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EXTRACELLULAR POLYSACCHARIDES PRODUCED BY TUBEROSE CALLUS

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Key Word Index—*Polianthes tuberosa*; Agavaceae; tuberose; callus culture; extracellular polysaccharide; glucuronomannan.

Abstract—A high yield of extracellular polysaccharide (ECP) was obtained from callus cultures of tuberose (*Polianthes tuberosa*), which could be separated into an unadsorbed and two acidic fractions (TPS-1, -2) by ion-exchange column chromatography. The yields of each fraction were markedly increased by the addition of 10^{-5} M 2,4-dichlorophenoxyacetic acid to the medium. Of the three fractions, the amount of TPS-1 accounted for over 60% of total yield of ECP, which was a predominant polysaccharide consisting of arabinose (Ara), mannose (Man) and galactose (Gal) as major neutral monosaccharides. Judging from the patterns of electrophoresis and ultra-centrifugation, TPS-1 was identified to be homogeneous. Methylation and GC-mass spectrometry analyses of this fraction revealed the presence of 1,2,3-linked Man, 1-linked Ara, 1,3-linked Ara, 1-linked Gal and 1,3,4-linked glucuronosyl (GlcUA) residues in a molar ratio of 1.0:1.08:0.85:0.75:1.08. Based on additional analyses of the mild acid hydrolysate and the absolute configuration of the constituent monosaccharides, a possible structure for TPS-1 was a glucuronomannan possessing the unit of →4)-β-D-GlcUA*p*-(1→2)-α-D-Man*p*-(1→ with branching at the C-3 position, where →1)-α-L-Ara*f*, →1)-β-D-gal*p*, →1)-α-L-Ara*f*-(3→1)-α-L-Ara*f* or →1)-α-L-Ara*f*-(3→1)-β-D-Gal*p* were attached randomly. About 35% of the GlcUA moieties were present as methyl esters. Further confirmation was made by ^{1}H and ^{13}C NMR spectroscopy.

INTRODUCTION

Numerous reports have been accumulated on the extracellular polysaccharide (ECP) produced by suspension-cultured cells and calli [1–11]. ECP is easily obtainable from culture media without the need for complicated processing of chemical extraction and purification. Taking this advantage, polysaccharides can be obtained avoiding any reduction in the degree of polymerization and the structural changes. If an efficient culture system for providing large quantities of ECP would be established, ECP might be a useful source for cosmetic, pharmaceutical and food uses.

We found that calli induced from flowers of tuberose (*Polianthes tuberosa*) are capable of producing large amount of ECP [12]; drastic increases in the apparent viscosity of culture media were observed during callus culture, confirming that certain polysaccharides were secreted from calli. In the present paper, we describe the characterization of ECP produced by tuberose calli in different media and the structural feature of a predominant component (TPS-1) containing ECP.

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RESULTS AND DISCUSSION

Production of ECP in different media

Suspension cultures of bush bean and *Mentha* form a dense, glue-like mass [1, 10]. This extremely thick suspension culture is caused by the release of large amounts of ECP into the medium. When tuberose calli were cultured, similar changes were observed in the medium. Therefore, a drastic increase in apparent viscosity of the medium was thought to be due to the release of ECP. In fact, ECP could be collected from the medium after precipitation with ethanol. In our previous report [12], we demonstrated that the use of D5-medium containing 10^{-5} M 2,4-dichlorophenoxy acetic acid (2,4-D) was effective in ECP production, while the N5B6-medium containing 10^{-5} M 1-naphthaleneacetic acid and 10^{-6} M 6-benzylaminopurine was suitable for callus growth. ECP production in tuberose was monitored using the selected media. Culturing in D5-medium gave 4 times more ECP production 1 month after inoculation, showing that the specific biosynthetic rate was around 360 mg g^{-1} dry callus weight per month. This value seemed to be much higher than that reported for other cell cultures of higher plants. The total ECP contained uronic acid with no quantitative differences between D5- and N5B6-media (Table 1). The presence of protein

Table 1. Contents of total sugar, uronic acid and protein in ethanol precipitates of different culture media

	Content (%)	
	N5B6	D5
Total sugar	66.6 ± 4.7	68.4 ± 10.3
Uronic acid	25.8 ± 7.0	26.1 ± 7.0
Protein	4.2 ± 1.8	4.7 ± 2.8

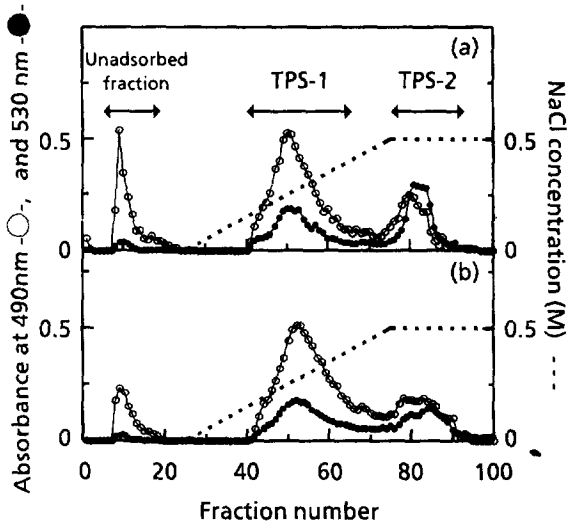

Fig. 1. Ion-exchange chromatographic profiles of ECP. ECP was harvested from D5 medium (a) and N5B6 medium (b) 4 weeks from the beginning of culture. (○) Total sugar (A_{490}); (●) uronic acid (A_{530}).

might be due to contamination from broken cells and/or extracellular proteins.

By ion-exchange chromatography, unadsorbed and acidic fractions were separated from ECP (Fig. 1). The acidic fraction was further separated into two fractions designated as tuberose polysaccharides (TPS)-1 and -2. As shown in Table 2, TPS-1 accounted for over 60% of the total ECP. When D5-medium was used, the yields of each fraction markedly increased, in particular the acidic polysaccharides. For each fraction, neutral sugar composition was determined (Fig. 2). All the fractions contained arabinose (Ara), mannose (Man), galactose (Gal), xylose (Xyl) and glucose (Glc) with variations in their contents. The major constituents of the unadsorbed fraction were Ara and Gal, whereas the increased amount of Man was present in the acidic fraction as an additional major sugar. Comparison between the fractions from N5B6- and D5-medium revealed that there were quantitative differences in Gal and Glc of the unadsorbed fraction as well as in Glc of TPS-2 fraction. In contrast, the sugar composition of TPS-1 was consistent regardless

Table 2. Yield of fractions obtained from ion-exchange chromatography

	Yield (mg/100 ml^{-1} medium)		Uronic acid (%)	
Fraction	N5B6	D5	N5B6	D5
Unadsorbed fraction	15.2	34.6	—	—
Acidic fraction				
TPS-1	57.1	243.4	31.2	25.2
TPS-2	17.7	82.1	56.6	47.6

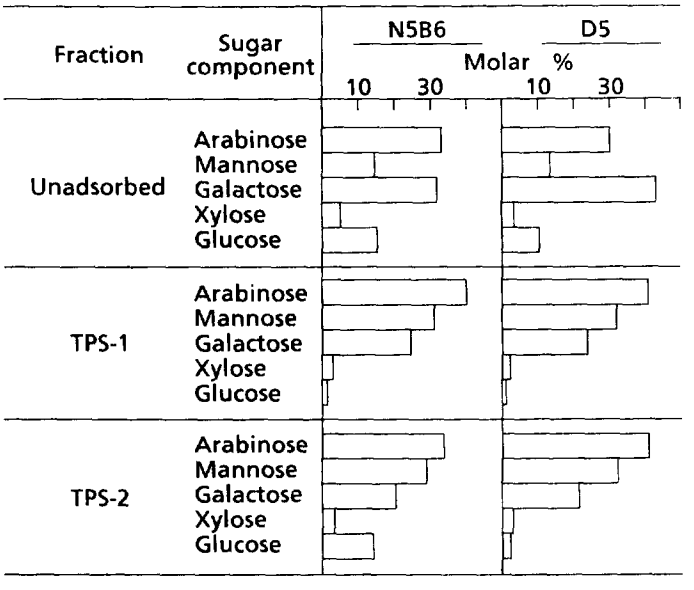

Fig. 2. Neutral sugar components of fractions separated from ECP produced in different media.

of medium. Based on electrophoresis and ultracentrifugal analyses, the isolated TPS-1 was shown to be homogeneous (Figs 3 and 4). These results indicate that the biosynthesis of ECP components was controlled by plant growth regulators supplemented in the media.

For a high yield of ECP, the addition of 2,4-D was found to be essential in the cultures of bush bean cells [2] and *Arabidopsis* callus [6]. The production of ECP from *Arabiopsis* calli was dependent on increasing concentration of 2,4-D, which was associated with callus morphology. Morphological differences in tuberose calli were observed between cultures in N5B6- and D5-media (data not shown), suggesting that ECP production is closely related to the dedifferentiation response induced by the relatively high concentration of 10^{-5} M of 2,4-D.

Structural analysis of TPS-1

Methylated TPS-1 was converted into alditol acetates for GC-mass spectrometric analysis. As shown in Table 3, four kinds of *O*-methyl sugars were assigned as follows [13]: 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methyl arabinitol, 1,3,4-tri-*O*-acetyl-2,5-di-*O*-methyl arabinitol, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-galactitol and 1,2,3,5-tetra-*O*-acetyl-4,6-di-*O*-methyl-mannitol in a molar ratio of 1.08:0.85:0.75:1.0 (Table 3). The occurrence of 2,3,5-*O*-methyl arabinitol and 2,3,4,6-*O*-methyl-galactitol indicated the presence of both terminal arabinofuranose (-1-Ara*f*) and galactopyranose (-1-Gal*p*) residues. For identification of uronic residues, partially methylated alditol acetates of carboxyl-reduced TPS-1 were also analysed by GC-mass spectrometry. In addition to the four kinds of alditol acetates detected, 1,3,4,5-tetra-*O*-acetyl-2,6-*O*-di-methyl glucitol was also identified in a relatively high proportion (Table 3), indicating the presence of glucuronosyl residues.

Because all of the arabinosyl residues were furanosidic, these units could be removed preferentially by mild acid hydrolysis. Following mild acid hydrolysis of TPS-1, dearabinosylated TPS-1 was separated into non-dialysable and dialysable fractions. Methylation analysis, after carboxyl reduction of the non-dialysable fraction, revealed the presence of 3,4,6-tri-*O*-methyl-mannitol and 2,3,6-tri-*O*-methyl glucitol in a molar ratio of 1.0:1.08. After removal of arabinofuranosyl residues, 1,2-linked mannosyl and 1,4-linked glucosyl residues could be detected, whereas 1,2,3-linked mannosyl and 1,3,4-linked glucosyl residues were now absent. This indicates that the arabinosyl residues is attached to both mannosyl and glucuronosyl residues at the C-3 position. From the dialysable fraction, Ara and Gal were identified by GC. In addition, TLC of this fraction showed that there were two components, the one having the same R_f as Ara, the other corresponding to that of maltose.

Considering the methylation results of both native and carboxyl-reduced TPS-1, monosaccharides were

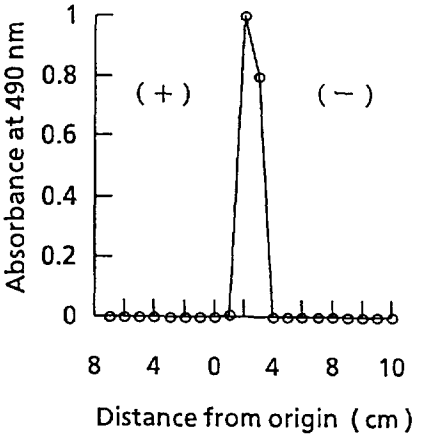

Fig. 3. Zone electrophoresis of TPS-1.

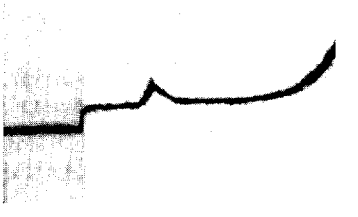

Fig. 4. Ultracentrifugal pattern of TPS-1.

Table 3. Molar ratio of methylated sugar fragments from hydrolysates of methylated native and carboxyl-reduced TPS-1

O-Methylated sugar (alditol acetate)	Mode of linkage	Molar ratio	
		Native	Carboxyl-reduced
4,6-Me_2-Man	→ 2)-Man-(1 → 3 ↑	1.0	1.0
2,3,5-Me_3-Ara	Ara-(1 →	1.08	1.08
2,5-Me_2-Ara	→ 3)-Ara-(1 →	0.85	0.85
2,3,4,6-Me_4-Gal	Gal-(1 →	0.75	0.75
2,6-Me_2-Glc	→ 4)-Glc-(1 → 3 ↑	—	1.08

assigned as 1-linked Ara*f* and 1-linked Gal*p*, and disaccharides as 1-Ara*f*-(3 → 1)-Ara*f* and 1-Ara*f*-(3 → 1)-Gal*p*. From the identities and proportions of the cleavage fragments from native, carboxyl-reduced and mild acid hydrolysates, a possible backbone could contain glucuronomannan with a branching point at C-3. As side-chain units, Ara, Gal, Ara–Ara and Ara–Gal were attached randomly to the backbone sugars. To determine the absolute configuration, constituent sugars were converted into trimethylsilyl ethers of the corresponding methyl 2-(polyhydroxyalkyl)-thiazolidine-4-carboxylates and subjected to GC analysis [14]. By comparison with authentic samples, the configurations were determined to be D for glucuronic acid (GlcUA), Man and Gal, and L for Ara. Anomeric configurations and conformations were also determined by ^{1}H NMR. The anomeric proton resonances at δ4.49, δ4.57, δ5.35 and δ5.45 were due to β-D-Gal, β-D-GlcUA, α-D-Man and α-L-Ara, respectively [15–18]. The ^{13}C NMR spectrum displayed two peaks at δ174.8 and δ170.9 which are attributed to GlcUA and esterified GlcUA respectively [19, 20]. In the anomeric carbon resonance region (δ90–110), four strong peaks were identified at δ108.8, δ103.9, δ102.5 and δ98.9. The sharp resonance at δ108.8 could be due to α-arabinofuranosyl residues [21], while the resonance at δ103.9, δ102.5, and δ98.9 could correspond to β-D-galactosyl, β-D-glucuronosyl acid and α-D-mannosyl residues, respectively [19, 22]. Another signal at δ54.4 could be due to the presence of a methyloxy group [21, 23], indicating that GlcUA was partially methoxylated. This result was confirmed by IR absorption at 1750 cm^{-1}. Furthermore, the content of methanol liberated by saponification with 1 N NaOH was measured by GC; 14.38 μg MeOH was generated from 1 mg TPS-1. Assuming that the weight proportion of GlcUA was *ca.* 22% of TPS-1, the degree of esterification was estimated to be 35%.

Thus, a possible structure of TPS-1 was tentatively assigned as follows. The backbone structure was composed of a repeating unit of

$$\left[\begin{array}{ll} \rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{UA-}(1 \rightarrow 2)\text{-}\alpha\text{-D-Man}p\text{-}(1 \rightarrow \\ \quad\widehat{3} \qquad\qquad\quad \widehat{3} \\ \quad\uparrow \qquad\qquad\quad \uparrow \\ \quad\mathrm{R} \qquad\qquad\quad \mathrm{R} \end{array}\right]$$

with *ca* 35% of GlcUA moieties as methyl esters. As side-chain units, (R), → 1)-α-L-Ara*f*, → 1)-β-D-Gal*p*, → 1)-α-L-Ara*f*-(3 → 1)-α-L-Ara*f* and → 1)-α-L-Ara*f*-(3 → 1)-β-D-Gal*p* were connected at the C-3 position of the backbone sugars.

It is known that polysaccharides possessing a glucuronomannan chain occur not only in the gums secreted by *Drosera* and *Angeissus* but also in ECP released from suspension-cultured tobacco cells [22, 24–27]. Including the described glucuronomannan produced by tuberose callus, the backbone structure was identical. However, the side-chain units varied among these glucuronomannan polysaccharides. The mode of linkage of glucuronomannan chain may be conservative, whereas the side-chain units may be variable according to plant species and/or culture conditions.

EXPERIMENTAL

Callus culture. Flowers of tuberose (*P. tuberosa* L.) were used as a source of explants. Calli were induced from explants on solid LS medium [29] supplemented with 10^{-5} M 1-naphthaleneacetic acid and 10^{-6} M 6-benzylaminopurine (N5B6-medium), and subcultured monthly in the liquid medium [12]. Separately, calli maintained in N5B6 liquid medium were transferred into LS liquid medium containing 10^{-5} M 2,4-D (D5-medium) and subcultured at 4-week intervals. For liquid culture, calli (1 g fr. wt) were inoculated into 30 ml vols of liquid media in 100 ml flasks and incubated in the dark at 120 rpm at 26°. After culturing for 4 weeks, liquid media were harvested for analyses of ECP.

Isolation of ECP. Liquid medium was separated from calli by filtration through a layer of cheese cloth and dild ×2 with H_2O in order to reduce the viscosity of the filtrate. After centrifugation at 10,000 *g* for 30 min, the supernatant was dialysed against H_2O and concd *in vacuo*. Four vols of EtOH were then added and the soln allowed to stand overnight at 4°. The ppt. obtained by centrifugation at 2000 *g* for 15 min was dissolved in 50 mM Na-Pi buffer (pH 6).

Ion-exchange chromatography of ECP. Approx. 20 ml of ECP (1 mg ml^{-1}) was loaded onto a column of DEAE-Sephadex A-25 (3.5 × 20 cm), PO_4 form. Frs were eluted sequentially with a linear gradient of 0–0.5 M NaCl at 250 ml hr^{-1}. Frs of 10 ml were collected. The two peaks in the acidic fr. were designated as tuberose polysaccharide (TPS)-1 and -2, respectively.

Zone-electrophoresis of TPS-1. The sample was electrophoresed on Whatman GF/A glass microfibre paper with 0.025 M Na tetraborate buffer (pH 9.3) at 1000 V for 15 min. After electrophoresis, the paper was cut into 1 cm strips from which the polysaccharides were eluted with H_2O. Total sugar content in each strip was measured colorimetrically.

Analysis of sugar composition of TPS-1. The major fr. (TPS-1, 2 mg) was lyophilized after EtOH pptn. Complete acidic hydrolysis was carried out with 4 N TFA at 125° for 2 hr. The hydrolysate thus obtained was flushed with N_2. Neutral sugars in the hydrolysate were converted into their corresponding aldononitrates for GC analysis [30].

Mild acid hydrolysis of TPS-1. A sample of TPS-1 (500 mg) was hydrolyzed with 0.1 N H_2SO_4 for 2 hr at 95°. The hydrolysate was neutralized with 1 N NaOH and dialysed against H_2O. Non-dialysable and dialysable hydrolysates were recovered and freeze-dried. The dialysable hydrolysate was used for TLC and GC, while the non-dialysable, after carboxyl-reduction and methylation, was used for GC and GC-MS.

Detection of mono- and disaccharides by TLC. Neutral sugars of the dialysable hydrolysate were separated by TLC (silica gel, Merck 13727), using *n*-BuOH–EtOH–H_2O (5:5:3). Sugars were detected with diphenylamine-

aniline reagent [31]. For the identification of separated sugars, Ara and maltose (Mal) were used as authetic markers for mono- and disaccharides, respectively.

Carboxyl-reduction of native and mild acid hydrolysate. Samples (native; 260 mg, hydrolysate; 130 mg) were reduced ×5 according to the method of ref. [32]. The resulting carboxyl-reduced TPS-1 (native; 110 mg, hydrolysate; 60 mg) was then hydrolysed with 4 N TFA and converted into the corresponding aldononitrates for identification of neutral sugars.

Methylation analysis. Samples (native; 56 mg, carboxyl-reduced; 50 mg) dissolved in DMSO were methylated with methylsulfinyl carbanion and MeI [33]. After methylation, samples were hydrolysed with 4 N TFA for 2 hr at 125° and converted into alditol acetates at 100° [34]. Partially methylated sugars thus obtained were analysed by GC and GC-MS. Assignments of sugars were made according to ref. [13].

Analytical methods. Uronic acid contents were determined by A 530 nm using the carbazole method [35] and expressed as GlcUA equivalents. Total sugar was measured at A 490 nm using the PhOH–H_2SO_4 method [36] and expressed as Glc equivalents. FID-GC was carried out using an Ultra-1 (25 m × 0.2 mm i.d., 0.33 μm film thickness) column. For aldononitrates of neutral sugars, the column temp. was prog. from 175° to 300° at 4° min^{-1}; for alditol acetates of neutral sugars the prog. was started at 120°. GC-MS was performed at 70 eV with an ion-source temp. of 200°. ^1H and ^{13}C NMR spectra were recorded in D_2O at 500 MHz and 125 MHz, respectively. Chemical shift are shown in δ values (ppm) with DDS (^1H) and dioxan (^{13}C) as int. standards. Centrifugal analyes were made at 60000 rpm using an analytical centrifuge. IR spectra were measured in KBr discs.

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