BIOLOGIA PLANTARUM 40 (3): 469-474, 1997/98

BRIEF COMMUNICATION

Hexokinases of tobacco leaves: influence of plant age on particulate and soluble isozyme composition

L. ŠINDELÁŘ, M. ŠINDELÁŘOVÁ and L. BURKETOVÁ

Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Na Karlovce 1a, CZ - 160 00 Praha 6, Czech Republic

Abstract

Changes in hexokinase particulate and soluble isozyme composition and activities in leaves of 65- and 115-d-old tobacco plants were determined by ion exchange chromatography on DEAE cellulose. During plant ageing, the activities of glucose and of fructose phosphorylating isozymes of particulate hexokinase decreased to 9.9 and 9.2 % of initial value, respectively. The activity of soluble hexokinase decreased to a lesser extent: that of glucose phosphorylating isozyme to 49.8 % and of fructose phosphorylating isozyme to 37.8 %. The activity of soluble fructokinase isozyme dropped to 34.8 %. Thus also the ratio of particulate and soluble isozymes was dependent on the age of leaf tissue.

Additional key words: glucokinase, fructokinase, *Nicotiana tabacum* L.

The complex of hexokinases controls the rate of utilization of the both storage and free sugars and consequently the rates of the glycolysis and of the oxidative pentose phosphate pathway (for review see, *e.g.*, 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 photosynthesizing plant tissues. First studies concentrated on non-photosynthesizing tissues (Medina and Sols 1953, Marré *et al.* 1968). Several studies have shown that plant tissue contains three to five different hexokinases (Turner and Turner 1980, Kruger 1990) distributed in soluble and particle cell fractions (Šindelářová and Šindelář 1988) which differ in their chromatographic and kinetic

Received 24 June 1997, *accepted* 1 September 1997.

Abbreviations: DEAE - diethylaminoethyl; FK - fructokinases; HK - hexokinases.

Acknowledgement: This study was supported by grant No. 522/96/0399 of the Grant Agency of the Czech Republic.

Fax: (+420) 2 24310113; e-mail: elsindel@site.cas.cz

properties. Previously investigated tissues included pea seeds (Turner *et al.* 1977a,b; Turner and Copeland 1981), spinach leaves (Baldus *et al.* 1981, Schnarrenberger 1990), maize endosperm (Doehlert 1990), soybean nodules (Copeland and Morell 1985), avocado (Copeland and Tanner 1988) and tomato fruits (Martinez-Barajas and Randall 1996), and barley leaves (Baysdorfer *et al.* 1989).

The functional significance of different hexokinase forms is only partly understood. In most cases, some of the isozymes were more or less specific for fructose. These so-termed fructokinases could be important during sucrose mobilization. Some of the complexity could be due to differing subcellular locations. Hexokinases have been reported on the outer membranes of mitochondria from spinach leaves (Baldus *et al.* 1981) and on the outer membranes of spinach leaf chloroplasts (Stitt *et al.* 1978). A hexokinase has also been described in the stroma of castor-bean leucoplasts (Miernik and Dennis 1983) and fructokinase has been attributed to spinach chloroplasts (Schnarrenberger 1990). Nevertheless, most studies have found several soluble isozymes, and enzymes bound on the outer side of organelles are also, functionally located in the cytosolic metabolic compartment, making it unlikely that function of the multiple enzyme forms can be explained solely in terms of subcellular compartmentation. Mitochondrial hexokinases are relatively loosely bound to the outer membrane. The properties and composition of the applied extraction and washing media are of great importance, because under unfavourable conditions they may cause extensive solubilization of hexokinases even during the isolation of intact mitochondria (Šindelářová and Šindelář 1988).

In this paper we present the changes in the content of particulate and soluble isozymes determined by ion exchange chromatography on DEAE cellulose. The activities were compared in photosynthetically active leaves of 65- and 115-d-old tobacco plants.

Tobacco plants (*Nicotiana tabacum* L. cv. Samsun) were grown in soil, at 16-h photoperiod, irradiance of 60 μmol m^{-2} s^{-1} (5 - 10 % of natural irradiance), and average temperature of 25 °C. Leaves (12 g) from similar insertion level at both plant ontogenetic stages were homogenized at 0 to 4 °C in mortar (without sea sand) with the 60 cm^3 IMT isolation medium (with the strict and consistent control of pH values during the isolation procedure) according to Tanner *et al.* (1983) and Šindelářová and Šindelář (1988). IMT contained 1 mM TES-KOH (pH 8.0), 0.3 M mannitol, 1 mM EDTA, 5 mM $MgCl_2$, and (added just before extraction) 50 mM 2-mercaptoethanol, 1 mM benzamidine, 1 mM ε-aminocaproic acid, 0.5 mM phenylmethylsulphonyl fluoride and 2 % (m/w) insoluble polyvinylpyrrolidone. The homogenate was filtered through four layers of *Miracloth* and one layer of nylon net with 100 μm meshes and centrifuged (15 000 *g*, 15 min). The pellet ("particle fraction") was washed with 10 cm^3 IMT, centrifuged, resuspended in 5 cm^3 buffer A (50 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$, 1 mM EDTA and 30 mM 2-mercaptoethanol) and quickly frozen at -80 °C. Combined supernatants were centrifuged at 105 000 *g* for 90 min and supernatant was precipitated by solid $(NH_4)_2SO_4$. The precipitate formed at 30 % to 80 % salt saturation ("soluble fraction") was collected by centrifugation, dissolved in 5 cm^3 buffer B (the same composition as buffer A, but with 20 mM Tris-HCl), desalted by centrifugation method through *Sephadex G-25 Fine* and applied to a

column of *DE-52* cellulose with 35 cm^3 bed volume previously equilibrated with buffer B. After washing with one bed volume of buffer B, the isozymes were eluted with 60 cm^3 of a linear gradient of Tris-HCl between 20 and 100 mM, followed by 240 cm^3 of a linear gradient of KCl between 0 and 500 mM in 100 mM buffer C (the same composition as buffer A, but with 100 mM Tris-HCl). Fractions of 6 cm^3 were collected, desalted by centrifugation passage through *Sephadex G-25 Fine* and assayed for enzyme activity. All operation were performed at 0 - 4 °C.

The 10 % *Triton X-100* (*Sigma Chemical Company,* St. Louis, USA) was added to the "particle fraction" to the final concentration 1 % and the suspension was stirred for 1 h in an ice bath. The supernatant was centrifuged (20 000 *g*, 10 min), then applied to a column of *DE-52* cellulose as described above, with the difference that all buffers contained 1 % *Triton X-100*. Active fractions (6 cm^3) were combined, desalted by centrifugation passage through *Sephadex G-25 Fine* and assayed for enzyme activity. All operations were done at 0 - 4 °C.

Enzyme activities were determined at 25 °C. Glucose phosphorylation was determined photometrically at 340 nm on the basis of $NADP^+$ reduction in the presence of an excess of glucose-6-phosphate dehydrogenase. The assay mixture (1 cm^3) contained 100 μmol of Tris-HCl buffer, pH 8.0, 5 μmol glucose, 2.5 μmol $MgCl_2$, 60 μmol KCl, 0.5 μmol $NADP^+$, 2.5 μmol ATP, 1 U of glucose-6-phosphate dehydrogenase, and from 0.05 to 0.1 cm^3 of enzyme solution. Fructose phosphorylation was determined similarly; the assay mixture contained 50 μmol of fructose and in addition 1.5 U of glucosephosphate isomerase.

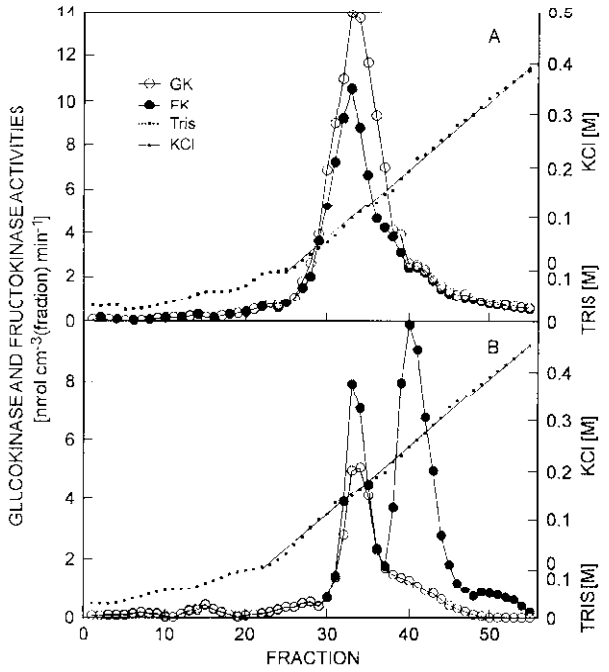

Fig. 1. Elution profile of particulate (*A*) and soluble (*B*) glukokinases (GK) and fructokinases (FK) obtained from leaves of 65-d-old tobacco plants.

The results are means of 4 independent experiments with 3 replicate determinations each.

In tobacco leaves, three main isozyme fractions in elution profiles from ion exchange chromatography on DEAE cellulose were obtained: one particulate fraction (hexokinase phosphorylating both glucose and fructose), and two soluble fractions (hexokinase, and fructokinase which phosphorylate exclusively fructose). All these isozymes had similar elution profiles and were released from the column at the same KCl concentrations. It is likely that these fractions represented the same isozymes. The content of the isozymes, however, differred in tobacco leaves of different age (the contents were determined by integration of eluting profile areas of individual isozymes with respect to fraction volumes; Table 1). The activity of glucose phosphorylating particulate hexokinase isozymes considerably decreased from 894.1 (in tobacco leaves of 65-d-old plants) to 88.6 nmol min^{-1} (115-d-old plants), *i.e.* to 9.9 % . Similarly, the activity of fructose phosphorylating isozymes fell from 596.3 to 54.8 nmol min^{-1}, *i.e.* to 9.2 % .The same course of activity was observed in soluble hexokinase isozymes; the decrease of glucose phosphorylating isozymes from 261.7 to 130.2 nmol min^{-1} (*i.e.* 49.8 %) and in fructose phosphorylating isozyme from 239.2 to 90.4 nmol min^{-1} (*i.e.* 37.8 %). The activity of soluble fructokinase isozyme dropped from 414.9 to 144.2 nmol min^{-1} (*i.e.* 34.8 %).

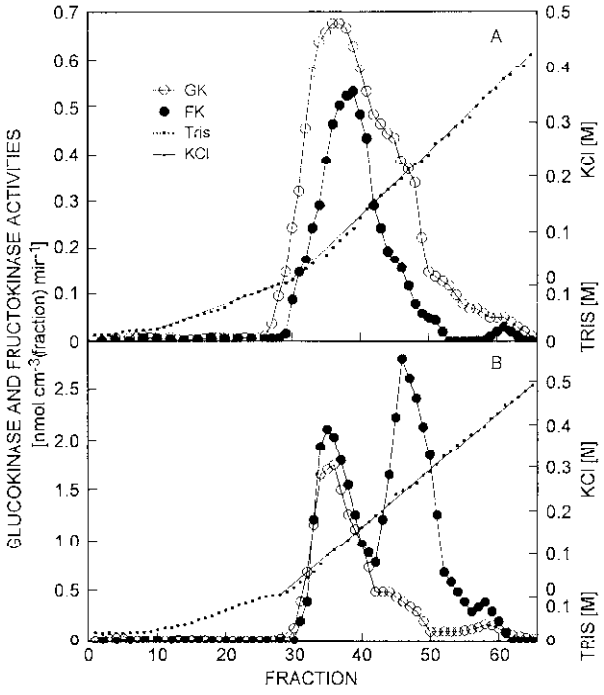

Fig. 2. Elution profile of particulate (*A*) and soluble (*B*) glukokinases (GK) and fructokinases (FK) obtained from leaves of 115-d-old tobacco plants.

The ratio of particulate and soluble isozymes was in dependence on the age of leaf tissue (Table 1). In leaves of 65-d-old plants, total phosphorylating activity of glucose was 1155.8 nmol min^{-1}, 77.4 % of which represented the activity of particulate isozymes. In leaves of 115-d-old plants, the total phosphorylating activity was 218.8 nmol min^{-1}, 40.5 % of which represented the activity of particulate isozymes. The total phosphorylating activity of fructose from leaves of 65-d-old plants was 1250.4 nmol min^{-1}, 47.7 % of which represented the activity of particulate isozymes, 19.1 % the activity of soluble isozymes of hexokinase type and 33.2 % represented the activity of isozyme of fructokinase type. In leaves of 115-d-old plants, the total phosphorylating activity was 289.4 nmol min^{-1} of which only 18.9 % represented particulate isozyme, 31.2 % the activity of soluble isozyme of hexokinase type and 49.8 % represented the activity of soluble isozyme of fructokinase type.

Table 1. Glucose and fructose phosphorylation activities in particulate and soluble fractions obtained from leaves of 65- and 115-d-old tobacco plants after DEAE-cellulose column chromatography (the values given are means ± standard errors of means).

Time after sowing [d]	Origin	Glucose phosphorylation [nmol min^{-1}]	Fructose phosphorylation [nmol min^{-1}]
65	particulate	894.1 ± 79.6	596.3 ± 102.4
	soluble	261.7 ± 52.4	239.2 ± 48.3
			414.9 ± 115.7
115	particulate	88.6 ± 15.3	54.8 ± 12.5
	soluble	130.2 ± 47.1	90.4 ± 28.6
			144.2 ± 37.3

All the isozymes showed a very broad pH optimum. In Tris-HCl buffer solutions the activities did not change within the pH range of 8.0 to 8.6 . All of these isozymes were Mg^{2+} dependent. The highest enzyme activity occurred at equimolar Mg^{2+} and ATP concentrations, at higher and/or lower concentration of Mg^{2+} the isozyme activities were decreased.

During the years 1994 - 1996, the isozyme composition of hexokinases and fructokinases in leaves of 60 to 125-d-old tobacco plants were determined six-times with similar results. Therefore it could be suggested that the activity of particulate and soluble isozymes considerably decreases during ageing of plants and reaches the minimum value between 90 and 100 d after sowing, since then it does not change.

References

Baldus, B., Kelly, G.J., Latzko, E.: Hexokinases of spinach leaves. - Phytochemistry **20**: 1811-1814, 1981.

Baysdorfer, C., Kremer, D.F., Sicher, R.C.: Partial purification and characterization of fructokinase activity from barley leaves. - J. Plant Physiol. **134**: 156-161, 1989.

Copeland, L., Morell, M.: Hexose kinases from the plant cytosolic fraction of soybean nodules. - Plant Physiol. **79**: 114-117, 1985.

Copeland, L., Tanner, G.J.: Hexose kinases of avocado. - Physiol. Plant. **74**: 531-536, 1988.

Doehlert, D.C.: Fructokinases from developing maize kernels differ in their specifity for nucleoside triphosphates. - Plant Physiol. **93**: 353-355, 1990.

Kruger, N.J.: Carbohydrate synthesis and degradation. - In: Dennis, D.T., Turpin, D.M. (ed.): Plant Physiology, Biochemistry and Molecular Biology. Pp. 467-492. Longman, Harlow 1990.

Marré, E., Cornaggia, M.P., Biandreti, R.: The effect of sugars on the development of hexose phosphorylating enzymes in the castor bean cotyledons. - Phytochemistry **7**: 1115-1123, 1968.

Martinez-Barajas, E., Randal, D.D.: Purification and characterization of fructokinase from developing tomato (*Lycopersicon esculentum* Mill.) fruits. - Planta **199**: 451-458, 1996.

Medina, A., Sols, A.: A specific fructokinase in peas. - Biochim. biophys. Acta **19**: 378-379, 1953.

Miernyk, J.A., Dennis, D.T.: Mitochondrial, plastid, and cytosolic isozymes of hexokinase from developing endosperm of *Ricinus communis*. - Arch. Biochem. Biophys. **226**: 458-468, 1983.

Schnarrenberger, C.: Characterization and compartmentation, in green leaves, of hexokinases with different specifities for glucose, fructose, and mannose and for nucleoside triphosphates. - Planta **181**: 249-255, 1990.

Šindelářová, M., Šindelář, L.: Hexokinases of tobacco leaves: subcellular localization and characterization. - Biol. Plant. **30**: 275-284, 1988.

Stitt, M., Bulpin, P.V., apRees, T.: Pathway of starch breakdown in photosynthetic tissues of *Pisum sativum*. - Biochim. biophys. Acta **544**: 200-214, 1978.

Tanner, G.J., Copeland, L., Turner, J.F.: Subcellular localization of hexose kinases in pea stems: mitochondrial hexokinase. - Plant Physiol. **72**: 659-663, 1983.

Turner, J.F., Copeland, L.: Hexokinase II of pea seeds. - Plant Physiol. **68**: 1123-1127, 1981.

Turner, J.F., Chensee, Q.J., Harrison, D.D.: Glucokinase of pea seeds. - Biochim. biophys. Acta **480**: 367-375, 1977a.

Turner, J.F., Harrison, D.D., Copeland, L.: Fructokinase (fraction IV) of pea seeds. - Plant Physiol. **60**: 666-669, 1977b.

Turner, J.F., Turner, D.H.: The regulation of glycolysis and the pentose phosphate pathway. - In: Davies, D.D. (ed.): The Biochemistry of Plants. Vol. 2. Pp. 297-312. Academic Press, New York - London - Toronto - Sydney - San Francisco 1980.