BIOSYNTHESIS OF WATER-SOLUBLE METABOLITES OF UDP-D-GALACTOSE CONTAINING D-GALACTOSE BY AN ENZYMIC PREPARATION ISOLATED FROM TISSUE CULTURE OF POPLAR (*Populus alba* L., var. *pyramidalis*)

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Biosynthesis of constituents of pectic polysaccharides containing D-galactose was investigated in poplar tissue culture in vitro. The particulate enzymic preparation isolated from the tissue culture of poplar catalyzed the transfer of D-[U-^{14}C]-galactose unit form UDP-D-[U-^{14}C]--galactose into various endogenous acceptors to form galactolipides, water-soluble metabolites and membrane-bound galactoproteins. The initial rate of formation of water-soluble metabolites depends on the substrate concentration and is time and amount of the enzymic protein directly proportional. Optimal formation of these metabolites proceeds at pH 7·6 and 40°C in the presence of 0·1 mM ATP and 10 mM $MgCl_2$. Long-term incubations afforded a mixture of three radioactive water-soluble metabolites: a neutral oligomer of $M_r \sim 620$ consisting at least of two β-D-[U-^{14}C]--galactose units linked by (1→6) bonds and two oligomers of $M_r \sim 1400$ and $\geqq 2950$, respectively, containing (1→6) and (1→4)-linked D-[U-^{14}C]-galactose units. Their relative proportion in the mixture changed in relation to incubation time and the substrate concentration. Structural features of these metabolites resembled those of D-galactans or L-rhamnogalacturonan side chains previously found in primary cell walls of some higher plants.

D-Galactose is an important constituent of the noncellulosic polysaccharides and glycoproteins found in primary cell walls of dicotyledonous plants. It has been reported in rhamnogalacturonans[1-4], xyloglucans[1,2], extensine[5,6], and in lectin-like proteins[7,8]. Suspension cultured sycamore primary cell walls contain two considerably different pectic polysaccharides - rhamnogalacturonan *I* (refs[1-4]) and rhamnogalacturonan *II* (refs[1,2]). Side chains of rhamnogalacturonan *I*, composed mainly of L-arabinose and D-galactose, contain D-galactose units substituted predominantly in positions 4 and 6 in an approximately equal ratio. So far, at least 30 types of side chains having different composition and structure were reported. Pectin polysaccharides similar to rhamnogalacturonan *I* were reported also in other plant species[2,9]. It is assumed that their side chains embodying D-galactose play a role in changes of physical properties of pectic polysaccharides, which precede the growth of primary cell wall of dicotyledonous plants[10-11].

Infrequent papers[12-15] dealing with the biosynthesis of biopolymers containing D-galactose report formation of various polymers from water[12] to alkali[13] soluble. The presence of (1 → 4)- and (1 → 3)-linked[13], as well as (1 → 6)-linked β-D-galactose units was found in a mixture of biosynthesized polymeric products of herbaceous materials[14,15]; nevertheless, no evidence on its biosynthesis in wood species was hitherto presented. Considering the fact that the side chains of highly branched acidic polysaccharide of primary cell walls of plants (L-rhamno-D-galacturonan *I*) are short (the average length about seven glycosyl residues[1-3]), their biosynthetic precursors might be the low-molecular water-soluble metabolites of UDP-D-galactose containing β-D-galactose units. Production of these metabolites has not been investigated. This paper is aimed to find optimal conditions for selective formation of water-soluble metabolites of UDP-D-galactose by a particulate enzymic preparation isolated from poplar tissue culture and to characterize their partial structure.

EXPERIMENTAL

Material and Methods

Chemicals and radiochemicals: UDP-D-galactose Na salt, UDP-D-glucose Na salt, NAD^+: nucleosidase (EC 3.2.2.5) from *Neurospora crassa*, β-galactosidase from *Aspergillus niger* and α-galactosidase from green coffee beans (Sigma, U.S.A.), catalase and D,L-glyceraldehyde (Reanal, Hungary), ATP Na_2 salt (Boehringer, F.R.G.). Maltose, maltotriose and maltopentaose (Koch-Light, England), Sephadex G-15 and Blue dextran (Pharmacia, Sweden), melibiose and lactose (Institute of Chemistry, Slovak Academy of Sciences, Czechoslovakia) Dowex 1X8 and 50WX8 (Bio Rad, U.S.A.), $NaBH_4$ (Baker, England), Miracloth (Calbiochem, Switzerland), D-galactose oxidase from *Dactylium dendroides* (Institute of Chemistry, Slovak Academy of Sciences, Czechoslovakia), L-arabino-D-galactan from *Larix sibirica* (Dr Š. Karácsonyi, Institute of Chemistry, Slovak Academy of Sciences, Czechoslovakia), pronase P (Kaken Kagaku, Japan). The purity of UDP-D-[U-^{14}C]-galactose ammonium salt (10 064 MBq $mmol^{-1}$, Amersham, England), was checked by procedure detailed in previous paper[16].

Chromatography: Whatman No 1 paper and solvent system S1 ethyl acetate—pyridine—water (8 : 2 : 1), S2 butanol—ethanol—water (10 : 1 : 2), S3 butanol—acetic acid—water (6 : 1 : 2) were employed; S2 was the solvent system for chromatography on 0-(carboxymethyl)cellulose paper (Whatman, England) in the lanthanum form[17].

High-voltage horizontal electrophoresis on Whatman No 1 paper proceeded in a 50 mM sodium tetraborate solution at pH 9·5, 45 V cm^{-1} and 10°C, picric acid being the referece[12].

The radioactivity was measured with a scintillation spectrometer Packard Tri-Carb 3 390 at an external standardization in scintillation solution SLD 31 (Spolana, Czechoslovakia) for water-soluble samples, and in toluene (3·5 g PPO and 50 mg POPOP in 1 dm^3 of toluene) for paper strips (1 × 4 or 0·5 × 4 cm).

Protein was assayed according to Lowry and coworkers[18] employing bovine serum albumine as reference.

The saccharide content was determined by the Dubois and coworkers[19] method (phenol–sulfuric acid).

Gel chromatography: the water-soluble mixture of metabolites was chromatographed on a Sephadex G-15 (1·3 × 99 cm) column with toluene saturated water. Radioactivity and the saccharide content were determined in an aliquot of 1-ml fractions. The relative molecular mass of water-soluble metabolites was estimated on the above-mentioned column from the selection curve according to Granath and coworkers[20]. Partition coefficients (K_{av}) of the individual water-soluble metabolites were calculated from the bed volume (V_t), elution volume of the completely excluded Blue Dextran (V_0) and from elution volumes (V_e) of D-galactose, maltose, maltotriose, and maltopentaose.

Plant material and isolation of the particulate enzymic preparation. The particulate enzymic preparation was isolated from the seven days old pigmented tissue culture of poplar[21]. The tissue was homogenized in a phosphate buffer solution (50 mM, pH 7·0) containing saccharose (0·4M) with an X-Press Cell Disintegrator (LKB-Biotec, Sweden) at −25°C and 200 MPa pressure. The homogenate was filtered through Miracloth and centrifugated to give the membrane fractions sedimenting between 500—40 000 *g* (protein content 30—54 mg) and between 40 000—100 000 *g* (protein content 11—14 mg per 100 g of fresh tissue culture mass. The afore-mentioned membrane fractions suspended in pH 7·0 phosphate buffer solution (25 mM) with saccharose (0·2M) were ready for immediate use.

Galactosyltransferase activities were assayed by measuring the amount of D-[U-^{14}C]-galactose transferred from UDP-D-[U-^{14}C]-galactose to endogenous acceptors under formation of radioactive galactolipides, water-soluble metabolites and galactoproteins. Aliquots of the membrane fraction 500—40 000 *g* (21 μg of protein) and 40 000—100 000 *g* (6·7 μg of protein) in pH 7·0 phosphate buffer solution (25 mM-KH_2PO_4–NaOH, 20 μl) containing saccharose (0·2M) were pre-incubated without substrate for 10 min and then incubated with 22·98 μM UDP-D-[U-^{14}C]-galactose (specific radioactivity 10 064 MBq mmol^{-1}) at 30°C for 15 min. The reactions were stopped by adding sodium tetraborate (50 mM, pH 9·5, 20 μl) and freezing. The reaction mixtures both without the enzymic preparation and with an inactivated (100°C, 15 min) preparation were concurrently incubated and their radioactivity was found to be negligible. The nonincorporated UDP-D-[U-^{14}C]-galactose was removed by electrophoresis on Whatman No 1 paper by the method of McNab et. al.[12]. The radioactive material immobile in sodium tetraborate at the electrophoresis condition was fractionally extracted; with chloroform–methanol (2 : 1), ref.[22] and water, fractions of glycolipides and water-soluble metabolites, respectively, were obtained. The 72-h action of pronase P (9 000 PUK) on the residual material in 50 mM pH 7·0 phosphate buffer solution containing 5% of ethanol at 25°C, followed by elution with water resulted in liberation of degraded glycoproteins[11]. No radioactivity was detected after the above-mentioned extractions in the electroforetogram. The individual fractions were hydrolysed with trifluoroacetic acid and analyzed by paper chromatography. Position of radioactivity on 1 × 4 cm chromatographic strips was localized by comparison with D-galactose and D-glucose standards.

Enzymic synthesis of water-soluble metabolites of UDP-D-[U-^{14}C]-galactose proceeded by incubation of the enzymic preparation (membrane fraction 500—40 000 *g*) with UDP-D-[U-^{14}C]-galactose under conditions plotted in Fig. 1 after a 10 min preincubation without the substrate in the reaction mixture (4·8 ml). A concurrent checking of the reaction mixture both without the enzymic preparation and with thermally incativated preparation showed a negligible radioactivity.

The water-soluble, paper electrophoresis immobile portion of products was purified by paper chromatography in S1 (34 h) and the radioactive material found near the starting line was chromatographically fractionated on Sephadex G-15. Each fraction was purified over a Dowex 1X8 (acetate form) packed column. The neutral and acid fractions eluable with water or 6M acetic acid were used for further characterization of products synthesized.

Hydrolysis of degradation products of glycoproteins, water-soluble metabolites of UDP-D-[U-^{14}C]-galactose, their reduction products or compounds obtained by periodate oxidation was effected with 2M trifluoroacetic acid at 120°C for 90 min (ref.[11]). Solutions of glycolipides in 50% propanol were hydrolysed[23] with 2M trifluoroacetic acid at 120°C for 90 min. The hydrolysate was neutralized with 2M-NaOH, a double volume of chloroform–methanol (2 : 1) was added, the aqueous layer was separated and deionized with Dowex 50WX8 (H^+).

Action of NAD^+: *nucleosidase* on the particulate enzymic preparation (42 μg of protein) in pH 7·0 phosphate buffer solution (100 mM) with 0·4M saccharose (20 μl total volume) was incubated with NAD^+: nucleosidase (333 pkat) at 30°C for 60 min. (ref.[16]) and with inactivated NAD^+: nucleosidase; all necessary tests complemented the experiment.

The effect of ATP, $MgCl_2$, UDP-D-*glucose and* L-*arabino*-D-*galactan* on the initial incorporation rate of D-[U-^{14}C]-galactose from UDP-D-[-U-^{14}C]-galactose into individual metabolites was investigated by incubation of the particulate enzymic preparation (membrane fraction 500—40 000 *g*, 20·2 μg of protein) with the substrate (22·98 μM, specific radioactivity 10 064 MBq . mmol^{-1}) at 30°C for 15 min.

Action of the saturated aqueous solution of sodium tetraborate on water-soluble metabolites of UDP-D-[U-^{14}C]-galactose (5 000—10 000 dpm) or their fragments obtained by a partial hydrolysis was examined at 25°C for 12 h. Unlabelled carriers (galactitol 50 μg and D-galactose 100 μg) were added after acidification with acetic acid and deionization with Dowex 50WX8 (H^+), and the borate ions were removed by a triple evaporation with methanol to dryness. The sample was hydrolysed with a 2M trifluoroacetic acid at 120°C for 90 min. The presence of D-[U-^{14}C]-galactose and [U-^{14}C]-galactitol was checked by chromatography on O-(carboxymethyl)-cellulose sheet in lanthanum form[17] in solvent system S2. The products were identified on 1 × 4 cm chromatographic strips by assaying the distribution of radioactivity and comparison with the mobility of D-galactose and galactitol.

Smith degradation. A sample of water-soluble metabolite of UDP-D-[U-^{14}C]-galactose (63 000—160 000 dpm) was oxidized with $NaIO_4$ (50 mM, 1 ml) in dark at 4°C for 96 h (ref.[13]). After addition of ethylene glycol (20 μl) the mixture was reduced with $NaBH_4$ by the already mentioned procedure and hydrolysed with trifluoroacetic acid. The hydrolysate was once more reduced with $NaBH_4$, the excess of the reducing agent was removed, carriers ethylene glycol (800 μg), glycerol (800 μg), galactitol (100 μg), threitol (100 μg), and arabinitol (50 μg) were introduced and the radioactive alditols were separated by paper chromatography with S2 (ref.[17]). The products were identified according the mobility of unlabelled references after locating the radioactivity.

Partial hydrolysis of neutral water-soluble metabolite of UDP-D-[U-^{14}C]-galactose. The sample was hydrolysed with 0·5M trifluoroacetic acid at 100°C for 15 min and the fragments formed were separated by paper chromatography in S1 for 72 h to remove D-[U-^{14}C]-galactose, and for 168 h to separate the radioactive fragment having the mobility of trisaccharides. Position of the radioactive fragments on chromatographic strips (1 × 4 and 0·5 × 0·4 cm) was detected by comparison with the mobility of unlabelled D-galactose and lactose, respectively.

Enzymic hydrolysis of neutral water-soluble metabolites of UDP-D-[U-^{14}C]-galactose. The sample (10 210 dpm) in acetate buffer solution (60 μl) (50 mM, pH 5·2) was incubated with β-galactosidase from *Aspergillus niger* (0·36 nkat) at 25°C for 72 h. The enzyme cleaved under this condition lactose. but not melibiose. The effect of α-galactosidase from green coffee beans (0·28 nkat) was investigated by incubation of a sample (10 900 dpm) in acetate buffer solution (50 mM, pH 6·8, 60 μl) at 25°C for 72 h. This enzyme hydrolysed melibiose exclusively. The

reaction was stopped by heating to 100°C for 5 min, the mixture was deionized (ion exchanger V, Merck) and the hydrolysates were analysed by paper chromatography in S1.

Action of D-*galactose oxidase* from *Dactylium dendroides* (12 nkat) on a neutral water-soluble metabolite of UDP-D-[U-^{14}C]-galactose (5 000 dpm) was monitored in the presence of catalase (1 200 nkat) according to Rogers and Thompson[24]. The sample was desalted by gel filtration (Sephadex G-15), then 0·1M-I_2 and 0·1M-KI in 0·5M-Na_2CO_3 were added according to Rosen and coworkers[25], and after hydrolysis and work-up the hydrolysate was analysed by paper chromatography in S3, where the mobility of D-galacturonic acid against D-galactose was R_{Gal} 0·76.

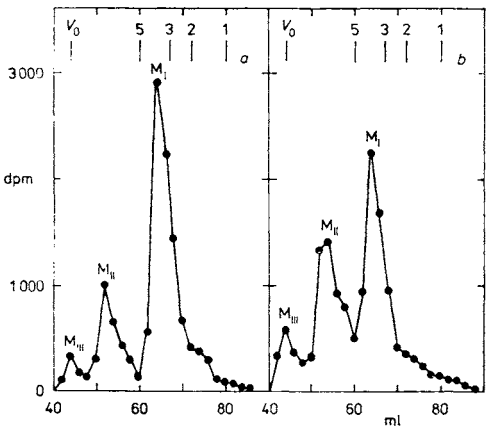

FIG. 1

Gel chromatography of water-soluble metabolites of UDP-D-[U-^{14}C]-galactose on a Sephadex G-15 column. Incubation time: *a* 1 h, *b* 6 h. The mixture contained the enzymic protein (membrane fraction 500—40 000 *g*, 39·5 μg), ATP (0·1 mM), $MgCl_2$ (10 mM), saccharose (0·2M), KH_2PO_4–NaOH (25 mM, pH 7·1), and UDP-D-[U-^{14}C]-galactose (23·8 μM, specific radioactivity 7 807 MBq mmol^{-1}); temperature 30°C, total volume 20 μl. V_0, 1, 2, 3 and 5 are the elution volumes of Blue Dextran, D-galactose, maltose, maltotriose, and maltopentaose, respectively. M_I neutral water-soluble metabolite of $M_r \sim 620$, M_{II} acidic water-soluble metabolite of $M_r \sim 1\,400$, M_{III} acidic water-soluble metabolite of $M_r \geqq 2\,950$. Ordinate: elution volume; abscissa: radioactivity of the metabolite

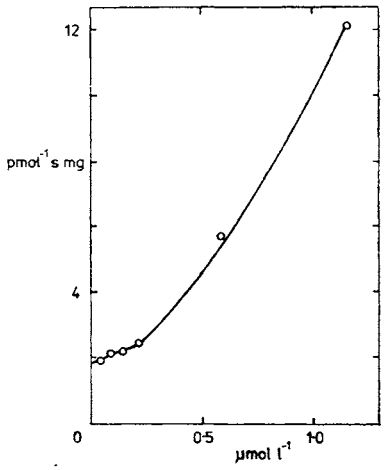

FIG. 2

Relationship between the initial rate of formation of water-soluble metabolites and concentration of the substrate. The mixture contained the enzymic protein (membrane fraction 500—40 000 *g*, 20·2 μg), UDP-D-[U-^{14}C]-galactose (0·77—22·98 μM, specific radioactivity 10 064 MBq mmol^{-1}), KH_2PO_4–NaOH (25 mM, pH 7·1), and saccharose (0·2 M) in a total volume 20 μl. Incubation time at 30°C: 15 min. Ordinate: reciprocal value of the substrate concentration; abscissa: reciprocal value of the initial rate of the enzymic reaction

RESULTS

Optimal Conditions for a Selective Formation of Water-Soluble Metabolites

Fractional centrifugation of disintegrated tissue culture of poplar afforded two membrane fractions sedimenting within 500 and 40 000 *g* and 40 000 and 100 000 *g*. Both fractions catalyse the transfer of D-[U-^{14}C]-galactose units from UDP-D-[U-^{14}C]-galactose to endogenous acceptors to form glycolipides containing D-[U-^{14}C]-galactose, water-soluble metabolites and glycoproteins (Table I); this finding indicates the presence of active galactosyltransferases. The membrane fraction sedimenting above 40 000 *g* was further not utilized, because water-soluble metabolites thus produced were contamined by D-[U-^{14}C]-glucose (20%). An attempt to block UDP-D-glucose-4-epimerase present in this fraction by hydrolysis of its coenzyme with NAD^+: nucleosidase[16] failed. Enzymic hydrolysis caused also the inhibition of water-soluble metabolites formation (by 93%) in addition to inactivation of UDP-D-glucose-4-epimerase. For further experiments only the fresh isolated fraction sedimenting within 500 and 40 000 *g* was employed as a particulate enzymic preparation, since it does not contain UDP-D-glucose-4-epimerase and therefore, the resulting products were not contamined with D-[U-^{14}C]-glucose.

Our preliminary experiments on formation of UDP-D-[U-^{14}C]-galactose metabolites in relation to time showed a short lag phase, which could be removed by a 10-min pre-incubation of the particulate enzymic preparation without any substrate; this procedure was applied in all further experiments.

To ascertain the optimal condition for formation of UDP-D-[U-^{14}C]-galactose metabolites the relationship between the initial rate of their production and the concentration of the substrate was examined. Since the particulate enzymic preparation probably contains some galactosyltransferases, the double reciprocal curve illustrating the influence of UDP-D-[U-^{14}C]-galactose concentration on the initial rate of water-soluble metabolites formation was not linear; nevertheless, it made it possible to calculate the maximal velocity of the enzymic reaction. The Hill plot[26] enabled to find linear dependences $\log (v/V_{max} - v)$ on the logarithm of the substrate concentration (v the initial rate, V_{max} the maximal velocity of the enzymic reaction) from which the particular apparent half-saturation constants of the enzyme by the substrate $S_{0.5}$ could be enumerated. The $S_{0.5}$ value, characterizing the highest affinity of galactosyltransferases responsible for the production of water-soluble metabolites to substrate under condition given in Fig. 2 was found to be 2·54 μM (V_{max} 0·53 pmol s^{-1} mg^{-1}, $\bar{n}$ 1·5) and the highest V_{max} value, but the lowest affinity of the substrate to the enzyme was observed when forming galactolipides ($S_{0.5}$ 8·18 μM, V_{max} 1·38 pmol s^{-1} mg^{-1}, $\bar{n}$ 0·9). The lowest V_{max} is indicative of production of galactoproteins ($S_{0.5}$ 4·12 μM, V_{max} 0·11 pmol s^{-1} mg^{-1}, $\bar{n}$ 1·7).

Possibilities were chosen to influence selectively the galactosyltransferase activities embodied in the particulate enzymic preparation aiming to enhance the yield of

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TABLE I

Incorporation of D-[U-^{14}C]-galactose in metabolites of UDP-D-[U-^{14}C]-galactose by various membrane fractions. The mixture contained the enzyme protein (fraction 40 000—100 000 *g*, 6·73 μg, or fraction 500—40 000 *g*, 21 μg), UDP-D-[U-^{14}C]-galactose (22·98 μM, specific radioactivity 10 064 MBq mmol^{-1}, KH_2PO_4–NaOH (25 mM, pH 7·1), and 0·2M saccharose in a total volume 20 μl. Incubation at 30°C: 15 min

Membrane fraction	Protein[a] mg	Incorporation[b], dpm min^{-1}			Specific activity[c] . 10^8, μkat mg^{-1}		
		glyco-lipides	water-soluble metabolites	glyco-proteins	glyco-lipides	water-soluble metabolites	glyco-proteins
500—40 000 *g*	41·36	273 230	64 732	20 743	23·95	5·67	1·81
40 000—100 000 *g*	13·24	163 727	60 133	13 786	44·83	16·46	3·77

[a] Protein content from 100 g of fresh tissue culture; [b] expressed by incorporation of D-[U-^{14}C]-galactose per 100 g of the fresh tissue culture at the 7th day of growth; [c] expressed by incorporation of D-[U-^{14}C]-galactose by unit amount of enzyme protein into the particular metabolites.

water-soluble metabolites. The optimal production of an unidentified water-soluble D-galactan requires, according to McNab and al.[12] the presence of 5 mM $MgCl_2$ and according to Mellor and Lord[23] recommendation an addition of 2mM ATP to avoid decomposition of UDP-D-[U-^{14}C]-galactose by traces of nucleotide pyrophosphatase probably also present in our tissue culture[16]. Application of this procedure to our system resulted in a decrease of initial rate of water-soluble metabolites formation (Table II). The maximal velocity of their origination requires the presence of 10 mM $MgCl_2$ and 0·1 mM ATP to suppress the partial production of galactolipides.

UDP-D-Glucose (6·96 μM) was found not to increase the incorporation of D-[U-^{14}C]-galactose from UDP-D-[U-^{14}C]-galactose into water-soluble metabolites and therefore, its participation in formation of these metabolites was not presumed. L-Arabino-D-galactan (0·9 μg μl^{-1}) as a possible primer of transfer does also not influence this origination.

TABLE II

Influence of Mg^{2+} and ATP on the initial rate of UDP-D-[U-^{14}]-galactose metabolites formation. Incorporation relates to incorporation of D-[U-^{14}C]-galactose into the mixture of metabolites (100%) without addition of Mg^{2+} and ATP

Addition		Incorporation of D-[U-^{14}C]-galactose, %			
$MgCl_2$ mmol l^{-1}	ATP mmol l^{-1}	total	galactolipides	water-soluble metabolites	galactoproteins
0	0	100	65·9	28·4	5·8
0	0·1	116·1	68·5	29·9	17·7
0	2	69·3	56·7	9·7	8·9
0·5	2	59·0	43·5	7·3	8·2
1	2	63·8	49·4	6·2	8·2
5	0	118·2	77·1	27·9	13·2
5	0·1	109·7	63·7	30·2	15·8
5	0·5	90·5	63·3	14·1	13·1
5	1	81·3	69·7	4·2	7·4
5	2	69·7	54·7	5·8	9·2
5	5	57·1	44·2	2·8	10·1
5	10	39·4	28·3	0	11·1
10	0	133·6	81·7	36·9	15·0
10	0·1	85·8	23·0	43·7	19·1
10	2	70·6	60·0	6·2	4·4
50	0	109·2	71·8	23·4	14·0
50	0·1	119·9	63·3	25·7	30·9
50	2	87·7	70·5	8·7	8·5

A linear relationship between incorporation of D-[U-^{14}C]-galactose into water-soluble metabolites, time and quantity of the enzymic protein during the initial 15 min of incubation was found under condition of saturation of galactosyltransferases by the substrate (Fig. 3).

It has been reported[12] that formation of the water-soluble D-galactan from UDP-D-galactose has its optimum at 30°C and pH 7·1; it is noteworthy that a like optimal pH (7·6) is also characteristic of production of our water-soluble metabolites by the particulate enzymic preparation isolated from the poplar tissue culture (Fig. 4). Nevertheless, the temperature optimum was around 40°C (Fig. 5).

Since the V_{max} of the formation of water-soluble metabolites from UDP-D-[U-^{14}C]--galactose is quite low, preparation of a sufficient amount needed for the structural

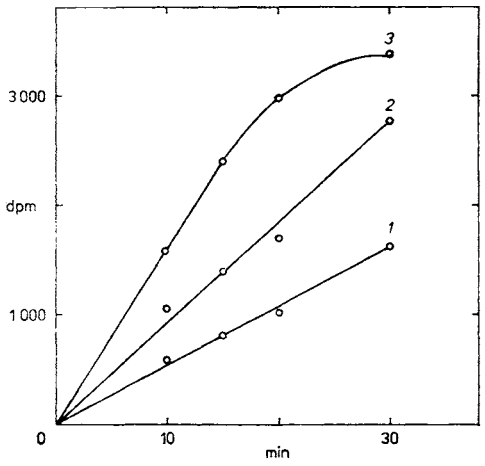

FIG. 3

Dependence of the initial rate of formation of water-soluble metabolites on time and amount of the enzymic protein. The mixture contained UDP-D-[U-^{14}C]-galactose (22·98 μM, specific radioactivity 10 064 MBq . . mmol^{-1}), enzymic protein (membrane fraction 500—40 000 *g* in KH_2PO_4–NaOH (25 mM, pH 7·1) and saccharose (0·2M) in a total volume 20 μl. Incubation temperature 30°C. Ordinate: incubation time; abscissa: radioactivity of water-soluble metabolites. Amount of the enzyme (μg): *1* 3·79, *2* 6·63, *3* 11·36

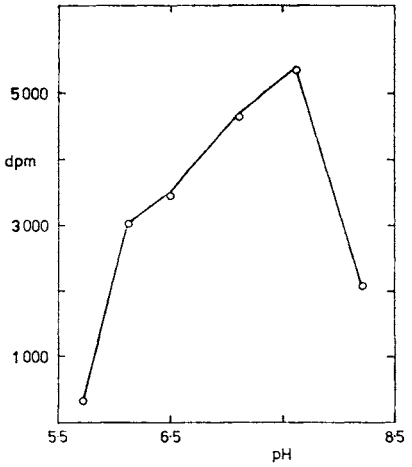

FIG. 4

Influence of pH on the V_{max} of water-soluble metabolites. The mixture contained UDP-D-[U-^{14}C]-galactose (22·98 μM, specific radioactivity 10 064 MBq mmol^{-1}), enzymic protein (membrane fraction 500—40 000 *g*, 22·2 μg), KH_2PO_4–NaOH (25 mM), or Na_2HPO_4–citric acid (25 mM, pH 5·71,, or TRIS–HCl (25 mM, pH 8·2), and saccharose (0·2M) in a total volume 20 μl. Incubation time at 30°C: 15 min. Ordinate: pH of the medium; abscissa: radioactivity of the water-soluble metabolites formed

studies required extension of the incubation time of the particulate enzymic preparation with the substrate up to 6 h. The portion of the water-soluble metabolites (15% incorporation of radioactivity), obtained under this condition, was indicative of low mobility at refractionation by paper chromatography in S1; this material was used for further study.

Partial Structural Characterization of Water-Soluble Metabolites

Gel chromatography of the afore-mentioned material over a Sephadex G-15 column (Fig. 1) showed the presence of three radioactive metabolites (M_I to M_{III}) differing in relative molecular masses ($M_r \sim 620$, $\sim 1\,400$, and $\geqq 2\,950$). The relative representation of the individual fractions in the mixture changed in relation to incubation time and concentration of the substrate (Table III). During the first hour of incuba-

TABLE III

The M_r distribution dependence of water-soluble metabolites upon incubation time and substrate concentration. The reaction mixture contained the enzymic protein (membrane fraction 500—40 000 *g*, 158 μg), ATP (0·1 mM), $MgCl_2$ (10 mM), saccharose (0·2M), KH_2PO_4–NaOH (25 mM, pH 7·1), and UDP-D-[U-^{14}C]-galactose (23·8 μM, specific radioactivity 7 807 MBq mmol^{-1}, or 130·9 μM and 620·2 MBq mmol^{-1}, respectively) in a total volume 80 μl; temperature: 30°C

Incubation time, h		1	6	1	6
Concentration of the substrate, μM		23·8	23·8	130·9	130.9
$M_r \sim 620$	Content in the mixture of water-soluble products, %	71·37	50·79	42·99	62·77
	Amount formed[a], pmol	161	142	153	563
$M_r \sim 1\,400$	Content in the mixture of water-soluble products, %	22·43	38·73	39·86	27·14
	Amount formed[a], pmol	51	108	142	243
$M_r \geqq 2\,950$	Content in the mixture of water-soluble products, %	6·20	10·48	17·15	10·09
	Amount formed[a], pmol	14	29	60	91
Total radioactivity incorporation into UDP-D-[U-^{14}C]-galactose metabolites[a], %		43·57	57·66	13·47	31·34
Total radioactivity incorporation into the mixture of water-soluble products[a], %		11·39	14·05	3·39	8·57
The total amount of mixture of water-soluble products formed[b], pmol		226	279	355	897

[a] In % of the incorporated D-[U-^{14}C]-galactose; [b] in pmol of D-[U-^{14}C]-galactose.

tion at lower concentrations of the substrate (23·8 μM) the substantial portion of D-[U-^{14}C]-galactose was incorporated into the metabolite M_I, whilst the ratio of radioactivity in the metabolite M_I decreases after 6 h and increases in metabolites M_{II} and M_{III}. At a high concentration of the substrate (130·9 μM), the ratio of D-[U-^{14}C]--galactose in the M_{II} and M_{III} does not increase with time, but their absolute amount does.

Behaviour of these fractions on Dowex 1X8 column in acetate form evidences one neutral (M_I) and two acid metabolites (M_{II}, M_{III}) to be involved. Whereas metabolite M_I passed the ion exchanger freely, the remaining M_{II} and M_{III} were trapped and afterwards eluted with 6M acetic acid.

A total hydrolysis of the neutral and acidic water-soluble metabolites afforded only D-[U-^{14}C]-galactose as a sole radioactive saccharide. Treatment with $NaBH_4$ followed by a hydrolysis led to D-[U-^{14}C]-galactose, as well; consequently, the

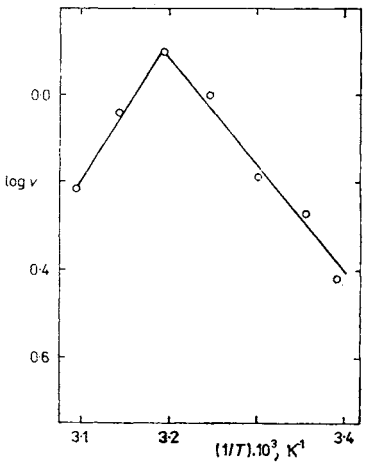

FIG. 5

Arrhenius plot of water-soluble metabolites formation. The mixture contained UDP-D--[U-^{14}C]-galactose (22·98 μM, specific radioactivity 10 064 MBq mmol^{-1}), enzymic protein (membrane fraction 500—40 000 *g*, 22.2 μg), KH_2PO_4–NaOH (25 mM, pH 7·1), and saccharose (0·2M) in a total volume 20 μl. The reaction velocity *v* (pmol s^{-1}) per 1 mg of the enzymic protein. Ordinate: log *v*; abscissa: reciprocal temperature value of the medium

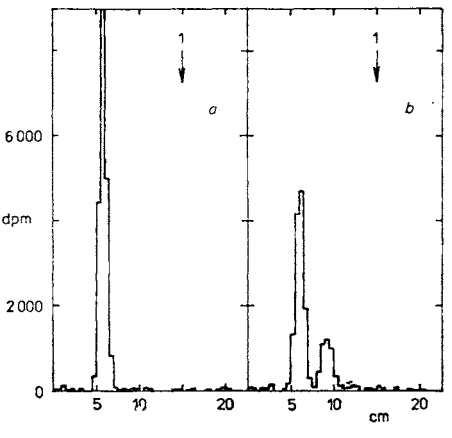

FIG. 6

Chromatographic separation of the mixture of the radioactive fragment of neutral water-soluble metabolite from the original compound after partial hydrolysis. The neutral water-soluble metabolite *a* without, *b* after treatment with trifluoroacetic acid (0·5M, 100°C, 15 min). Solvent system ethyl acetate–pyridine–water (8 : 2 : 1), paper Whatman No 1, development 168 h. References: 1 lactose. Ordinate: the distance from the starting point; abscissa: radioactivity of the product

reducing end of all three metabolites is substituted by non-radioactive components to which D-[U-^{14}C]-galactose was transferred.

Partial hydrolysis of M_I followed by paper chromatography on S1 revealed D-[U-^{14}C]-galactose and a radioactive fragment containing D-[U-^{14}C]-galactose and having the mobility of trisaccharide (R_{Gal} 0·13) (Fig. 6). Treatment with $NaBH_4$ and hydrolysis of this fragment did not yield [U-^{14}C]-galactitol, but only D-[U-^{14}C]--galactose. A radioactive reducing disaccharide was not obtained by this partial hydrolysis. The results of fragmentation and the course of partial hydrolysis in time (Fig. 7) let propose that M_I contains at least two D-[U-^{14}C]-galactose units linked head-to-tail to an unknown aglycone. Release of D-[U-^{14}C]-galactose and a further radioactive intermediate by β-galactosidase from *Aspergillus niger* (Fig. 8) from the material under investigation and resistance towards the action of α-galactosidase from green coffee beans indicate the presence of β-glycosidic bonds between D--[U-^{14}C]-galactopyranose units at the non-reducing end of the chain. The Smith degradation afforded [U-^{14}C]-glycerol only, what eliminates the appearance of

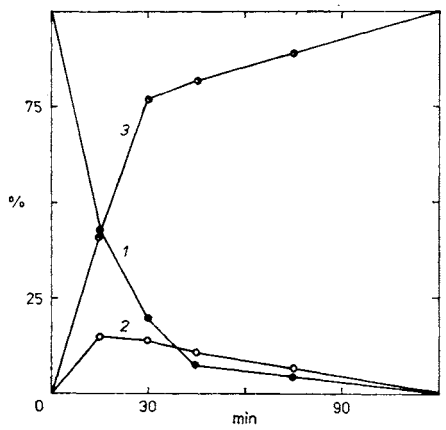

FIG. 7

The time dependence of the partial hydrolysis with trifluoroacetic acid (0·5M, 100°C) of the neutral water-soluble metabolite. *1* The original metabolite, *2* the radioactive fragment having mobility of trisaccharide on paper chromatography in ethyl acetate–pyridine–water (8 : 2 : 1), *3* D-[U-^{14}C]-galactose. Ordinate: hydrolysis time; abscissa: % of the total radioactivity present in the original metabolite

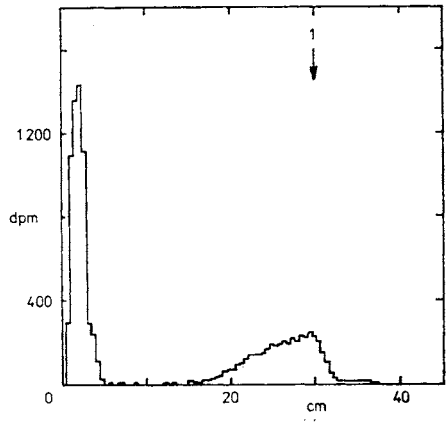

FIG. 8

Chromatographic separation of the mixture of products after action of β-galactosidase from *Aspergillus niger* on a neutral water-soluble metabolite. Solvent system: ethyl acetate–pyridine–water (8 : 2 : 1), paper Whatman No 1, development 72 h. Reference: 1 D-galactose. Ordinate: distance from the origin; abscissa: radioactivity of the product

(1 → 3) and (1 → 4)-linked D-[U-^{14}C]-galactose units in the metabolite chain. The metabolite M_1 does not undergo transformation by D-galactose oxidase from *Dacty-*

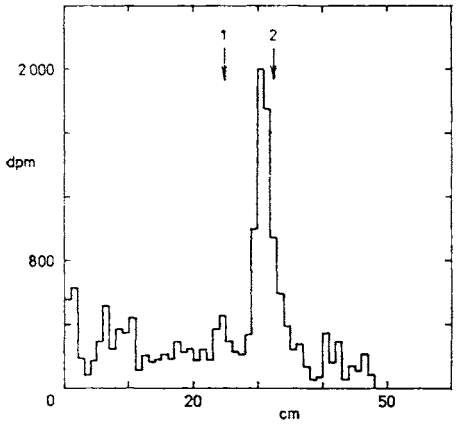

FIG. 9

Paper chromatography of the hydrolysate of the neutral water-soluble metabolite after oxidation by D-galactose oxidase and hypoiodite. References: 1 galacturonic acid, 2 D-galactose. Ordinate: distance from the origin; abscissa: radioactivity of the product

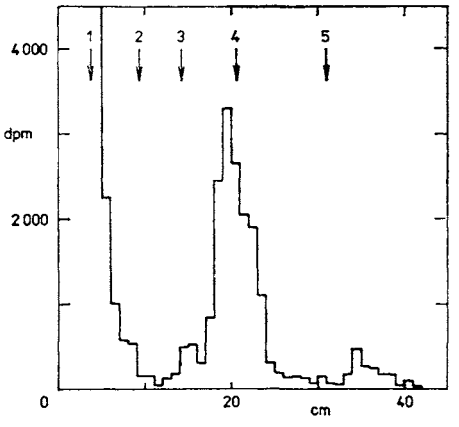

FIG. 10

Paper chromatography of products of Smith degradation of the acidic water-soluble metabolite of $M_r \sim 1\,400$. References: 1 galactitol, 2 arabinitol, 3 threitol, 4 glycerol, 5 ethylene glycol. Solvent system butanol–ethanol–water (10 : 1 : 2). Ordinate: distance from the origin; abscissa: radioactivity of the product

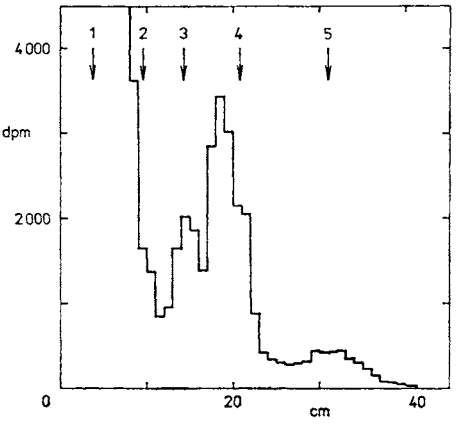

FIG. 11

Paper chromatography of products of Smith degradation of the acidic water-soluble metabolite of $M_r \geqq 2\,950$. References: 1 galactitol, 2 arabinitol, 3 threitol, 4 glycerol, 5 ethylene glycol. Solvent system: butanol–ethanol–water (10 : 1 : 2). Ordinate: distance from the origin; abscissa: radioactivity of the product

lium dendroides and therefore, appearance of (1 → 2)-linked D-[U-^{14}C]-galactose units to which the enzyme exhibits activity[27,28] is not presumed in it. This follows from the absence of significant amounts of D-[U-^{14}C]-galacturonic acid in hydrolysates of M_I after action of the above-mentioned enzyme and potassium hypoiodite (Fig. 9). These results let us conclude that the neutral water-soluble metabolite M_I of $M_r \sim 620$ contains at least two β-D-[U-^{14}C]-galactose (1 → 6)-linked units to an endogenous acceptor.

Investigation of further two water-soluble metabolites M_{II} and M_{III} led to partially different results (Figs 10 and 11). The Smith degradation of these metabolites of relative molecular masses $M_r \sim 1\,400$ and $\geqq 2\,950$ furnished [U-^{14}C]-glycerol and [U-^{14}C]-threitol in molecular ratios 10 : 0·83 and 10 : 2·95, respectively, thus indicating the M_{II} and M_{III} probably to consist mainly of (1 → 6), but also of (1 → 4)-linked D-[U-^{14}C]-galactose units. Treatment of M_{III} with pronase P did not result in change of its M_r.

DISCUSSION

Our results show that the particulate enzymic preparation isolated from the poplar tissue culture is able to incorporate D-[U-^{14}C]-galactose from UDP-D-[U-^{14}C]-galactose into metabolites having structural features of D-galactans[29,30], whilst their side chains display those of rhamnogalacturonans[1-4]. These metabolites are dissimilar to oligosaccharides related to raffinose[31], where the α-D-galactose units are substituted in position 6. The chain extension of metabolites under study starts from the non-reducing end of the saccharide chain involving UDP-D-galactose as a donor of D-galactose units; according to Robins and coworkers[32] this process does not suppose any participation of lipidic intermediates.

An open question is, whether the metabolite M_I is involved in formation of a D-galactan chain as an acceptor of further D-[U-^{14}C]-galactosyl residue transferred from UDP-D-[U-^{14}C]-galactose by UDP-D-galactose: metabolite M_I-4-β-D-galactosyltransferase. In contrast to M_{II} and M_{III}, the absence of (1 → 4)-linked D-[U-^{14}C]-galactose units in M_I is in favour of this possibility; this is also in line with the finding that at low concentration of UDP-D-[U-^{14}C]-galactose a part of the radioactivity was transferred from M_I to M_{II} and M_{III} at an extended incubation time. It could be, therefore, concluded that the particulate enzymic preparation contains at least two positionally specific UDP-D-galactose-acceptor-β-D-galactosyltransferases. Although the existence of these enzymes was presumed[33] no evidence has been presented as yet. A more detailed study is needed to throw more light on the mechanism of formation of pectic polysaccharides containing D-galactose.

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