UDP-D-GLUCOSE 4-EPIMERASE ACTIVITY OF THE TISSUE CULTURE OF WHITE POPLARS (*Populus alba* L. *var. pyramidalis*)

Ladislav BILISICS, Štefan KARÁCSONYI and Marta KUBAČKOVÁ

Institute of Chemistry,
Slovak Academy of Sciences, 809 33 Bratislava

Received November 18th, 1980

The presence of UDP-D-glucose 4-epimerase (EC 5.1.3.2) in the culture tissue of white poplar was evidenced. As found, the partially purified enzyme preparation contained UDP-D-glucose glucosyltransferase, UDP-D-galactose galactosyltransferase and non-specific enzymes able to cleave the uridine-diphosphate saccharides into the appropriate hexose monophosphates. The activity change of UDP-D-glucose 4-epimerase in tissue culture cells during the growth was in accord with changes in D-galactose content in cell walls and indicated the possibility to regulate the formation of polysaccharides containing D-galactose at the level of production of UDP-D-galactose in cells.

The enzyme UDP-D-glucose 4-epimerase (EC 5.1.3.2), catalyzing the reversible transformation of UDP-D-glucose to UDP-D-galactose, has been found in animal tissues[1], microorganisms[2,3] and also in various plant materials[4-7]. Considering the fact that UDP-D-galactose resulting from its action is an important donor of galactosyl residue for the biosynthesis of polysaccharides[8] and galactolipides[9] containing D-galactose, the changes in its activity might be involved in the regulation of biosynthesis of these cell-wall polysaccharides[7,10,11]. This paper deals with examination of conditions for activity determination of this enzyme and with comparison of its features with those of other resources; an emphasis is imposed on the examination of changes in its activity in cells with respect to the D-galactose content in cell walls during the growth of white poplar tissue cultures.

EXPERIMENTAL

Material

Chemicals: UDP-D-glucose Na salt, α-D-glucose 1-phosphate Na_2 salt, α-D-galactose 1-phosphate K_2 salt, D-glucose 6-phosphate Na_2 salt, β-NAD, NADH Na_2 salt. NAD^+ nucleosidase (EC 3.2.2.5) of *Neurospora crassa* (Sigma, USA). Kinetin and adenosine (Koch-Light, Great Britain), adenine and benzylaminopurine (Lachema, Czechoslovakia) and Miracloth (Calbiochem, Switzerland). UDP-D-(U-^{14}C)-glucose ammonium salt (specific activity 10 952 MBq/mmol, Amersham, Great Britain) was diluted to the requested specific activity by addition of unlabelled compound. Radiochemical purity of the employed UDP-D-(U-^{14}C)-glucose was checked after

hydrolysis of the substrate on the presence of radioactive D-galactose by descending paper chromatography in solvent S_1.

Green culture tissue of white poplar[12], subcultivated in 21-day intervals on an inductive Diaz-Colon medium[13] at 60–70% relative humidity and 25 ± 1°C in diffuse light (800 lx, 8 h periods), was used in 20–50 g amounts for isolation of the enzyme preparation or cell walls.

Methods

Growth of the tissue was monitored by the increase of the number of cells in ten-membered sets. Cells of the tissue were counted with a hemocytometer from suspensions of cells obtained by the action of 0·1M-EDTA Na_2 salt in 0·3M saccharose at 70°C (ref.[14]).

Cell walls were isolated by a modified Kivilaan method[16] at temperatures up to +2°C. Tissue cultures washed with 100 volumes of water (v/w) were disintegrated and suspended in 2·5 volumes (v/w) of glycerol after de-freezing in liquid N_2. After a multiphase homogenization with the Potter–Elvehjem homogenizer (4 500 rpm, 6 min) and with a turmix-blender (12 000 rpm, 3 min) in glycerol, the isolated cell walls, trapped with a layer of Miracloth, were successively washed with a ten-fold volume of glycerol (v/w), which was, in turn successively removed with a thirteen-fold volume of water. It was not succeeded to remove further nitrogen- or phosphorus-containing compounds from the obtained microscopically pure preparation of cell walls with glycerol or water. Cell walls after extraction of lipides[17], washing with chloroform–methanol (2 : 1), acetone and drying above P_2O_5 to a constant weight were hydrolyzed with sulfuric acid[18].

The content of D-*galactose* in cell-wall hydrolysates was determined by gas chromatography of the corresponding alditoltrifluoro acetate[19] using myo-inositol as an internal reference. Hewlett–Packard chromatograph, model 5 711 A, equipped with a flame-ionizing detector and a stainless-steel column (200 × 0·3 cm) packed with 3% SP-2 340 over Chromosorb W AW DMCS (80 to 100 mesh), was employed for gas chromatography at 180–210°C (4 min), 2°C/min. The carrier-gas flow (N_2) was 28·5 ml/min.

*Preparation and partial purification of UDP-*D-*glucose 4-epimerase.* The tissue culture homogenate was obtained by disintegration of cells with a X-Press Cell Disintegrator (LKB-Biotec, Sweden) at −25°C and 200 MPa pressure. A complete disintegration was achieved by a six-fold repetition of the homogenization process. The enzyme was prepared at 0 ± 2°C according to[10]. The de-freezed homogenate was stepwise centrifuged in a 0·1M phosphate buffer of pH 7·03 containing 10 mM of 2-mercaptoethanol and 2 mM of EDTA at 3 000 *g*/20 min and at 40 000 *g*/20 min; 0·5M $MnCl_2$ was added to the supernatant with stirring to a 0·15 mmol l^{-1} final concentration. The precipitate was centrifuged at 20 000 *g*/20 min. The partially purified enzyme was obtained from the supernatant by a successive precipitation with ammonium sulfate at a 35–65% saturation of the solution followed by centrifugation of the precipitate at 20 000 *g*/20 min. The obtained preparation was stored at −30°C in a 0·2M glycine–NaOH buffer of pH 9·03 containing glycerol (20%).

The protein content was determined spectrophotometrically according to[15] using bovine serum albumin as a standard.

Inactivation of UDP-D-*glucose 4-epimerase* (6 pkat) with NAD^+ nucleosidase (333 pkat) was effected at pH 7·03 and 30°C for 60 min. The enzyme activity was estimated from an aliquot after its adjustment to pH 8·91.

Activity of UDP-D-*glucose 4-epimerase* was estimated from the conversion of UDP-D-(U-^{14}C)-glucose to UDP-D-(U-^{14}C)-galactose in 0·2M glycine–NaOH (pH 9·03) buffer in a 20 µl volume

at 30°C during 5 and 10 min (ref.[10]). After the reaction stopped (100°C, 2 min) the nucleoside-diphosphate saccharides present were hydrolyzed with 0·1M-HCl. Solutions containing radioactive saccharides were extracted with pyridine (20 μl, 4 times) after removal of the solvent *in vacuo* and drying in an desiccator. The radioactivity of D-(U-^{14}C)-galactose was determined after separation of D-(U-^{14}C)-glucose by paper chromatography in solvent S_1.

The radioactivity was measured with a scintillation spectrometer Packard TriCarb 3 390 (68% effic.). Paper chromatograms cut into 1 × 4 or 0·5 × 4 cm strips were dipped into measuring vessels containing the scintillation mixture (5 ml) PPO (3·5 g) and POPOP (50 mg) in toluene (1 dm^3).

Identification of products of the enzyme conversion of UDP-D-(U-^{14}C)-glucose proceeded in a 0·1M phosphate buffer of pH 7·03 at 30°C. Incubation mixtures containing 11·75 μg of the enzyme protein (fraction at a 35—65% saturation with ammonium sulfate), 43·51 nm of UDP--D-(U-^{14}C)-glucose (specific activity 106·56 MBq/mmol, or 65 μg of the protein, 2 nmol of UDP-D-(U-^{14}C)-glucose (specific activity 2 312·5 MBq/mmol), were incubated in a 20 μl volume for 10 min and 1—30 h, respectively. The fraction of the uridine diphosphate saccharides and hexose monophosphates was divided into three aliquot portions after separation from immobile fraction of water-soluble mono-, oligo- and polysaccharides by electrophoresis on paper in solvent S_4. The first portion showed, after hydrolysis with HCOOH or HCl, the presence of D-(U-^{14}C)-galactose and D-(U-^{14}C)-glucose by paper chromatography in S_1, the second one in S_2 the uridine-diphosphate saccharides and a mixture of hexose monophosphates containing D-(U-^{14}C)-glucose and D-(U-^{14}C)-galactose, which were identified after hydrolysis in S_1. The third fraction was shown to contain D-(U-^{14}C)-glucose and D-(U-^{14}C)-galactose in the uridine-diphosphate saccharides and in the mixture of D-glucose 1-phosphate and D-galactose 1-phosphate, respectively, after hydrolysis in the solvent system S_1, the presence of radioactive glucose 6-phosphate being excluded (checked by chromatography in S_3). Hydrolysates of the water-soluble oligo- and polysaccharides displayed the presence of D-(U-^{14}C)-glucose and D-(U-^{14}C)--galactose as evidenced by paper chromatography in S_1.

For chromatography and electrophoresis paper Whatman No 1 and following systems were used: S_1 ethyl acetate–pyridine–water (8 : 2 : 1), S_2 ethanol–1M-ammonium acetate (5 : 2, pH 3·73), S_3 isobutyric acid–ammonia–water (66 : 1 : 33), and S_4 8%-CH_3COOH–2%-HCOOH of pH 1·80 at 10°C and 50 V/cm for 60 min.

Radioactive products were extracted from the chromatographic paper with water in a glass vessel saturated with water vapours at 10° during 12 h at a continuous flow of water through the paper.

Nucleoside diphosphate saccharides were hydrolyzed with a 90%-HCOOH at 100°C for 3 h, and after dilution with H_2O in a 1 : 8 ratio for 1·5 h, or in a 0·1M-HCl at 100°C for 15 min (ref.[10]).

Radioactive oligo- and polysaccharides were hydrolyzed with a 90% HCOOH at 100°C for 6 h, and after dilution with H_2O in a 1 : 8 ratio at 100°C for 1·5 h.

Hexose monophosphates were hydrolyzed with a 1M-HCl at 100°C for 12 min, or with 90%--HCOOH at 100°C for 3 h, and after dilution with H_2O in a 1 : 8 ratio for additional 1·5 h.

RESULTS AND DISCUSSION

Results concerning the isolation and partial purification of UDP-D-glucose 4-epimerase of white poplar tissue culture are listed in Table I. The fraction precipitating at a 35—65% saturation with $(NH_4)_2SO_4$ contained up to 87% of the total activity.

The presence of the full activity in the supernatant above 40 000*g* indicated that a soluble cytoplasmatic enzyme close to that isolated from *Vicia faba*[6], *Acer pseudoplatanus*[10], and *Populus robusta*[10] was involved. As found, the reaction rate is, under the given conditions linear both with time and enzyme proteins during the first 10 min (Fig. 1). Investigation of the relationship between the initial velocity of the enzymic reaction and concentration of UDP-D-glucose revealed, in accordance with data reported in[10], that it is linear at low concentrations of the substrate in contrast to the enzyme of *Saccharomyces fragilis*, which displayed a sigmoid dependence[20] under these conditions. The apparent Michaelis constant for UDP-D--glucose $K_M = 0{\cdot}15$ mM has a similar value with that of wheat germ[5] of *Phaseolus aureus*[4], but is lower than with a purified enzyme of *Populus robusta*[10]. The effect of pH of the medium, upon the activity of UDP-D-glucose 4-epimerase of white poplar tissue culture was studied and the optimum pH was ascertained to be 9·03 (Fig. 2). The enzyme is stable for 15 days in 0·2 M glycine–NaOH containing in 20% of glycerol at the optimum pH and −30 °C. The decrease of the original activity after a 4 month period was 46%. As it follows from results both of inactivation of the enzyme by NAD^+ nucleosidase and conversion of UDP-D-(U-^{14}C)-glucose to UDP--D-(U-^{14}C)-galactose without addition of NAD^+, the endogenous activator of the enzyme is the tightly bound NAD^+ similarly as with enzymes of *Saccharomyces fragilis*[2], *Escherichia coli*[3,21], *Phaseolus aureus*[4], *Populus robusta*[10] and *Acer pseudoplatanus*[10]. Activity of UDP-D-glucose 4-epimerase of white poplar tissue culture cannot be influenced by an external addition of $2{\cdot}5 \cdot 10^{-4}$ M-NAD^+, or $2{\cdot}5 \cdot 10^{-4}$ M-NADH and is similar, in this aspect, to enzymes of various resour-

TABLE I

Purification of UDP-D-glucose 4-epimerase of the white poplar tissue culture

Purification degree	Total activity[a] nkat	Total protein[a] mg	Specific activity μkat . mg^{-1} . 10^5	Yield %
Extract above 3 000*g*	30·45	836	3·64	100
Supernatant above 40 000*g* after treatment with $MnCl_2$	32·10	654	4·91	105·4
35%–65% saturation with $(NH_4)_2SO_4$	26·55	208	12·76	87·2

[a] Expressed per 100 g of the tissue culture at the 15th day of cultivation.

ces[2-4,10,21] the endogenous activator of which is the bound NAD^+. The possibility to influence the enzyme activity by addition of various purine derivatives into the incubation mixture was investigated with $2{\cdot}5 \cdot 10^{-5}$ M benzylaminopurine (109% relative activity) and $2{\cdot}5 \cdot 10^{-5}$ M kinetin (108% relative activity); these did not substantially influence the activity. Addition of $2{\cdot}5 \cdot 10^{-5}$ M adenosine (123% relative activity), or $2{\cdot}5 \cdot 10^{-5}$ M adenine (147% relative activity) stimulated, however, the activity of UDP-D-glucose 4-epimerase.

The principal substance of the identified products of the enzymic conversion of UDP-D-(U-^{14}C)-glucose at pH 7·03 (Table II) was UDP-D-(U-^{14}C)-galactose. D-(U-^{14}C)-glucose 1-phosphate, D-(U-^{14}C)-galactose 1-phosphate and a mixture of radioactive oligo- and polysaccharides containing D-(U-^{14}C)-glucose and D-(U-^{14}C)-galactose were also isolated to a lesser extent. The enzyme preparation UDP-D-glucose 4-epimerase contained, therefore, also UDP-D-glucose glucosyltransferase,

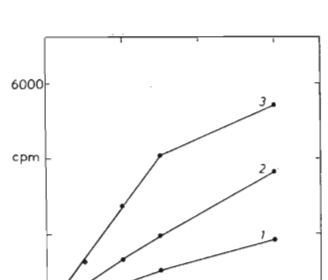

FIG. 1

Dependence of the UDP-D-(U-^{14}C)-galactose upon time of incubation and enzyme protein. Reaction mixture contained 43·51 nmol UDP-D-(U-^{14}C)-glucose (specific activity 53·28 MBq/mmol) and enzyme protein (fraction at a 35—65% saturation with $(NH_4)_2 \cdot SO_4$) in a 0·2M glycine–NaOH of pH 9·03 in an over-all volume 20 µl at 30°C. Ordinate: incubation time, abscissa: UDP-D-(U-^{14}C)-galactose formed. *1* 3·92 µg of protein, *2* 7·84 µg of protein, *3* 15·68 µg of protein

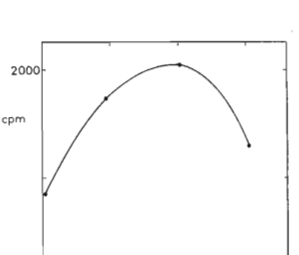

FIG. 2

Effect of pH of the medium on the activity of UDP-D-glucose 4-epimerase. The reaction mixture contained 43·51 nmol of UDP-D-(U-^{14}C)-glucose (specific activity 53·28 MBq/mmol), 1·5 pkat of enzyme (protein at a 35—65% saturation with $(NH_4)_2SO_4$) in 20 µl total volume. Reaction time 10 min at 30°C. Ordinate: pH of the medium. Abscissa: UDP-D-(U-^{14}C)-galactose. Buffers: 0·1M phosphate of 7·03 or 7·96; 0·2M glycine–NaOH of pH 9·03 or 10·05

UDP-D-galactose galactosyltransferase and enzymes capable to cleave the uridine-diphosphate saccharides present in the reaction mixture into the corresponding hexose monophosphates. The effect of the contaminative enzymes was negligible under the given conditions of UDP-D-glucose 4-epimerase determination (Fig. 1).

Results of activity changes of UDP-D-glucose 4-epimerase during the growth of tissue culture are plotted in Fig. 3. The activity of the given enzyme underwent changes during the growth and reached its maximum in accordance with the D-galactose content in cell walls in the period of the most intense tissue culture growth. During this period an enhanced offer of UDP-D-galactose in cells to enzymes res-

TABLE II

Conversion of UDP-D-(U-^{14}C)-glucose by a purified enzyme preparation UDP-D-glucose 4-epimerase of white poplar tissue culture

Compound	The product formation rate[a] $\mu mol\ s^{-1}\ mg^{-1}$
UDP-D-(U-^{14}C)-galactose	$6{\cdot}13 \cdot 10^{-5}$
D-(U-^{14}C)-glucose 1-phosphate	$8{\cdot}19 \cdot 10^{-7}$
D-(U-^{14}C)-galactose 1-phosphate	$9{\cdot}74 \cdot 10^{-8}$
Radioactive oligo- and polysaccharides	$2{\cdot}48 \cdot 10^{-8}$

[a] Calculated per 1 mg of the enzyme protein (fraction at a 35—65% saturation (with $(NH_4)_2SO_4$).

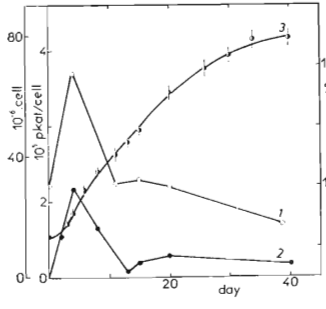

FIG. 3

Change of the activity of UDP-D-glucose 4-epimerase in cells (*1*) in relation to the D-galactose content in cell walls (*2*) during growth of the white poplar tissue culture (*3*). The reaction mixture contained 11·75 μg of the enzyme (protein at a 35—65% saturation with $(NH_4)_2SO_4$), 43·51 nmol of UDP-D-(U-^{14}C)-glucose (specific activity 53·28 MBq/mmol) in 0·2M glycine–NaOH (pH 9·03, total volume 20 μl). Reaction time 5 or 10 min at 30°C. The number of cells of the tissue culture refers to the mean error

ponsible for the synthesis of polysaccharides[8] and galactolipides[9] containing D-galactose was encountered. These results let us suggest that UDP-D-glucose 4-epimerase in cells is involved in the control of biosynthesis of cell-wall polysaccharides containing D-galactose.

Our thanks are due to Dr V. Farkaš for valuable hints and discussions.

REFERENCES

1. Langer R., Glaser L.: J. Biol. Chem. *249*, 1126 (1974).
2. Maxwell E. S., de Robichon-Szulmajster H.: J. Biol. Chem. *235*, 308 (1960).
3. Wilson D. B., Hogness D. S.: J. Biol. Chem. *244*, 2132 (1969).
4. Druzhinina T. N., Novikova M. A., Shibaev V. N.: Biokhimiia *34*, 108 (1969).
5. Fan D. F., Feingold D. S.: Plant. Physiol. *44*, 599 (1969).
6. Koenigs B., Heinz E.: Planta *118*, 159 (1974).
7. Dalessandro G., Northcote D. H.: Planta *134*, 39 (1977).
8. Panayatos N., Villemez C. L.: Biochem. J. *133*, 263 (1973).
9. Axelos M.: Biochimie *60*, 35 (1978).
10. Dalessandro G., Northcote D. H.: Biochem. J. *162*, 267 (1977).
11. Dalessandro G., Northcote D. H.: Biochem. J. *162*, 281 (1977).
12. Blanáriková V., Karácsonyi Š.: Biol. Plant. *20*, 14 (1978).
13. Diaz-Colon J. D., Bowery R. W., Davis F. S., Bauer J. R.: Physiol. Plant. *27*, 60 (1972).
14. Bilisics L., Karácsonyi Š., Kubačková M.: Biol. Plant. *21*, 390 (1979).
15. Lowry O. H., Rosenbrough N. J., Farr L., Randall R. J.: J. Biol. Chem. *193*, 265 (1951).
16. Kivilaan A., Beaman T. C., Bandurski R.: Nature (London) *184*, B. A. 81 (1959).
17. Miller D. H., Mellman I. S., Lamport D. T. A., Miller M.: J. Cell. Biol. *63*, 420 (1974).
18. *Carbohydrate Composition of Extraactive-free Wood and Wood Pulp by Gas–Liquid Chromatography*. TAPPI Standard-Provisional Method T-249, pm-75 (1975).
19. Borchardt L. G., Piper C. V.: TAPPI *53*, 257 (1970).
20. Ray M., Bhadury A.: J. Biol. Chem. *250*, 4373 (1975).
21. Nelsestuen G. L., Kirkwood S.: J. Biol. Chem. *246*, 7533 (1971).

Translated by Z. Votický.