Hexokinases of Tobacco Leaves: Subcellular Localization and Characterization

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Abstract. Subcellular localization of the enzymes which phosphorylate hexoses was studied in photosynthesizing tobacco leaves by means of differential centrifugation and centrifugation in sucrose gradient. More than 80 % of the total hexokinase activity of leaf tissues were found to be associated with the particulate fraction of mitochondria; however, the ratio of the particulate hexokinase fraction to the soluble fraction was influenced by the extraction medium applied. The particulate hexokinases showed a high affinity to glucose ($K_m = 26.8 \, \mu M$) and a relatively low affinity to fructose ($K_m = 17.6 \, \text{mM}$). They had a broad pH optimum, because 81 % of the phosphorylating activity obtained for glucose and 75 % of the activity obtained for fructose occurred in pH range from 7.9 to 9.1 (Tris-HCl buffer). The hexokinases were Mg$^{2+}$ dependent with the highest activity occurring at equimolar Mg$^{2+}$ and ATP concentrations. Their activity was enhanced by KCl, NaCl, and (NH$_4$)$_2$SO$_4$ at 30 to 120 mM concentrations.

The complex of hexokinases controls the rate of the utilization of both storage and free sugars and in this way also the rates of the glycolysis and of the oxidative pentose phosphate pathway (TURNER and TURNER 1980). Considerable attention was devoted to the study of their properties in animal tissues, whereas relatively little has so far been known about their subcellular localization and other properties in plant tissues. The first studies connected with plant material concentrated on non-photosynthesizing tissues (MEDINA and SOLS 1953, MARRE et al. 1968); nevertheless, more recent studies have demonstrated that the enzymes by which hexoses are phosphorylated are also in photosynthesizing tissues partitioned into soluble and particulate fractions (BALDUS et al. 1981, DRY et al. 1983, TANNER et al. 1983, COSIO and BUSTAMANTE 1984). Because so far no complex study of the properties of hexokinases was carried out in tobacco plants, we present here the first part of our results on the subcellular localization of hexokinases and their characterization, which will be followed by a report on their submitochondrial localization, solubilization, and their isoenzyme pattern.

MATERIAL AND METHODS

Experimental tobacco (Nicotiana tabacum L. cv. Samsun) plants were grown in soil under constant conditions, at 7000 lx (12L : 12D; Philips HLRg
400 W discharge lamps), and average temperature of 25 °C. Leaf blades of 60-day-old plants (from sowing) were used in the experiments.

**Preparation of Crude Homogenates**

Tobacco leaves were homogenized at 0 to 4 °C in mortar with the IMP isolation medium (Pierpoint 1959), or with IMT (Tanner et al. 1983) in the ratio of 1 : 5 (m/v), and the homogenate was filtered through nylon cloth with 100 μm meshes. IMP contained 0.4 M sucrose; 0.2 M Tris; 10 mM KH₂PO₄; 20 mM citrate; 5 mM EDTA (pH 7.7); IMT consisted of 1 mM TES-KOH buffer (pH 7.2) which contained 0.3 M mannitol; 1 mM EDTA; 0.1 % BSA fraction V (Sigma); and 10 % Polyclar AT.

**Fractionation by Differential Centrifugation**

According to Pierpoint (1959), the crude homogenate obtained from 30 g of leaf tissues was centrifuged for 10 min at 1000 g, the pellet was washed with 20 ml of IMP and resuspended in 20 ml of a washing medium (WMP) which contained 0.2 M sucrose; 0.1 M Tris; 5 mM KH₂PO₄; 5 mM citrate; and 2.5 mM EDTA (pH = 7.2). Combined supernatants were centrifuged for 15 min at 15 000 g, the pellet washed with 20 ml of IMP, centrifuged, and resuspended in 10 ml of WMP. Combined supernatants were centrifuged at 105 000 g for 90 min and the resulting pellet was resuspended in 2.5 ml of WMP.

In case the procedure described by Tanner et al. (1983) was employed, nearly the same fractionation scheme was applied except that only IMT was used (with a strict and consistent control of pH values during the isolation procedure) instead of the isolation and washing media and that pellets were not washed. Both fractionation procedures were carried out at 0 to 4 °C.

**Fractionation in Sucrose Gradient**

A 10 ml portion of the crude homogenate prepared in IMT was layered on the discontinuous sucrose gradient prepared according to Douce et al. (1972), containing sucrose at concentrations of 1.8—1.45—1.2 (6 ml of each solution) and 0.9—0.6 M (3 ml of each), in a buffer consisting of 1 mM TES-KOH, pH 7.5, containing 1 mM EDTA and 0.1 % BSA. The gradient was centrifuged at 20 000 rev. min⁻¹ for 60 min in a Beckman L8-80 ultracentrifuge and the SW 28 rotor and then fractions with a volume of 1.36 ml were withdrawn from the bottom of the centrifugation tubes.

**Determination of Enzyme Activities**

Enzyme activities were determined at 25 °C. Glucose phosphorylation was determined on the basis of NADP⁺ reduction photometrically at 340 nm in the presence of an excess of glucose-6-phosphate dehydrogenase. The assay mixture (1 ml) contained 100 μmol of Tris-HCl buffer, pH 8.0; 5 μmol glucose; 2.5 μmol MgCl₂; 60 μmol KCl; 0.5 μmol NADP⁺; 2.5 μmol ATP; 1 U of glucose-6-phosphate dehydrogenase; and 50 to 100 μl of enzyme preparation. Fructose phosphorylation was determined similarly; the assay mixture contained 5 μmol of fructose instead of glucose and in addition 1.5 U of glucosephosphate isomerase.