity for fructose. The K_m value for fructose was 4-fold higher than for glucose and was significantly higher than reported for maize scutellum and maize endosperm enzyme [3]. The loranthus enzyme resembles hexokinase from sugar beet [7] in being associated with a higher K_m value for fructose than that observed for the phosphorylation of glucose. The loranthus enzyme had a still lower affinity for galactose; the K_m value being 2-fold higher compared to fructose.

The loranthus enzyme was inhibited powerfully by ADP and slightly by AMP. The activity of hexokinase will vary depending upon the relative levels of adenine nucleotides available to the enzyme, since ADP and AMP are competitive inhibitors with respect to ATP. It appears that the activity of the loranthus enzyme is regulated by adenylate energy charge [15]. The activity of mammalian hexokinase is regulated by glucose-6-phosphate [16–18]. In contrast, the loranthus enzyme is insensitive to G-6-P regulation, like hexokinase from yeast [19]. The enzyme from maize endosperm [5,20] and wheat germ [21] is reported to be slightly inhibited by the sugar phosphate.

EXPERIMENTAL

Plant. The loranthus plant (*D. falcata*) parasitizing the mango tree (*Mangifera indica*) was found on the campus.

One step grinding procedure for enzyme extraction. Leaf stalks and midribs from healthy and soft leaves were removed and remaining leaf tissue (10 g) was blended with the medium for 1 min at low speed and 2 min at high speed, allowing two intervals of half min each in between. The dispersion was strained through 4 layers of muslin and made up to 100 ml. Tris-medium consisted of 0.1 M Tris–HCl buffer, pH 7.5, 20 mM 2-mercaptoethanol, 50 mM sucrose and 20 mM EDTA (pH 7.5). PVP-supplemented medium was prepared by dissolving 1 g soluble PVP (MW 40000) in 100 ml of Tris-medium. Triton-supplemented media were made by dissolving 0.5, 1 and 2 ml of Triton X-100 separately each in 100 ml of Tris-medium to give 0.5, 1 and 2% soln of the detergent.

Two steps grinding procedure for enzyme extraction. 10 g of leaf tissue was blended for 1 min at low speed followed by 2 min at high speed with 100 ml of "elimination medium", consisting of 0.1 M Pi buffer, pH 7.5, 50 mM 2-mercaptoethanol, 20 mM EDTA (pH 7.5) and 50 mM sucrose. The dispersion was centrifuged at 1600 *g* for 30 min, and the residue was blended for 3 min with 110 ml of "extraction medium" which differed from the above in containing also 1% Triton X-100. The homogenate was filtered through 4 layers of muslin cloth and centrifuged at 1600 *g* for 30 min.

Enzyme assay. Based on glucose-6-phosphate estimation. Hexokinase activity was determined according to the method of ref. [22], with slight modifications. The standard reaction mixture contained 30 μmol Tris–HCl buffer (pH 8.5), 2 μmol $MgCl_2$, 6 μmol glucose, 2 μmol ATP, 0.15 μmol NADP, 0.1 unit glucose-6-phosphate dehydrogenase (Boehringer and Mannheim), enzyme preparation and water in a total vol of 3 ml. The reaction was started by the addition of ATP and *A* measured at 340 nm for 10 min at an interval of 1 min, using a 1 cm cuvette. The reaction was linear at least up to 20 min.

Based on ADP estimation. Enzyme activity was determined by coupling the ADP production to a pyruvate kinase and lactate dehydrogenase system and measuring the oxidation of NADH by pyruvate formed from phosphoenolpyruvate. The reaction mixture in a total vol of 3 ml contained 30 μmol Tris–HCl buffer, pH 8.5, 2 μmol $MgCl_2$, 30 μmol KCl, 6 μmol glucose, 2 μmol ATP, 0.06 μmol PEP, 1 μmol NADH, 2.5 units of pyruvate kinase, 1.5 units lactate dehydrogenase, enzyme preparation and H_2O. The reaction was started by the addition of sugar and *A* measured at 340 nm at intervals of 1 min for 10 min.

Indirect spectrophotometric method. In some expts, the enzyme activity was determined by indirect spectrophotometry. The assay system consisted of 30 μmol buffer, 2 μmol $MgCl_2$, 6 μmol glucose, 2 μmol ATP, enzyme preparation and H_2O in a total vol of 3 ml. The reaction was started by the addition of ATP and incubated for 10 min at the desired temp or pH, to determine the optimum temp or the optimum pH. After 10 min, the reaction was stopped by heating the tubes for 2 min at 100°. The amount of G-6-P formed was determined by transferring an aliquot to a 1 cm cuvette containing 20 μmol of Tris–HCl buffer pH 8.5, 0.15 μmol NADP and 0.2 unit of glucose-6-phosphate dehydrogenase. The *A* at 340 nm was measured till a constant value was obtained. A unit of the enzyme catalyzed the formation of 1 μmol of G-6-P/ADP in 10 min under the above conditions. The sp act of the enzyme was expressed as units per mg protein.

Protein measurement. Protein was determined in TCA ppts according to the method of ref. [23], with modifications described in ref. [24]. BSA was used as a standard.

Enzyme purification. The 1600 *g* supernatant obtained by two steps grinding procedure was passed through a column of Sephadex G-25 (coarse), equilibrated with buffer A (50 mM Pi buffer pH 7.5, 50 mM 2-mercaptoethanol and 50 mM sucrose) to remove low MW compounds. $(NH_4)_2SO_4$ was added to the pooled active fraction with constant stirring to obtain 20% satn. The suspension was centrifuged at 15000 *g* for 30 min and the residue discarded. $(NH_4)_2SO_4$ was added to the supernatant to 75% satn and after 18 hr, the ppt was spun at 15000 *g* for 30 min and suspended in 5 ml of buffer A. The fraction was desalted by passing through a column of Sephadex G-25 equilibrated with buffer B (5 mM Pi buffer, pH 7.5, 50 mM 2-mercaptoethanol and 50 mM sucrose) and loaded onto a DEAE-cellulose column which had been previously equilibrated with buffer B. The column was washed with 3 bed vol of equilibrating buffer. The enzyme recovered in the washing was treated with Ca phosphate gel (gel: protein ratio = 15 : 1) which had been washed once with buffer B. The enzyme was kept in contact with the gel for 15 min and then centrifuged at 700 *g* for 10 min. The supernatant contained 10% of the original activity was discarded and the enzyme eluted with buffer A.

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