BIOLOGIA PLANTARUM (PRAHA)

33 (2) : 150–155, 1991

Subcellular Localization of Glucose-6-Phosphate Dehydrogenase in Tobacco Mesophyll Protoplasts

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Abstract. Subcellular localization of glucose-6-phosphate dehydrogenase (EC 1.1.1.49.) isoenzymes was determined in mesophyll protoplasts prepared from *Nicotiana tabacum* L. cv. Samsun. Intact chloroplasts and soluble cytosolic proteins were obtained by means of differential centrifugation. The 1 000 g pellet contained 97 % of chloroplasts and 16.8 ± 2.1 % of the total activity of glucose-6-phosphate dehydrogenase. The rest of the enzyme was localized in the cytosol which also contained 91 % of the total activity of phosphoenolpyruvate carboxylase.

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49.) plays an important role in the regulation of the rate of the oxidative pentose phosphate pathway (Kanamori *et al.* 1979, Turner and Turner 1980). This enzyme is present in plant tissues in the form of two isoenzymes, one of which is localized in the chloroplasts, and the other in the cytosol. Some mechanisms involved in its coarse and fine regulation by light and darkness also are known (Heber *et al.* 1967, Schnarrenberger *et al.* 1973, Anderson *et al.* 1974, Anderson and Duggan 1976, Srivastava and Anderson 1983, Eickenscher and Scheibe 1986 and others). Both isoenzymes show very similar physico-chemical properties (they have nearly the same pH optima, relative molar masses, kinetic constants K_m and V, *etc.*), and thus they cannot be distinguished from each other by means of conventional methods (Schnarrenberger *et al.* 1973, Herbert *et al.* 1979). More advantageous in studying the subcellular localization of glucose-6-phosphate dehydrogenase isoenzymes and their physico-chemical properties, and in determining their kinetic parameters than homogenates prepared from leaf tissues of experimental plants (Herbert *et al.* 1979) is the use of mesophyll protoplasts, becouse they make it possible to obtain in a short time intact cell organelles and simultaneously to minimize mechanical stress caused by the destruction of intact

Received November 10, 1989; accepted March 16, 1990

cells. In this paper, we present results obtained by studying subcellular localization of glucose-6-phosphate dehydrogenase isoenzymes in tobacco protoplasts, which served us as a basis for the study of changes in the activity of this enzyme in protoplasts infected with phytoviruses, described earlier (Šindelář 1986).

MATERIAL AND METHODS

Experimental tobacco (*Nicotiana tabacum* L. cv. Samsun) plants were grown under constant conditions in soil, at irradiance of 60 $\mu E\ m^{-2}s^{-1}$ (12L : 12D, Philips HLRg 400 W discharge lamps) and average temperature of 25 °C.

Preparation and Purification of Protoplasts

Tobacco leaves (90 days after sowing) were cut to 1 mm wide strips which were subjected to plasmolysis for 1 h at 25 °C in CPW medium (Cocking and Peberdy 1974) containing 0.45 M mannitol. Then the solution was replaced with the same medium containing 1 % cellulase "Onozuka R–10" and 0.25 % "Macerozyme R–10" (Serva); this mixture was vacuum infiltrated into the leaf strips. Six grams of leaf strips were incubated with 60 ml of the medium. The medium was decanted after 15 minutes of incubation at 30 °C, centrifuged for 10 min at 2 000 *g*, and the supernatant again returned onto leaf strips. Leaf tissues were then incubated for 3 to 4 h at 30 °C, protoplast suspension was filtrated through a 100 μm net, and after combining it with the same volume of TEMES buffer (20 mM Tris-HCl buffer, 1 mM EDTA, 2.5 mM $MgCl_2$, 30 mM 2-mercaptoethanol, and 1.4 M sucrose, pH 7.0) centrifuged for 3 min at 100 *g*. The floated protoplasts were washed twice with TEMEM medium (the same as TEMES, but sucrose was replaced with 0.45 M mannitol) and resuspended in 9 ml of TEMEM medium.

Breaking of Protoplasts and Fractionation of Cell Organelles

This operation was carried out at a temperature of 0 to 4 °C. Protoplasts were passed three times through a net with 20 μm meshes (Pharmacia) which resulted in their complete disintegration; this operation was checked using a light microscope. A 5 ml aliquot of cell organelle suspension, designated S_0, was used for the determination of total protein content, chlorophyll content, phosphoenolpyruvate carboxylase activity, and glucose-6-phosphate dehydrogenase activity.

The remaining 4 ml of cell organelle suspension were centrifuged for 10 min at 1 000 *g*. The P_1 pellet, containing the substantial part of chloroplasts, was resuspended in 4 ml of TEME medium (TEMES medium without the osmoticum), and the supernatant was centrifuged for 15 min at 15 000 *g*. In this way, the supernatant S and the pellet P_2 were obtained. The P_2 pellet was resuspended in 4 ml of TEME medium.

Determination of Enzymic Activities

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49.) activity was determined spectrophotometrically, NADPH generation rate was monitored at 340 nm. The assay mixture (0.3 ml) contained 100 µl of TM buffer (100 mM Tris-HCl buffer; 12.5 mM $MgCl_2$; pH 8.0 which is the pH optimum of the enzyme); 1 µmol of $NADP^+$; 0.1 ml of homogenate; and 1 µmol of glucose-6-phosphate. The results obtained (Table 1), have been expressed as the half-level of nanomoles of the generated NADPH, with respect to a high activity of phosphogluconate dehydrogenase in homogenates.

In determining the activity of this enzyme Triton X-100 (final concentration 0.05 %) was added in all fractions to the homogenate, and the activity was determined after 30 min, when all chloroplasts were already completely disintegrated. Triton X-100 did not affect the activity of the enzyme.

Phosphoenolpyruvate carboxylase activity (EC 4.1.1.31.) was determined according to Downton *et al.* (1971).

Enzyme activities were determined at 25 °C.

Protein content was determined according to Bradford (1976) using bovine serum albumin as a standard, and chlorophyll content according to Arnon (1949).

Protoplasts number was determined in a haematocytometer according to Bürker,

Table 1.

Subcellular localization of glucose-6-phosphate dehydrogenase in protoplasts prepared from *Nicotiana tabacum* cv. Samsun leaves.

Fraction	Proteins	Chlorophyll	Phospho-enolpyru-vate carboxylase	Glucose-6-phosphate dehydroge-nase
Crude homogenate	395.0 100.0 %	90 100.0 %	26.4 100.0 %	6.0 100.0 %
1000 *g* pellet	173.0 43.8 %	87.2 96.9 %	1.5 5.5 %	1.0 16.8 %
15000 *g* pellet	15.8 4.0 %	1.0 1.1 %	0.6 2.2 %	0.3 4.2 %
15000 *g* supernatant	209.8 53.1 %	0.5 0.5 %	24.0 90.8 %	4.6 77.9 %
(Σ	100.9 %	98.5 %	98.5 %	98.9 %)

Protein content and chlorophyll content are expressed in µg per 10^6 protoplasts, enzyme activities in nmol min^{-1} per 10^6 protoplasts.

and the number of viable protoplasts by staining with methylene blue according to Hooley and McCarthy (1980).

The results presented in Table 1 are means of three to five determinations in four independent experiments.

RESULTS AND DISCUSSION

Subcellular localization of glucose-6-phosphate dehydrogenase in tobacco mesophyll protoplasts was determined by the fractionation of cell organelles and of the cytosol using the method of differential centrifugation. The results presented in Table 1 and illustrated in Fig. 1 show the advantages of utilizing the protoplast release technique when studying subcellular localization of enzymes. These data clearly show that intact chloroplasts (chlorophyll was used as a marker) were almost completely present in the 1 000 *g* pellet, and that 91 % of the cytosol (including vacuole contents), with the marker phosphoenolpyruvate carboxylase, were present in the 15 000 *g* supernatant. About 76 % (75.9 ± 2.1) of glucose-6-phosphate dehydrogenase activity was present in the 15 000 *g* supernatant, which contained the cytosol plus vacuolar contents, and about 17 % (16.8 ± 2.2) were present in the 1 000 *g* pellet containing 96.9 % of intact chloroplasts. The percentages of glucose-6-phosphate dehydrogenase activity found in chloroplasts ranged from 13.2 to 20.4 %.

In the 15 000 *g* pellet, about 4.2 % of the total amount of this enzyme were detected. This value can be explained in terms of a 2.2 % proportion of the cytosol in this fraction (as characterized by phosphoenolpyruvate carboxylase) and in terms of a 1.1 % proportion of the chloroplast enzyme (as indicated by chlorophyll content); the remaining 0.9 % can be characterized as a contamination resulting from the method employed.

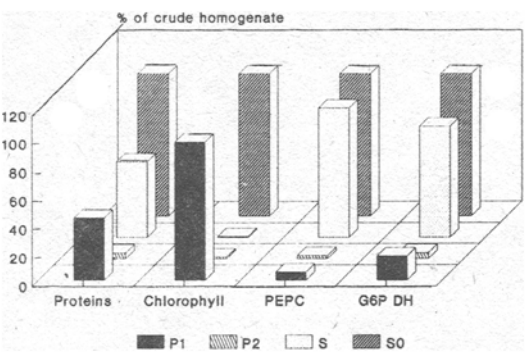

Fig. 1. Distribution of proteins, chlorophyll, phosphoenolpyruvate carboxylase, and glucose-6-phosphate dehydrogenase in subcellular fractions prepared from tobacco mesophyll protoplasts by means of differential centrifugation. The results are expressed as percentages of contents or activities recorded in the initial crude homogenate S_0.

The properties of glucose-6-phosphate dehydrogenase isoenzymes and the mechanisms of their coarse and fine regulation were studied by a number of authors who are referred to in the introduction. Much less is known about their content in the chloroplasts and in the cytosol; Heber *et al.* (1967) concluded that this enzyme was localized mainly in the cytosol and that only a small proportion, about 10 to 20 %, was present in the chloroplasts. A similar value (round 20 %) also was found in the chloroplasts by Fickenscher and Scheibe (1986). Our results obtained with tobacco mesophyll chloroplasts are in good agreement with data reported by the above authors, as shown by data presented in Table 1 and Fig. 1.

Acknowledgement

The authors wish to thank Dr. S. Rakouský for valuable advice relating the preparation of tobacco mesophyll protoplasts and the techniques of their processing.

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Action of different bioactive compounds in diverse organisms is based on common molecular mechanisms. From this point of view understanding of interactions of ligands and corresponding receptors is equally important for solving pharmacological, agrochemical and environmental problems. Despite to the fact that the first modern investigation of quantitative-structure-activity relationships (QSAR) of physiologically active molecules was focused on action of auxin derivatives on plant systems the present state of knowledge of QSAR in medicine has far outstripped that in agrochemistry. For this reason methods and conceptual app oaches developed in QSAR studies of drugs are inspiring for scietists investigating mechanisms of action of phytohormones and developing new pesticides.

The book is based on contributions presented on a symposium sponsored by Division of Agrochemicals of the American Chemical Society which was held in Los Angeles, California, In September, 1988. It consists from four sections containing 24 papers. Section 1 (View on the Field) is focused on progress in design of bioactive molecules, prediction of activity and molecular modelling, Section 2 (Ways and Means) on computer and statistical modelling and sequence – function relationships of proteins. The third section (Agrochemical Mechanisms) contains papers dealing with QSAR studies of agrochemicals as *e.g.* inhibitors of insect juvenile hormone esterase, fenvalerate and ether-type pyrethroids and photosysteme II inhibitors and more conceptual contributions focused on agrochemical design strategies and molecular designs of fungicide development. Sections 4 and 5 are dedicated to drug and toxicity mechanisms, respectively.

The book is a good example of sucessful multidisciplinary approach to solution of problems of general biological importance. It clearly shows how combination of statistical and computer-based surways of known structures of receptors and ligands can minimize slassical testing of broad spectrum of ligand analoques and predict characters of the most promissing chemical structures. This elegant and inspirative text can be recommended to scientists studying mechanisms of action of plant growth regulators and developing new agrochemicals.

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