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CELL-WALL BIOSYNTHESIS IN DIFFERENTIATING CELLS OF PINE ROOT TIPS

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Key Word Index—*Pinus halepensis*; Gymnospermae; pine (root tips); cell-wall biosynthesis; root differentiation; cell-wall polysaccharides.

Abstract—The patterns of incorporation of radioactivity from D-[U-^{14}C]glucose into pectins, hemicelluloses and cellulose synthesised in differentiating cells of pine root-tips were analysed after sequential solvent extractions. The percentage composition of the radioactive glycosyl residues of hot-H_2O- and Na_2EDTA-+NH_4-oxalate-+$NaBH_4$-extractable cell-wall polymers was very similar. All sections of pine roots investigated synthesise a remarkably large amount of polymers rich in galactosyl and arabinosyl residues. These polymers may create in the wall a highly hydrated gel compartment structurally and physiologically important for the roots. The KOH-extractable polymers are rich in glucosyl and xylosyl residues. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

The postembryonic architecture of higher plants is formed from two meristems in the shoot and root, which are laid down during embryogenesis. The activity of the shoot meristem gives rise to leaves, stems and floral organs, whereas the root meristem produces the root, which is characterized by a fairly uniform development. The continuous growth and differentiation of roots appear in all developmental stages, being present in three distinct but overlapping zones along the length of the root [1]. The meristematic zone is usually protected by root cap cells which slough off according to a defined programme of terminal differentiation. The elongation zone follows to the meristematic zone and continues with the differentiated zone. Because of these characteristics, the root represents an ideal model system for molecular, physiological, biochemical and morphological studies of postembryonic development.

The synthesis, transport and incorporation of pectins, hemicelluloses and cellulose into the cell-wall have been analysed during the differentiation of root meristem in monocotyledons [2] and dicotyledons [3, 4]. In addition, particular attention has been given to the synthesis and transport of highly hydrated polysaccharides and/or glycoproteins in specialized slime-producing areas of roots in maize [5–8], wheat [9] and rice [10].

We have undertaken a biochemical investigation on the biosynthesis of cell-wall polysaccharides in root of pine seedlings, in order to understand the dynamic changes of cell-wall polymers during cellular differentiation. The results obtained indicate that the type of polysaccharide incorporated into cell-wall is directly correlated with the state of differentiation of the root.

RESULTS AND DISCUSSION

The uptake of D-[U-^{14}C]glucose by 20-day-old pine root seedlings during 1 h of incubation was ca 70% with respect to the initial radioactivity (227 kBq). Roots of the labelled seedlings were rapidly washed in an ice-cold glucose solution and carefully sectioned, as shown in Fig. 1. Each fraction, composed of pooled corresponding sections of four roots, was depleted of the soluble endogenous pool, dried and used for analysis. Dried sections of each fraction were sequentially treated with hot water, Na_2EDTA, NH_4-oxalate, $NaBH_4$ and KOH at two different concentrations for the extraction of cell-wall matrix polymers. The cell-wall polymers extracted with Na_2EDTA, NH_4-oxalate and $NaBH_4$ were combined and analysed. The same was done for the cell-wall polymers extracted by weak and strong alkali. The solid residue of each fraction

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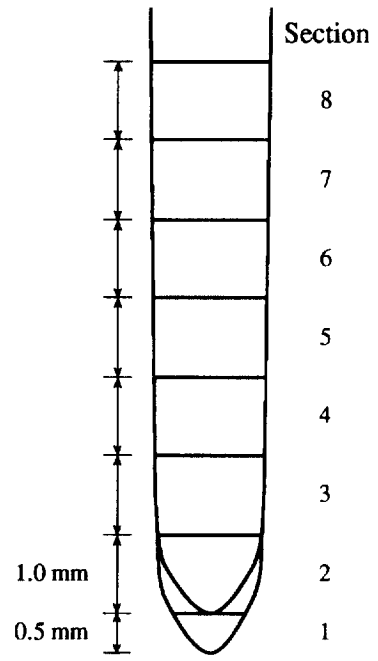

Fig. 1. Sections of pine root tips that were analysed.

remained after the strong alkali extraction was considered to be α-cellulose. Preliminary experiments have shown that no radioactive polymeric material was secreted in the incubation medium, even when pine-root seedlings were incubated in the presence of D-[U-^{14}C]glucose for up to 4 h. This observation indicates, that in pine-roots, neither root-cap cells nor rhizodermal cells secrete polymeric materials into the medium in the short term. In this respect, pine-roots may be similar to pea-roots, cultured tomato and *Convolvulus* roots which secrete small amounts of polymeric materials that accumulate between the cell-wall and the protoplast, and remain there throughout the subsequent stages of development [11]. This contrasts with the well-known secretory activity of root-cap cells of maize [5] and wheat [9], in which large amounts of secretory material accumulate externally to the cell-walls as slime droplets.

Table 1 shows the percentage of radioactive polymers sequentially extracted from each fraction with different extractants. The highest percentage of hot-H_2O-extractable polymers was recorded from root-cap cells; this value decreased progressively during the differentiation of meristematic cells. The data demonstrate that the synthesis of hot-H_2O-extractable polymers is peculiar of the root-cap cells and to a less extent of the cells which are near to the meristematic zone.

The percentage composition of the radioactive glycosyl residues of hot-H_2O-extractable polymers is shown in Table 2. In root-cap cells, as well as in meristematic and elongating cells (fractions 1–4), the major radioactive glycosyl residues incorporated into hot-H_2O-extractable cell-wall polymers were galactose, arabinose and galacturonic acid. Glucose, galactose, arabinose and galacturonic acid were present in elongated and differentiated cells (fractions 5–8). The sharp increase found in the percentage of radioactive glucosyl residues in fractions 6–8 was accompanied by a decrease of the percentage of galactosyl and arabinosyl residues. It is unlikely that the presence of radioactive glucosyl residues can be attributed to starch contamination, because each fraction was extensively treated with α-amylase. In all fractions, the percentage of mannosyl, xylosyl, fucosyl, rhamnosyl and glucosyluronic acid residues accounted for less than 5%.

The results in Table 1 show that the percentage of Na_2EDTA- + NH_4-oxalate- + $NaBH_4$-extractable cell-wall polymers from root-cap cells, meristematic and differentiating cells (fractions 1–6) showed little variation, whereas a marked decrease of the percentage of these polymers was observed in differentiated cells (fractions 7 and 8). The percentage distribution of the radioactive glycosyl residues of Na_2EDTA- + NH_4-oxalate- + $NaBH_4$-extractable polymers is reported in Table 3. The pattern of sugars was similar to that for the hot-H_2O-extractable polymers. However, in fractions 6–8, the ratio between galactosyluronic acid and rhamnosyl residues was very close to one. The amount of galacturonic acid was obtained by electrophoretic separation of uronic acids (Fig. 2). The similar percentage of radioactive glycosyl residues found in the hot-H_2O- and Na_2EDTA- + NH_4-oxalate- + $NaBH_4$-extractable cell-wall polymers indicates that they could have a different capability of interaction among cell-wall polymers. In addition, our data indicate that all fractions of pine-roots investigated synthesise, with some variations, a large amount of polymers rich in arabinosyl and galactosyl residues. Galactose and arabinose have been found to be incorporated into galactans, arabinans, arabinogalactans, arabinogalactan-proteins and, to a lesser extent, in extensins, xyloglucans and glucuronoarabinoxylans [12–14]. The capability of hot-H_2O, Na_2EDTA, NH_4-oxalate and $NaBH_4$ to extract mainly pectins from the walls would suggest that, in pine-roots, the newly synthesised polymers rich in galactosyl and arabinosyl residues are likely to be galactans, arabinans and/or arabinogalactans, which are commonly linked as neutral side-chains to the rhamnosyl residues of rhamnogalacturonan I [13]. The attachment of neutral polymers, such as galactans, arabinans and/or arabinogalactans to rhamnogalacturonan I would increase the extent of branching of these polysaccharides and, consequently, their degree of hydration. Thus, branched rhamnogalacturonan I, besides its structural role, may create in the wall of pine-root a highly hydrated gel compartment physiologically important not only for root growth and differentiation but also for all the interactions between root and soil. The presence of polymers rich in arabinosyl and galactosyl residues has also been dem-

Table 1. Percentages of radioactive cell-wall polymers sequentially extracted from root fractions isolated from 20-day-old pine seedlings incubated in the presence of 5 μCi of D-[U-^{14}C]glucose for 1 h, at 25°. Results are from one representative experiment of three

Fraction	Radioactivity (%)				
	Hot H_2O	2% Na_2EDTA + 0.5% NH_4-ox. + 1% $NaBH_4$	0.1 N KOH + 4.0 N KOH	Residue	Total dpm · 10^{-3}
1	33.2	32.6	24.4	9.8	21.8
2	21.9	34.5	26.5	17.1	62.3
3	18.5	33.0	31.2	17.3	91.4
4	14.1	29.3	33.1	23.5	93.9
5	9.4	29.2	20.3	41.1	97.0
6	7.4	27.2	19.3	46.1	84.3
7	6.4	18.2	13.1	62.3	87.3
8	6.3	14.6	12.1	67.0	60.1

Table 2. Radioactive-sugar composition of cell-wall polymers extracted in hot H_2O from root fractions isolated from 20-day-old pine seedlings incubated in the presence of 5 μCi of D-[U-^{14}C]glucose for 1 h, at 25°. Results are from one representative experiment of three

Sugar	Radioactivity (%)							
	Fraction							
	1	2	3	4	5	6	7	8
Galactose	47.2	38.3	38.4	33.1	34.2	23.0	23.2	22.8
Glucose	5.1	3.8	4.3	6.7	18.3	37.4	31.7	28.6
Mannose	4.7	2.6	2.3	3.0	1.6	2.0	1.7	2.1
Arabinose	20.4	27.3	27.2	26.7	25.5	17.4	17.3	17.3
Xylose	4.4	4.4	3.9	4.1	3.5	2.9	2.6	3.5
Fucose	1.6	4.6	3.2	2.7	2.2	1.0	2.1	2.6
Rhamnose	1.3	2.6	3.5	3.2	1.4	3.1	4.7	7.5
Galacturonic acid	12.1	12.9	14.9	17.0	10.0	10.3	12.8	11.9
Glucuronic acid	3.2	3.5	2.3	3.5	3.3	2.9	3.9	3.7
Total dpm · 10^{-3}	7.2	13.6	16.9	13.2	9.1	6.2	5.6	3.8

Table 3. Radioactive-sugar composition of cell-wall polymers extracted in 2% Na_2EDTA + 0.5% NH_4-oxalate + 1% $NaBH_4$ from root fractions isolated from 20-day-old pine seedlings incubated in the presence of 5 μCi of D-[U-^{14}C]glucose for 1 h, at 25°. Results are from one representative experiment of three

Sugar	Radioactivity (%)							
	Fraction							
	1	2	3	4	5	6	7	8
Galactose	39.4	39.4	38.4	38.6	31.1	31.8	29.1	30.0
Glucose	6.2	6.5	6.5	5.9	17.0	18.5	22.4	18.0
Mannose	4.7	4.2	3.4	2.1	2.1	2.1	2.7	3.9
Arabinose	26.5	26.2	27.6	28.3	23.4	20.3	20.1	21.0
Xylose	3.6	3.1	3.6	3.0	2.9	2.5	3.3	1.1
Fucose	2.9	2.6	2.2	2.2	0.2	—	—	—
Rhamnose	4.3	3.8	2.8	3.9	7.5	10.8	10.4	10.0
Galacturonic acid	11.0	12.6	13.3	14.7	14.1	12.2	10.4	14.5
Glucuronic acid	1.4	1.6	2.2	1.3	1.7	1.8	1.6	1.5
Total dpm · 10^{-3}	7.1	21.5	30.2	27.5	28.3	22.9	15.9	8.8

onstrated in cell-walls of sycamore [3] and pea [4] root tips.

Under our experimental conditions, it is not possible to prepare purified cell-walls because of the small amount of starting material. Therefore, our data do not exclude the synthesis of arabinogalactan-proteins which are localized in cytoplasmic organelles [15, 16], on the plasma membrane [17–21], in the cell-wall [22] and in the medium of suspension-cultured cells [23], in stylar secretions [24, 25] and in plant gums and mucilages [26]. Arabinogalactan-proteins, as do branched rhamnogalacturonan **I**, possess a high water-holding capacity which may contribute, among other functions, to the interactions between root and soil. Recent studies on maize have excluded the possibility that root-cap mucilage acts as a buffer against desiccation in the manner of shoot mucilages [27]. Our data suggest that this role may be performed by specific cell-wall polymers, such as highly branched rhamnogalacturonans and arabinogalactan proteins. Nevertheless, analysis of the glycosyl residue and glycosyl linkage compositions of different polysaccharides and glycoproteins is required in order to establish the type of the newly synthesised polymers in pine-roots. This may help to clarify the possible interaction between arabinogalactan-proteins and pectic polysaccharides in the cell-wall.

The presence of galactosyluronic acid and rhamnosyl residues in the hydrolysates of hot-H_2O- and Na_2EDTA-+ NH_4-oxalate-+ $NaBH_4$-extractable polymers indicates the synthesis of homo-

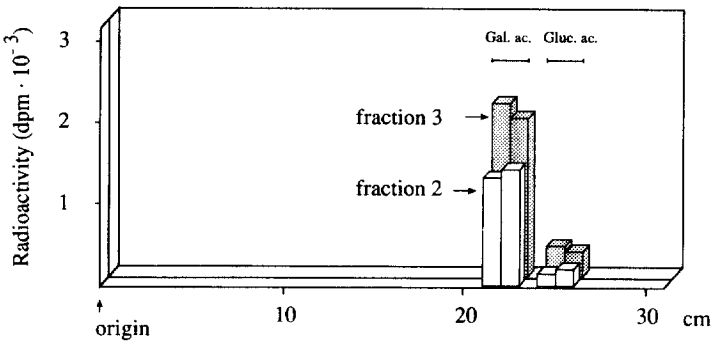

Fig. 2. Electrophoretic separation of uronic acids from the cell-wall polysaccharides extracted in 2% Na_2EDTA + 0.5% NH_4-oxalate + 1% $NaBH_4$ from root fractions (2 and 3) isolated from 20-day-old pine seedlings incubated in the presence of 5 μCi of D-[U-^{14}C]glucose for 1 h, at 25°. Gal. ac., galacturonic acid; gluc. ac., glucuronic acid.

galacturonans and/or rhamnogalacturonan I. Judging by the ratio between galactosyluronic acid and rhamnosyl residues it would appear that root-cap, meristematic and elongating cells synthesise mainly homogalacturonans, whereas elongated and differentiated cells (fractions 5–8) synthesize mainly rhamnogalacturonan I. The presence of a high percentage of glucosyl residues only in the elongated and differentiated cells suggests the synthesis of non-cellulosic β-glucans. The possible presence of glucose-rich polysaccharides other than xyloglucan has been reported in pine hypocotyls [28] but not in Douglas fir cell walls [29]. In particular, it has been demonstrated in pine hypocotyls that glucose-rich polysaccharides are involved in an active turnover during growth [30]. A similar situation has been described for monocot cell walls [13, 14].

The radioactive sugar composition of cell-wall polymers extracted using two different concentrations of KOH (0.1 and 4 N) is reported in Table 4. The presence of radioactive glucosyl and xylosyl residues in the hydrolysates of KOH-extractable polysaccharides suggests the synthesis of hemicelluloses of xyloglucan type, as well as xylans and β-glucans. Xyloglucans play an important role during growth and differentiation of pine hypocotyls [31], as well as in dicots [32].

In the hydrolysates of the hemicellulosic fraction, we have still found high percentages of radioactive galactosyl and arabinosyl residues and, to a lesser extent, galactosyluronic acid and rhamnosyl residues. By attributing all these glycosyl residues to pectic polysaccharides, it can be calculated that in root-cap, as well as near the meristematic zones, pectins extracted by KOH comprised more than 65%. In differentiated cells (fractions 5–8), the ratio between pectins and hemicelluloses was ca 1:1. These data indicate a strong interaction between pectins and other polymers in pine-root cell-walls. These interactions can be achieved in the wall through different types of cross-links [33].

The biosynthesis of cellulose, calculated as a percentage of the total amount of radioactive polysaccharides, increased markedly from the root-cap cells (9.8%) to the differentiated cells (67%). On the contrary, the synthesis of matrix polysaccharides was percentually highest in root-cap cells (ca 90.2%) and then decreased from the meristematic cells (ca 80%) to the differentiated cells (fraction 8 ca 33%) (Table 1). Thus, differentiation of pine-root meristematic cells is accompanied by marked changes in the relative synthesis of cell-wall polymers.

The cell-wall residue (α-cellulose) remaining after all extractions was contaminated by non-cellulosic polysaccharides that were incompletely extracted by the methods used. Galactosyl, mannosyl, arabinosyl, xylosyl, rhamnosyl, galactosyluronic acid and glucosyluronic acid residues were still present in these

Table 4. Radioactive-sugar composition of cell-wall polymers first extracted in 0.1 N KOH and then reextracted in 4.0 N KOH from root fractions isolated from 20-day-old pine seedlings incubated in the presence of 5 μCi of D-[U-^{14}C]glucose for 1 h, at 25°. Results are from one representative experiment of three

	Radioactivity (%)							
	Fraction							
Sugar	1	2	3	4	5	6	7	8
Galactose	33.1	30.7	27.4	26.9	22.8	19.9	19.2	21.5
Glucose	17.9	19.9	20.4	19.6	25.2	30.1	26.1	28.9
Mannose	2.9	1.3	1.3	1.0	2.8	2.4	2.9	2.9
Arabinose	21.6	20.7	21.8	21.3	20.4	19.1	20.9	15.2
Xylose	8.2	8.7	11.0	11.0	8.8	11.0	10.8	10.8
Fucose	1.0	2.8	2.3	2.4	1.9	2.0	2.5	2.6
Rhamnose	5.1	5.7	5.3	6.2	6.8	5.2	5.4	6.4
Galacturonic acid	8.3	8.1	9.3	9.5	9.5	8.7	10.2	10.0
Glucuronic acid	1.9	2.1	1.2	2.1	1.8	1.6	2.0	1.7
Total dpm · 10⁻³	5.3	16.5	28.5	31.1	19.7	16.3	11.4	7.3

Table 5. Radioactive-sugar composition of cell-wall polymers present in the insoluble residue (α-cellulose) of root fractions isolated from 20-day-old pine seedlings incubated in the presence of 5 μCi of D-[U-^{14}C]glucose for 1 h, at 25°. Results are from one representative experiment of three

	Radioactivity (%)							
	Fraction							
Sugar	1	2	3	4	5	6	7	8
Galactose	3.7	1.7	2.1	1.5	2.5	3.8	3.1	1.8
Glucose	74.5	78.7	79.0	77.1	74.1	75.3	73.9	85.5
Mannose	3.4	2.0	2.6	3.3	1.7	4.1	2.8	3.8
Arabinose	5.2	5.2	7.7	7.0	6.9	4.2	6.4	3.6
Xylose	2.6	2.1	1.7	2.6	2.3	2.2	3.5	1.8
Fucose	—	—	—	—	—	—	—	—
Rhamnose	1.7	0.5	0.1	0.8	3.2	1.0	1.0	0.8
Galacturonic acid	5.9	6.5	4.5	5.5	7.5	7.3	7.4	2.9
Glucuronic acid	3.0	3.3	2.3	2.2	1.8	2.1	1.9	0.7
Total dpm · 10^{-3}	2.1	10.7	15.8	22.1	39.9	38.9	54.4	40.3

fractions (Table 5). The difficulty in obtaining pure cellulose after sequential extractions has been reported by different authors in different plant materials and probably reflects the complex heterogeneity in the bonding between cell-wall polymers [34, 35].

EXPERIMENTAL

Plant material, labelling of the seedlings and sectioning of the roots

Seeds of *P. halepensis* L. were soaked in running H_2O (12 h), planted in damp vermiculite and grown for 20 days in darkness at 26 ± 1°. Four uniform seedlings having primary roots 30 mm long were washed in sterile bidist, H_2O and incubated in vials (5 × 20 mm) containing 5 μCi of D-[U-^{14}C]glucose (sp. act. 10.5 GBq mmol^{-1}) in 100 μl of H_2O for 1 h at 25°, in the dark. At the end of labelling, roots were rapidly washed in an ice-cold glucose soln (2mM) and sectioned with a razor blade under a dissecting microscope. After the removal of the root-cap (500 μm) (section 1), other sections were sequentially cut into 1 mm long sections (Fig. 1). Each fr. composed of pooled corresponding sections of four roots, showed a different level of differentiation. Frs 2, 3 and 4 were mainly composed of meristematic cells, their immediate derivatives and slightly elongating and differentiating cells, respectively. (Protoxylem and protophloem, and the beginning of metaxylem and metaphloem were visible in fr. 4.) Frs 5–8 were composed of cells at a different level of elongation growth and differentiation. (The primary structure appeared to be almost completely differentiated in fr. 8.) Each fr. was dropped into 5 ml of boiling EtOH (96%) and refluxed for 15 min. This was repeated twice in order to remove all soluble sugars. Frs were dried in a vacuum desiccator over NaOH.

Fractionation of cell-wall polymers

Extraction of cell-wall polymers was performed as described in Ref. [36], as modified in Ref. [4]. Sections (alcohol-insol. residue) of each fr. were sequentially extracted with H_2O, 1 h, 100° (twice); 2% Na_2EDTA, 1 h, 100° (twice); 0.25% NH_4-oxalate, pH 4.5, 1 h, 100° (twice); 1% $NaBH_4$, under N_2 and with constant stirring, 24 h, 25°; 0.1 N KOH + 3 mg ml^{-1} $NaBH_4$, under N_2 and with constant stirring, 24 h, 25°; 4 N KOH + 3 mg ml^{-1} $NaBH_4$, under N_2 and with constant stirring, 24 h, 25°. The insol. material remaining after strong alkali extraction, considered as α-cellulose, was hydrolysed with cold 72% H_2SO_4 (1 h) followed by hot 3% H_2SO_4 at 120°, for 1 h and neutralized with a 15% (v/v) soln of methyl-*d*-*n*-octylamine in $CHCl_3$. Excess amine was removed by washing × 5 with $CHCl_3$.

Hot-H_2O extracted polymers were incubated with salivary amylase [37] in the presence of 100 μl of toluene and 3 mg of NaCl for 12 h, in order to allow the digestion of starch and then dialysed (Spectrapor membrane tubing M_r cut off: 3500) for 24 h against 5 l of dist. H_2O under stirring. Since preliminary expts showed that the radioactive polymers extracted from each fr. with Na_2EDTA, NH_4-oxalate and $NaBH_4$ had a very similar qualitative and quantitative glycosyl residue composition, extracts of these three treatments were combined. For the same reason, we combined the weak and strong alkali extracted polymers. The Na_2EDTA- + NH_4-oxalate- + $NaBH_4$-extract and the alkali-extract were dialysed (Spectrapor membrane tubing M_r cutoff: 3500) for 24 h against 10 l of dist. H_2O with vigorous stirring. All dialysates were taken to dryness in a rotary evaporator and hydrolysed in sealed ampoules with 2 N TFA at 120° for 90 min. TFA was removed using a speed-vac concentrator.

The dried hot-H_2O- and Na_2EDTA- + NH_4-oxalate- + $NaBH_4$-extract, alkali-extract and α-cellulose were dissolved in H_2O and electrophoresed on Whatman No. 1 paper in HOAc (8%, v/v)–HCO_2H (2%, v/v) buffer, pH 2.0, 5 kV, 45 min to separate any remaining amine, peptides and amino acids from neutral sugars and uronic acids [9]. Radioactive neutral sugars and uronic acids were eluted from the origin of the electrophoretogram and separated by descending PC (solvent A). The radioactivity present on the starting line of the chromatogram, corresponding to uronic acid markers, was eluted with H_2O, taken to dryness, dissolved in H_2O and electrophoresed on Whatman No. 1 paper in pyridine-HOAc-H_2O (1:10:89) buffer, pH 3.5 at 5 kV, 70 min, to separate galacturonic from glucuronic acid [38]. When the radioactive galactose, glucose and mannose peaks were not completely separated on the chromatogram, the radioactivity present in the region of these markers was eluted with H_2O, taken to dryness, dissolved in

H_2O and rechromatographed (solvent A for 48–60 h). To separate xylose from fucose, the radioactivity present in the region of xylose marker was eluted with H_2O, taken to dryness, dissolved in H_2O and rechromatographed (solvent B).

Paper chromatography

Descending PC was performed on Whatman No. 1 paper in the following solvent system: Solvent A, EtOAc–pyridine–H_2O (8:2:1); Solvent B, H_2O–satd PhOH (39:100, w/v). Marker sugars were detected with aniline hydrogen phthalate reagent [39].

Radioactivity counting procedure

Radioactivity on paper chromatograms and electrophoretograms was estimated as previously described [40].

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