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Extracellular Polysaccharides from the Culture Medium of Cell Suspension Cultures of Carrot*

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The extracellular polysaccharides have been fractionated from the culture medium of carrot (*Daucus carota*) cell cultures by precipitation with ethanol and by chromatography on DEAE-Sepharose CL-6B and DEAE-Trisacryl M ion-exchange and Bio-Gel A-1.5m gel-permeation. The sugar composition and molecular mass of purified neutral and acidic polymers were determined. The neutral and acidic polymers were treated with purified endo-β-glucanase from *Trichoderma viride* and pectic depolymerases, such as endo-pectate lyase from *Erwinia carotovora Er.* and endo-polygalacturonase from *Kluyveromyces fragilis*, respectively. The "hairy" (ramified) regions of acidic polymer were sequentially treated with purified α-L-arabinofuranosidase and β-galactosidase from carrot cell cultures, and were further hydrolyzed with 50 mM trifluoroacetic acid for 1 hr at 100 ℃. From these results, the extracellular polysaccharides secreted from carrot cell cultures are characterized.

Key words : Carrot, Cell suspension culture, Cell wall, Extracellular polysaccharides, Pectic polymer.

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INTRODUCTION

In suspension cultures of plants, the cell wall-derived polysaccharides are accumulated in their culture media during growth[31]. Using plant cell cultures, a large amount of these polysaccharides can be readily obtained on the soluble form without the extraction with chemical reagents and enzyme action from the cell wall materials. Therefore, many, if not most, researchers working on the cell walls are interested in elucidating the structure of the extracellular polysaccharides in the growth medium of cell cultures. So far several neutral polymers in the extracellular polysaccharides isolated from the culture medium have been studied extensively in some detail[4, 5, 11, 29, 31, 32]. The acidic pectic polymers have also been detected in the extracellular polysaccharides of suspension cultures of sycamore[2, 27, 31], bush bean[21], and tobacco[30], but no analysis of the polysaccharides from carrot cell cultures has been done. Recently, we have ascertained that uronic acid-containing material is accumulated in the medium of cell suspension cultures of carrot during cell growth[20]. The study presented herein describes fractionation and characteristics of the extracellular polysaccharides released into the growth medium of cell suspension cultures of carrot, using enzymic degradation and mild acid hydrolysis.

MATERIALS AND METHODS

1. *Isolation of the extracellular polysaccharides.*

Cells of carrot (*Daucus carota* L. cv. Kintoki) were cultured in the basal medium of Murashige and Skoog[23] containing 3 % (w/v) sucrose as the carbon source and 4.5 μM 2,4-dichlorophenoxyacetic acid as a growth regulator. The culture conditions were as described previously in detail[12]. The cells were removed from 15-day-old cultures by filtering the culture medium through a glass fiber filter paper (Type CG-90, Toyo Roshi Co.), and the medium was concentrated by a Pellicon Lab Cassette (PTGC membrane, Millipore Corp., Bedford, MA, USA). The concentrated culture medium was poured into absolute ethanol (3-vol), and the turbid solution was left overnight at 4 ℃. The precipitated material was collected by centrifugation, redissolved in 50 mM K-phosphate buffer (pH 6.0), and dialyzed against the same buffer with several changes of the buffer. A solution was then dialyzed twice against distilled water. Any precipitate formed during dialysis was removed by centrifugation, and the supernatant solution was kept frozen until required.

2. *Ion-exchange chromatography.*

An aliquot (ca 250 mg sugar content) of the extracellular polysaccharides was dialyzed against 20 mM K-phosphate buffer (pH 6.0), and applied

to a DEAE-Sepharose CL-6B column (2.0 x 20 cm), previously equilibrated with the same buffer. After sample application, the column was washed with 160 ml of equilibration buffer, and then eluted with a linear gradient of buffer from 20 mM to 0.6 M. For the DEAE-Trisacryl M (IBF Biotechnics, Villeneuve-la-Garenne, France) column chromatography, the polysaccharides were applied, following the conditions described above, and eluted sequentially with 20 mM K-phosphate buffer and buffer containing 0.125, 0.25, and 0.5 M NaCl. The eluate was collected as 4-ml fractions and assayed for total sugars.

3. *Gel-permeation chromatography.*

The sample (5-10 mg sugar content) was applied to a column (1.5 x 90 cm) of Bio-Gel A-1.5m or Bio-Gel P-2, previously equilibrated with 50 mM Na-acetate buffer (pH 5.2) containing 20 mM EDTA, and eluted with the same buffer. The eluate was collected as 1.8-ml fractions and assayed for total sugars.

4. *Sources of enzymes.*

Endo-β-glucanase (EC 3.2.1.4) was purified from a cellulase preparation from *Trichoderma viride* (Calbiochem, San Diego, Cal., USA) as described[18]. Endo-pectate lyase (EC 4.2.2.2) and endo-polygalacturonase (EC 3.2.1.15) were purified to electrophoretic homogeneity from cell homogenates of *Erwinia carotovora Er.*[13] and from the culture filtrate of *Kluyveromyces fragilis* (IAM 4763)[18], respectively. β-Galactosidase (EC 3.2.1.23) and α-L-arabinofuranosidase (EC 3.2.1.55) were purified to electrophoretic homogeneity from cell homogenates of carrot cell cultures, as described[17, 19].

5. *Enzymic degradation.*

For the endo-pectate lyase treatment, the sample (5-10 mg) was suspended in 10 ml of 50 mM Tris-HCl buffer (pH 8.6) containing 0.9 unit of enzyme and 0.5 mM $CaCl_2$. After incubation at 30 ℃ for 48 hr with continuous stirring in the presence of a drop of toluene or 0.02 % (w/v) Na-azide acting as bacteriostatic, the reaction mixture was adjusted to pH 5.0 and stopped by heating at 100 ℃ for 3 min. For the other enzyme treatments, each reaction mixture contained the sample and enzyme (10 units) in 10 ml of 50 mM Na-acetate buffer (pH 5.0). The reaction conditions were just as described. After the enzyme treatment, the digestion product was reduced to a small volume with evaporation *in vacuo* under 40 ℃, and chromatographed on a Bio-Gel A-1.5m column.

6. *Mild acid hydrolysis.*

The sample was treated with 50 mM trifluoroacetic acid in a sealed tube for 1 hr at 100 ℃, and the acid was evaporated *in vacuo*. The dried material was redissolved in 1 ml of water, and the solution was chromatographed on

a Bio-Gel P-2 column (1.5 x 90 cm).

7. *Saponification.*

The methoxyl and acetyl groups in acidic polysaccharides were saponified with 10 mM NaOH. After gently stirring for 90 min in an ice-water bath, the solution was adjusted to pH 5.0 with 50 mM glacial acetic acid and dialyzed against water.

8. *Analyses.*

The contents of total sugars and galacturonic acid in each sample were estimated by the phenol-H_2SO_4[7] and *m*-hydroxydiphenyl[3] methods, respectively. Polysaccharides were hydrolyzed with 2 M trifluoroacetic acid for 1 hr at 121 ℃, and neutral sugars in the hydrolysates were analyzed by gas-liquid chromatography (GLC) as their alditol acetate derivatives according to the technique of Albersheim et al.[1]. GLC was performed with a Hitachi instrument (Model 163, Hitachi Ltd.), equipped with a Chromato-Processor (Model 834-30), as described[14].

RESULTS AND DISCUSSION

The culture medium used in this experiment was obtained from a cell culture corresponding to the end of the exponential phase of growth cycle. As reported previously[15], the cell population density was 8.1 mg cell dry weight per ml culture medium from 15-day-old cultures. The cell-free culture medium (2500 ml) was concentrated by a Pellicon Lab Cassette using a membrane with 10 kDa cut-off.

The extracellular polysaccharides from the concentrated culture medium were precipitated with ethanol. The yield was 0.4 mg (total sugar content) per ml of the starting culture medium. All the uronosyl residues in the complete acid hydrolysate of the ethanol-insoluble material appeared to be galacturonic acid ; glucuronic acid was not detected by paper chromatography with a solvent system of 1-butanol : acetic acid : water (4 : 1 : 2, v/v), although the sensitivity of the method is not high. The culture filtrate passed through the membrane with 10 kDa cut-off contained 124 mg (galacturonic acid equivalent) of *meta*-hydroxydiphenyl-positive (*i.e.*uronic acid containing) material, which would be galacturonic acid and/or oligogalacturonides, because glucuronic acid was not present in the cell wall preparation derived from carrot cell cultures[16]. However, since polygalacturonase acting exo-fashion has been isolated in the culture medium of carrot cell cultures[20], it would be difficult to say for certain whether the galacturonic acid and oligogalacturonides in the filtrate were produced by this enzyme action from the galacturonan-like polymer(s) released into the medium or the galacturonic acid itself was excreted into

the medium during cell growth.

The sugar composition analysis (Table 1) shows that the ethanol-insoluble material contained 97 % neutral sugars and only 3 % galacturonic acid. Among the neutral sugars, arabinose and galactose were the main components with xylose and glucose being the next two abundant sugars.

Table 1. Sugar composition of fractions of the extracellular polysaccharides from the culture medium of cell suspension cultures of carrot.

Fraction	Weight % of total sugars							
	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	GalUA
Ethanol-insoluble material	2.42	Tr	34.4	17.4	Tr	32.2	10.7	2.84
S-1 and T-1	5.49	—	10.9	44.6	—	15.7	20.0	3.31
S-2	3.24	—	45.3	2.20	—	45.1	Tr	4.14
S-3	7.08	—	50.2	6.22	—	23.6	0.97	11.9
T-2	6.03	—	65.1	Tr	—	13.7	—	15.2
T-3	2.24	—	42.8	4.92	—	24.2	0.40	25.5
S-1A	6.32	—	4.61	44.7	—	10.4	32.3	1.69
S-1B	2.43	—	15.7	34.0	—	22.0	23.3	2.63
Acid-treated fraction T-2	6.94	—	66.2	Tr	—	11.8	—	15.1

Data represent the mean of two analyses, each run in duplicate. Tr ; trace, Rha ; rhamnose, Fuc ; fucose, Ara ; arabinose, Xyl ; xylose, Man ; mannose, Gal ; Galactose, Glc ; glucose, GalUA ; galacturonic acid.

The ethanol-insoluble material was then fractionated by the anion-exchange column chromarography. The chromatography on anion-exchangers used for fractionation of pectic polysaccharides often gives a relatively low recovery of the polysaccharides. As pointed out by Redgwell and Selvendran[25], the results would be presumably due to the irreversible adsorption of uronic acid-rich polymers on the anion-exchangers. Therefore, in this experiment, we used DEAE-Sepharose CL-6B and DEAE-Trisacryl M.

Half of the ethanol-insoluble material did not bind to the DEAE-Sepharose CL-6B column, and the rest of the material retained on the column was eluted around 0.1 M buffer following the "tail", as illustrated in Fig. 1. The three fractions (S-1, S-2, and S-3) were pooled separately and dialyzed against water. The yields were 119 mg of fraction S-1, 84.6 mg of fraction S-2, and 34.9 mg of fraction S-3 from 250 mg of the ethanol-insoluble material and, thus, the recovery from the column was ca 95 %. For the chromatography of DEAE-Trisacryl M, on the other hand, the ethanol-insoluble material was fractionated by sequential elution with 20 mM K-phosphate buffer and buffer containing 0.125 M, 0.25 M, and 0.5 M NaCl. The elution profile is shown in Fig. 2, and the four fractions (T-1, T-2, T-3,

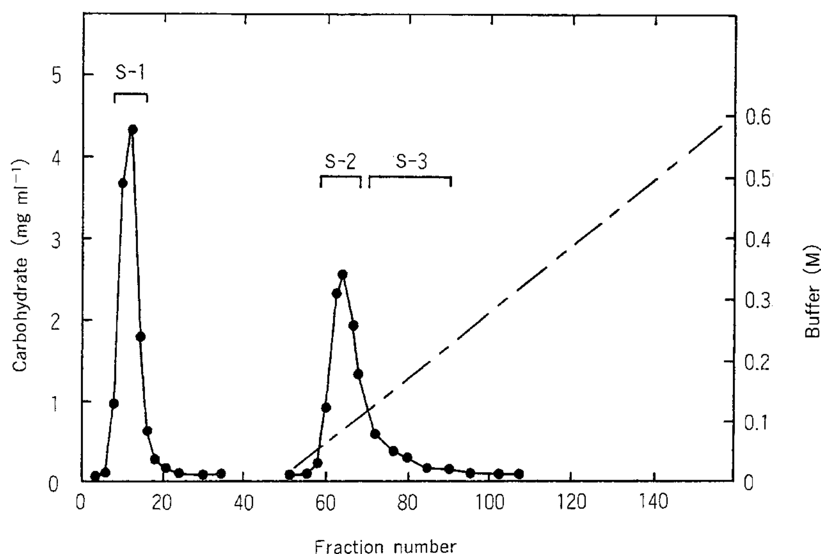

Fig. 1. Ion-exchange chromatography of the extracellular polysaccharides on DEAE-Sepharose CL-6B. Experimental conditions as described in MATERIALS AND METHODS. The column fractions were pooled as indicated.

and T-4) were pooled and dialyzed against water. The yields were 137 mg of fraction T-1, 92.3 mg of fraction T-2, 18.6 mg of fraction T-3, and 3.3 mg of fraction T-4 from 260 mg of the ethanol-insoluble material and the recovery from the column was ca 97 %.

Fractions S-1 and T-1 did not bind to the anion-exchangers, and accounted for the range 48–53 % of the ethanol-insoluble material. The fractions contained xylose, glucose, galactose, and arabinose as the main sugars (Table 1), indicating that the fractions would consist of a mixture of several neutral polysaccharides[31]. Whereas the other fractions were retained on the column and had the different sugar composition. Fractions S-2 and T-2 were the majority of acidic polysaccharides obtained from the chromatography. Fraction S-2 had the high contents of arabinose and galactose, while fraction T-2 was composed of arabinose, galacturonic acid, galactose, and rhamnose. It appears that the acidic fractions, in particular T-2, contain so-called "hairy" or ramified fragments[6] which were comprised mostly of the arabinose and galactose side-chains, such as arabinogalactan and/or galactan, and short-chains.

Fraction S-1 was further fractionated by gel-permeation on a Bio-Gel A-1.5m column into subfractions S-1A and S-1B (Fig. 3A). These subfractions were pooled separately, concentrated, and then purified by the same Bio-Gel A-1.5m gel-permeation procedure. The carbohydrate in each

subfraction eluted as a symmetrical peak almost overlapping with each peak in the initial Bio-Gel A-1.5m gel-permeation. Fraction T-2 was also subjected to the same Bio-Gel A-1.5m column, the carbohydrate was eluted as a single peak (Fig. 3B). The apparent molecular masses of subfractions S-1A and S-1B and fraction T-2 were 52 kDa, 40 kDa, and 80 kDa, respectively, as estimated by gel-permeation on a Bio-Gel A-1.5m column by comparison with the elution volume of linear dextrans (T-500, T-70, and T-40; Pharmacia, Uppsala, Sweden). The subfraction S-1A was enriched in xylose and glucose (Table 1), suggesting a sugar composition typical for a xyloglucan[9]. When subfraction S-1A was submitted to the action of endo-β-glucanase, the treated subfraction S-1A caused a slight reduction in molecular mass (profile not shown). However, since the products were scarcely detected in the inclusion limit of the Bio-Gel A-1.5m column, subfraction S-1A was not degraded extensively by the enzyme treatment. Perhaps the action of endo-β-glucanase would be blocked by the presence of some short-chains in

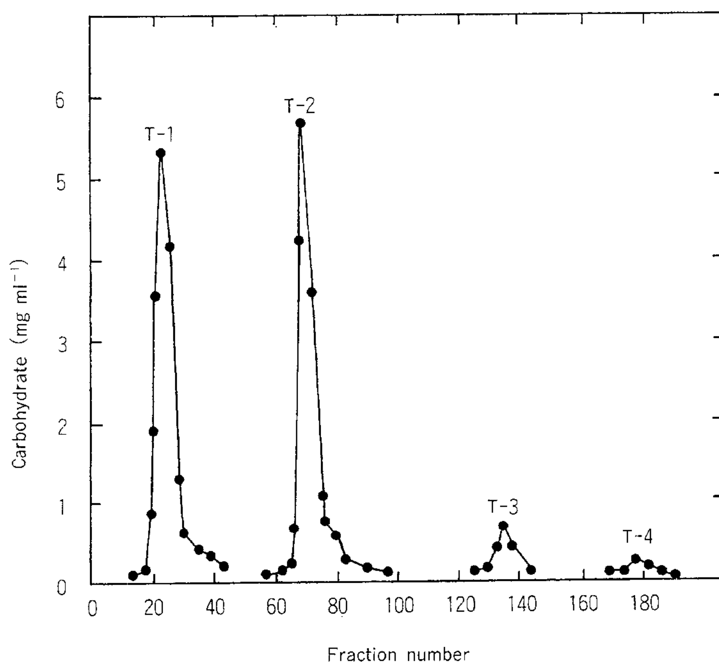

Fig. 2. Ion-exchange chromatography of the extracellular polysaccharides on DEAE-Trisacryl M. The column was eluted with 20 mM K-phosphate buffer (pH 6.0) (fractions 1-50), buffer containing 0.125 M NaCl (fractions 51-110), buffer containing 0.25 M NaCl (fractions 111-150), and buffer containing 0.5 M NaCl (fractions 151-190), respectively.

subfraction S-1A. In this experiment, fraction S-1 was not investigated further.

In order to characterize the pectic polymer, fraction T-2 was submitted to the action of endo-pectate lyase and endo-polygalacturonase, after de-esterification with cold dilute alkali, as described[16, 18]. Even after an exhaustive reaction, the elution profile of fraction T-2 on a Bio-Gel A-1.5m column was the same as that of native fraction T-2 ; no enzymic digestion products were released, namely, fraction T-2 underwent little or no degradation by either pectic degrading enzyme. These findings confirm the pre-

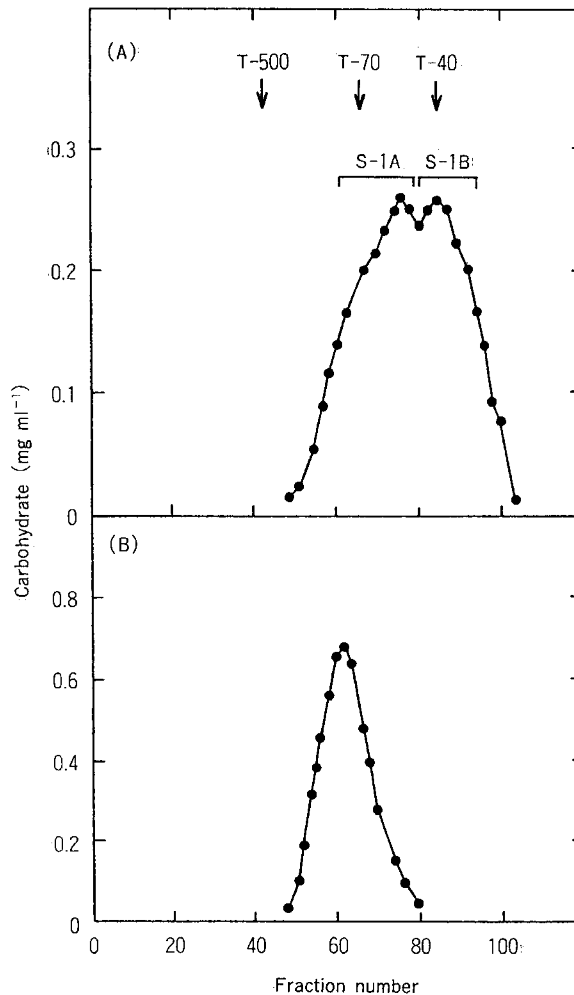

Fig. 3. Gel-permeation chromatography of fractions S-1 (A) and T-2 (B) on Bio-Gel A-1.5m. Experimental conditions as described in MATERIALS AND METHODS. The arrows index the elution positions of Dextran T-500 (molecular mass, 500 kDa), Dextran T-70 (70 kDa), and Dextran T-40 (40 kDa), respectively, used for the column calibration.

sence of the "hairy" fragments in fraction T-2. It is possible that fraction T-2 is similar to the arabinose-rich "hairy" regions solubilized from carrot cell walls by endo-pectate lyase, as described[16].

To analyze for the "hairy" fragments, fraction T-2 was degraded sequentially with α-L-arabinofuranosidase and β-galactosidase. The α-L-arabinofuranosidase used in this experiment is known to be active preferentially on p-nitrophenyl-α-L-arabinofuranoside and on (1→5)-linked sugar beet α-L-arabinan[19]. This enzyme is also able to degrade ca 20 % of arabinosyl linkages in the arabinan-like polymer (arabinose residues: ca 95 weight %) located in the pectic fraction prepared from the cell walls of carrot cell cultures. After incubation with α-L-arabinofuranosidase, the enzyme-treated sample was applied to a Bio-Gel P-2 gel permeation column,

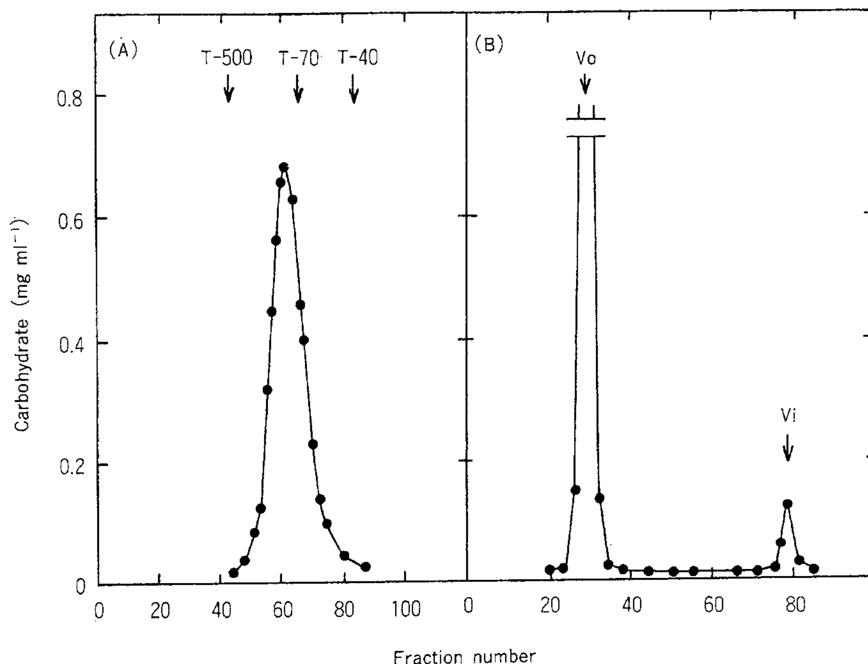

Fig. 4. Gel-permeation chromatography of the enzyme-treated fraction T-2 on Bio-Gel A-1.5m (A) and of the acid-degraded fraction T-2 on Bio-Gel P-2 (B). Experimental conditions as described in MATERIALS AND METHODS. The void volume (Vo) and included volume (Vi) were determined with Blue Dextran and glucose, respectively.

but no digestion products could be detected by this chromatography. It is conceivable that the galactosyl units occur as short-chains attached to the arabinose side-chains. The treated fraction T-2 was then degraded by

β-galactosidase, which was active on p-nitrophenyl-β-D-galactopyranoside and on (1→4)-linked citrus β-D-galactan[17]. Even after prolonged incubation, the elution profile of the treated fraction T-2 did not change, and no digestion products were released (Fig. 4A). Thus, the existence of the enzyme-unsusceptible linkages in the "hairy" fragments is suggested. In addition, the feruloyl groups hindered the binding of the enzymes may be located at part of the non-reducing end of arabinose side-chains as described for spinach pectin[8]. Finally, the enzyme treated fraction T-2 was hydrolyzed with 50 mM trifluoroacetic acid for 1 hr at 100 ℃. The products eluted at an inclusion volume of the Bio-Gel P-2 column accounted for ca 4 % of fraction T-2 (Fig. 4B), and were mainly composed of galactose, as identified by GLC. Actually, the content of galactose in the acid-treated fraction T-2 was slightly reduced (Table. 1), but the arabinose side-chains were fairly resistant. The arabinose residues can be removed from "hairy" regions in sugar-beet pulp[10] and apple juice[26] pectins by mild acid treatment. In general, the hydrolysis by the acidic condition is useful for the solubilization of pectic fraction from the cell wall preparation[24], but only small amounts of the polysaccharides are solublilized from carrot cell walls[22]. In the light of these findings, it is suggested that fraction T-2 isolated from the medium of carrot cell cultures, as well as pectic fractions of carrot cell walls[16, 22], is the rhamnogalacturonan backbone with very complicated side-chains. Further linkage analysis of the extracellular polysaccharides of carrot cell cultures is being pursued.

It has been thought that the structural changes in native cell walls already present are accomplished by the action of endogenous hydrolytic enzymes at a stage of vigorous growth and differentiation[28]. As mentioned in the introduction, the cell wall-derived polysaccharides and hydrolytic enzymes are accumulated in the medium of carrot cell cultures during growth[20]. It can be deduced, therefore, that the extracellular polysaccharides form potential substrates for the hydrolytic enzymes during carrot cell culture. Evidently, the characteristics of polysaccharides containing the growth medium must be helpful in explaining metabolic events *in situ* of the matrix-polymers of cell walls.

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ニンジン懸濁培養細胞の培養液に分泌される細胞外多糖成分

今野晴義・山崎良樹・加藤研治

植物培養細胞は懸濁（液体）培養を行うことによって，細胞壁構成多糖成分と構造的に類似している多糖成分を，その培地中に分泌することが知られており，これまで数種の植物培養細胞について報告されている．そこで，これまで研究例のないニンジン培養細胞を使用して，その細胞外多糖成分について検討した．

ニンジン懸濁培養細胞の対数増殖期後期の培養濾液からエタノール分画によって多糖成分を集め，DEAE-Sepharose CL-6B および DEAE-Trisacryl M カラムクロマトグラフィーによって，数種の多糖画分に分画し，それら画分の構成糖組成を検討した．また，上記カラムに吸着しない中性多糖画分は，分子量 52 kDa と 40 kDa の 2 種の画分から成り，*Trichoderma viride* から精製したエンド-β-グルカナーゼによってわずかに分解された．これらの結果から，分子量 52 kDa の画分はキシログルカンであると示唆された．一方，上記カラムに吸着する酸性多糖画分は，DEAE-Trisacryl M カラムクロマトグラフィーによって数種の画分に分画された．その中で分子量 80 kDa を示す主要画分は，*Erwinia carotovora Er.* から精製したエンド-ペクチン酸リアーゼ，*Kluyveromyces fragilis* から精製したエンド-ポリガラクチュロナーゼ，ニンジン培養細胞から精製した α-L-アラビノフラノシダーゼや β-ガラクトシダーゼなどによって分解されなかった．しかし，50 mM のトリフルオロ酢酸による分解にて，4％に相当するガラクトースを含む生成物が遊離された．これらの結果から，この酸性多糖画分は，複雑な中性糖側鎖の結合したラムノガラクチュロナン領域（“Hairy” region）であることが示唆された．

キーワード：ニンジン，懸濁培養細胞，細胞壁，細胞外多糖，ペクチン質