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ARTICLE *in* JOURNAL OF NATURAL PRODUCTS · MAY 2006

Impact Factor: 3.8 · DOI: 10.1021/np058043m · Source: PubMed

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Bioactive Exopolysaccharides from the Cultured Cells of Tomato, *Lycopersicon esculentum* var. San Marzano

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Received March 18, 2005

Three exopolysaccharides, EPS(**1**), EPS(**2**), and EPS(**3**), were isolated from suspension-cultured cells of tomato (*Lycopersicon esculentum*, var. San Marzano). The partial primary structures were determined on the basis of spectroscopic analyses. EPS(**2**) was a heteropolysaccharide with a tetrasaccharide repeating unit constituted by sugars having one residue in α-manno, one residue in β-manno, and two different residues in β-gluco/galacto configurations. EPS(**3**) was a heteropolysaccharide with a pentasaccharide repeating unit with sugars having three residues in α-manno, one residue in α-gluco/galacto, and one residue in β-gluco/galacto configurations. The anticytotoxic activities of exopolysaccharides were tested in a brine shrimp bioassay.

The polysaccharides extracted from natural sources were of particular interest with regard to their practical applications. Some of the most common polysaccharides are used for their chemical−physical properties. These include high molecular weight, viscosity, resistance to high temperatures, the ability to interact with numerous molecules, structural ramifications and smaller substituents, as support for resins, as a component of artificial plasma, and in the food industry. Others presented strong antigenic activity, and they are used by the pharmaceutical industry in the production of vaccines. They are able to contain many biological messages and, accordingly, are able to perform a number of functions due to their unusual multiplicity and structural complexity.

Increasing attention has been paid to the exopolysaccharides produced in large quantities by a wide range of bacteria and cyanobacteria due to their commercial applications as industrial food additives and to their participation in pathogenic and symbiotic processes in plants and animals and the general interactions between microorganisms and their environment.[1]

Limited data are available regarding the polysaccharides from the cell cultures of *Solanaceae*.[2] The determination of the pharmacological effect of polysaccharides primarily depends on defined sterile cultivation conditions. At the same time, high space−time yields of the production of biomass and valuable substances have to be guaranteed.

The present paper deals with the isolation and chemical characterization of the water-soluble polysaccharides released from suspension-cultured cells of tomato (*Lycopersicon esculentum* L. var. San Marzano). Two new purified exopolysaccharides were tested for their biological activities.

Cell culture of *L. esculentum* produced two main exopolysaccharides that seem to be rather different from polysaccharides obtained from the walls of tomato fruit as reported by Walker.[2]

The presence of extracellular polysaccharides was observed from the high viscosity of the culture medium. A polysaccharide fraction was collected from the culture medium of tomato suspension cells (1 L) growing in the presence of 3% sucrose wt/v. After four weeks of growth the cell suspension was filtered and the exopolysaccharide fraction (260 mg) was obtained by EtOH precipitation of free cells in culture broth. The raw material, tested for sugar content (70%), protein content (10%), and nucleic acid content (1%), was purified by gel chromatography (Sepharose CL-6B DEAE) with a yield of 89%, and the resulting compounds comprised three different fractions, EPS(**1**) 9%, EPS(**2**) 60%, and EPS(**3**) 31%, all containing less than trace amounts of protein and nucleic acids (Figure 1). EPS(**1**) was eluted in H_2O, representing the neutral fraction, while EPS(**2**) and EPS(**3**) were eluted at different salt concentrations (0.3 and 0.4 mol of NaCl, respectively) representing the acidic fractions.

Sugar mixtures of each fraction, native and carboxyl reduced, were identified by HPAE-PAD of hydrolyzed polysaccharides and by GLC and GC-MS of alditol acetates and methyl glycoside acetates.

As showed in Table 1, sugar analysis of native EPSs indicated that EPS(**1**) was composed of Ara:Gal:Glc:Man in a relative ratio of 0.7:1.0:0.4:0.9, respectively; EPS(**2**) was composed of Ara:Gal in a relative ratio of 0.3:1.0, respectively; and EPS(**3**) was composed of Ara:Man in a relative ratio of 1.0:0.5, respectively. EPS(**1**) was composed of neutral sugars; the sugar compositions of EPS(**2**) and EPS(**3**) demonstrated their acidic nature. The results of sugar analyses of methyl glycoside acetates indicated that EPS(**2**) was constituted of L-Ara:D-Gal:L-AraA (0.5:1.0:0.2, respectively) and EPS(**3**) of L-Ara:D-Man:L-AraA (0.5:0.3:1.0, respectively).

Only EPS(**2**) and EPS(**3**) were further analyzed because EPS(**1**) was less pure and recovered in a low yield. EPS(**2**) and EPS(**3**) were analyzed by chemical and spectroscopic analysis (Table 2).

The UV spectra of exopolysaccharides did not indicate any strong absorption peaks in the range of 350 to 210 nm. The quantities of uronic acid varied with different preparations, reaching 170 μg/mg EPS(**2**) and 240 μg/mg EPS(**3**). The specific rotations of EPS(**2**) and EPS(**3**) were $[\alpha]^{25}_{D}$ −50.40 and −60 (concentration of 5 mg/mL H_2O), respectively. The absolute configuration of carbohydrates was shown to be D-Gal and L-Ara for EPS(**2**) and D-Man and L-Ara for EPS(**3**), when analyzed as their respective acetylated (+)-2-butyl glycosides from methylation analysis and NMR spectra. It was evident that the sugar residues in both EPSs were pyranosidic, while arabinose was furanosidic.

The molecular weight of EPSs was estimated from the calibration curve of standard dextrans obtained by gel filtration on Sepharose

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10.1021/np058043m CCC: $33.50
Published on Web 02/15/2006

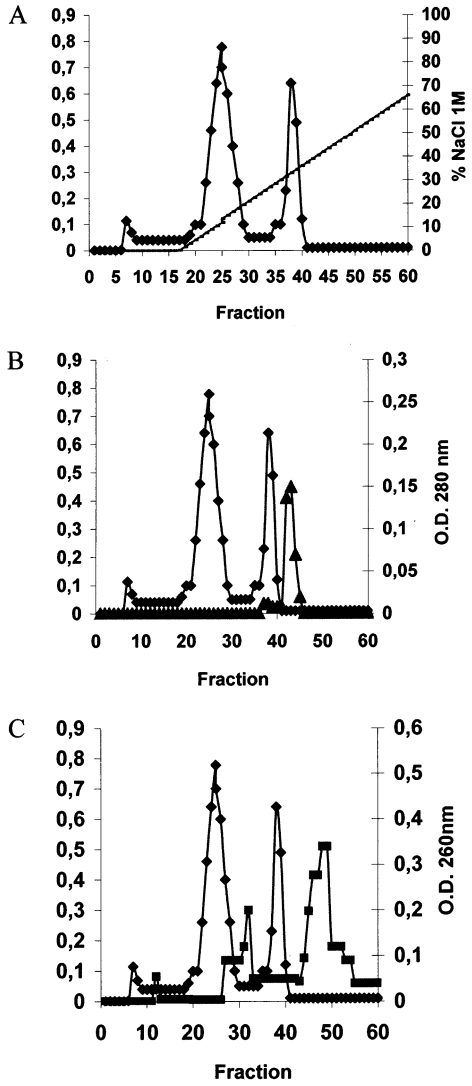

Figure 1. Chromatography of polysaccharide fractions on Sepharose DEAE CL-6B. The bed dimension was 1.5 × 40 cm. The eluant was H_2O with a linear salt gradient to 1 M NaCl. The flow rate was 12 mL h^{-1}, and the volume fraction was 10 mL. (A) Optical density by Dubois assay for carbohydrate detection; (B) optical density at 280 nm for protein detection; (C) optical density at 260 nm for nucleic acid detection: ◆ carbohydrate; ▲ protein; ■ nucleic acid.

Table 1. Sugar Compositions of Exopolysaccharides from Tomato Cell Suspension[a]

	component (relative ratio %)					
fraction	Ara	Gal	Glc	Man	AraA	Xyl
EPS(**1**)n	0.7	1.0	0.4	0.9		
EPS(**2**)n	0.3	1.0		tr		tr
EPS(**2**)cr	0.6	1.0		tr		tr
EPS(**2**)m	0.5	1.0			0.2	tr
EPS(**3**)n	1.0	tr	tr	0.5		tr
EPS(**3**)cr	1.0	tr	tr	0.3		tr
EPS(**3**)m	0.5	tr		0.3	1.0	tr

[a] Hydrolysis of EPSs was performed with 2 M trifluoroacetic acid at 120° C for 2 h. n = native; cr = carboxyl reduced; m = methanolysis; tr = trace amount.

CL-6B and also by density gradient centrifugation. In both methods, the molecular weights were approximately 8.0 × 10^5 for EPS(**2**) and 9.0 × 10^5 for EPS(**3**) (Table 2). The infrared spectra of EPSs were similar to those of bacterial polysaccharides.[3] A broad absorption band attributable to OH was observable at 3400 cm^{-1}. The absence of sulfate groups in EPS(**2**) as well as in EPS(**3**) was confirmed by IR spectra and also by a negative color reaction with sodium rhodizonate.

Table 2. Chemical Characterization of Exopolysaccharides from Tomato Cells

parameter	EPS(**2**)	EPS(**3**)
MW	800.000	900.000
$[\alpha]_D^{25}$	−50.40 (H_2O)	−60.0 (H_2O)
UV	no absorption	no absorption
assay: uronic acid (μg/mg EPS)	170	240
Bio-Rad (%)	<1	<1
sulfate	absent	absent
piruvic acid	negative	negative
absolute configuration	D-Gal; L-Ara	D-Man; L-Ara

Analysis of the partially methylated alditol acetates, obtained from the permethylated EPSs after acid hydrolysis, showed that in both polysaccharides there is the presence of hexose chains linked on C1−C2, C1−C3, and C1−C6 and the presence of side chains on C1−C2−C6.

The ^1H and ^{13}C NMR spectra recorded in H_2O at 343 K were quite complex. In the nonanomeric proton region, several overlapping spin systems were evident. The ^1H NMR spectrum of EPS(**2**) showed, in the anomeric region, four major signals at δ 5.26 (1H, d, J = 3.0 Hz), 4.71 (1H, d, J = 1.5 Hz), 4.52 (1H, d, J = 8.0 Hz), and 4.49 (1H, d, J = 8.3 Hz) (Table 3). The ^1H NMR spectrum of EPS(**3**) showed five major anomeric signals at δ 5.47 (1H, d, J = 2.9 Hz), 5.44 (1H, d, J = 1.5 Hz), 5.39 (1H, d, J = 2.0 Hz), 5.32 (1H, d, J = 3.9 Hz), and 4.55 (1H, d, J = 7.8 Hz) (Table 3). The ^{13}C NMR spectrum of EPS(**2**) showed four signals at δ 111.9, 106.0, 106.3, and 106.4 in the anomeric region, confirming the presence of four residues in the repeating unit, and a small signal at δ 178.4 (COOH) due to the presence of uronic acid in minute quantities. The ^{13}C NMR spectrum of EPS(**3**) showed the presence of five signals at δ 111.5, 110.7, 105.1, 105.0, and 100.9, confirming the presence of five residues in the repeating unit, and an intense signal at δ 176.9 (COOH) indicative of the presence of uronic acid. The ^1H and ^{13}C chemical shifts and the C−H coupling constants of each anomeric carbon were assigned by HMQC experiments. Sugar residues were labeled from A to D for EPS(**2**) and from A to E for EPS(**3**) in decreasing order of proton chemical shifts. The comparison of these values gave information about the anomeric configuration of some residues (Table 3). In the ^1H NMR spectrum of EPS(**2**) the signal of residue B at δ 4.71 was a β-manno (J = 1.5 Hz), while the signals at δ 4.52 and 4.49 of residues C and D were typical of a β-gluco/galacto (J = 8.0−8.3 Hz) configuration. In the ^1H NMR spectrum of EPS(**3**) at δ 5.44 an α-manno (J = 1.5 Hz) configuration for residue B, an α-gluco/galacto at δ 5.32 for residue D (J = 3.9 Hz), and a β-gluco/galacto (J = 7.8 Hz) configuration at δ 4.55 for residue E were observed. The downfield ^{13}C chemical shift observed for residues A of both EPS(**2**) and EPS(**3**) and residue C of EPS(**3**) may be indicative of a furanosidic form instead of pyranosidic. This was also confirmed by the presence of signals belonging to ring carbons in the region at δ 88−80 ppm, attributable to an arabino furanosidic residue present in both exopolysaccharides.

From these data it was evident that both polysaccharides presented a very complex primary structure. EPS(**2**) was a heteropolysaccharide with a tetrasaccharide repeating unit constituted by sugars having one residue in an α-manno, one residue in a β-manno, and two different residues in β-gluco/galacto configurations. EPS(**3**) was a heteropolysaccharide with a pentasaccharide repeating unit with sugars having three residues in an α-manno, one residue in an α-gluco/galacto, and one residue in a β-gluco/galacto configuration.

Exopolysaccharides have been reported to inhibit a variety of host defense mechanisms. It has been suggested that the production of exopolysaccharides may be a major factor in the pathogenesis

Table 3. Chemical Shifts and Coupling Constants of Anomeric Signals in ^{1}H and ^{13}C Spectra of EPSs[a]

	EPS(**2**)			EPS(**3**)		
residue	δ H-1/C-1	$^3J_{H-1,H-2}$	$^1J_{H-1,C-2}$	δ H-1/C-1	$^3J_{H-1,H-2}$	$^1J_{H-1,C-2}$
A	5.26/111.9	3.0	175.05	5.47/110.7	2.9	172.5
B	4.71/106.3	1.5	161.4	5.44/100.9	1.5	163.4
C	4.52/106.0	8.0	n.d.	5.39/111.5	2.0	176.0
D	4.49/106.4	8.3	n.d.	5.32/105.1	3.9	168.3
E				4.55/105	7.8	170.6

[a] Sugar components of EPS(**2**) are labeled from A to D, and those of EPS(**3**) are labeled from A to E, in both cases with decreasing chemical shifts. Coupling constants are in Hz. n.d.= not detected.

Table 4. Inhibition of Avarol Toxic Activity on *Artemia salina*

	avarol 10 ppm polysaccharide 0 ppm	avarol 10 ppm polysaccharide 5 ppm	avarol 10 ppm polysaccharide 50 ppm	avarol 10 ppm polysaccharide 500 ppm	IC_{50}[a] ppm
raw polysaccharide	30/0[b]	25/5	22/8	7/23	113
polysaccharide EPS(**2**)	30/0	14/16	3/27	1/29	3
polysaccharide EPS(**3**)	30/0	19/11	7/23	5/25	11

[a] 50% inhibiting concentration of avarol toxicity. [b] Deaths/survivals *Artemia salina*. The values are the average of at least three determinations.

of induced infections. Polysaccharide production can protect bacteria or more generally cells from antibiotics,[4] antibodies,[5] and protein-ase.[2]

We have studied the effect of exopolysaccharides produced by tomato suspension cultures on the inhibition of the cytotoxic effects produced by avarol. The ability of the exopolysaccharides obtained in this study to induce inhibition of avarol (10 μg/mL) toxicity tested in the brine shrimp (*Artemia salina*) bioassay was evaluated. Avarol is a sesquiterpene hydroquinone that showed strong toxicity (LC_{50} 0.18 μg/mL or 0.57 nM) in a brine shrimp bioassay, which gives results that correlate well with cytotoxicity in cancer cell lines such as KB, P388, L5178y, and L1210.[6] As summarized in Table 4, EPS(**2**) was a potent anticytotoxic compound in this bioassay; in fact, the inhibition of the avarol toxicity of 50% (IC_{50}) was observed at a concentration of 3 and 11 μg/mL for EPS(**2**) and EPS(**3**), respectively.

Further developmental studies in our laboratory are directed toward using these novel exopolysaccharides to test other biological activities and toward continuous production using a bioreactor.

Experimental Section

Cell Cultures. Commercial seeds of *L. esculentum* were purchased from Bulsem Salerno (Italy). For callus induction, plant sections from sterile grown *L. esculentum* were cultured on MS basal medium supplemented with (mg L^{-1}) myoinositol (100), nicotinic acid (0.5), pyridoxine hydrochloride (0.5), thiamine hydrochloride (0.1), glycine (2), sucrose (30 000), and agar (9000). This medium was supplemented with (M) *p*-chlorophenoxyacetic acid (10^{-5}), 2,4-dichlorophenoxyacetic acid (2×10^{-6}), and 6-benzylaminopurine (10^{-6}). The initial callus was transferred to fresh medium after 4 weeks, and the resulting culture was maintained as above. Suspension cultures were initiated from fourth generation callus by transfer of ca. 3 g of callus into 100 mL of liquid medium. Suspension cultures were maintained in 250 mL flasks, by transfer of ca. 1 g of fresh weight tissue (ca. 10 mL) into 100 mL of fresh medium every 21 days. Cultures were maintained at 24 °C, 150 rpm, in continuous light.[7]

EPS Recovering. Cells were harvested by centrifugation (9800*g*, 20 min). The liquid phase (1 L) was treated with 1 volume of cold EtOH added dropwise under stirring. The alcoholic solution was kept at −18 °C overnight and then centrifuged at 15300*g* for 30 min. The pellet was dissolved in hot H_2O (1/10 initial volume). The same procedure was repeated twice. The final aqueous solution was dialyzed against tap H_2O (48 h) and distilled H_2O (20 h), lyophilized, and weighed (260 mg).

Chromatographic Conditions. The polysaccharide fraction (raw polysaccharide) was purified by gel chromatography (Sephadex G-50; 2.5 × 50 cm) using H_2O−pyridine−HOAc (500:5:2) as eluant. Fractions were collected with a flow rate of 6 mL h^{-1} (5 mL each fraction), followed by anion exchange chromatography (DEAE-Sepharose CL-6B; 1.5 × 40 cm) eluted with 100 mL of H_2O and 1 L of a NaCl gradient from 0 to 1 M with a flow rate of 12 mL h^{-1}, collecting fractions of 10 mL each. Each fraction was tested for the presence of carbohydrates by a spot test on TLC sprayed with α-naphthol and quantitatively by the method of Dubois.[8] The α-naphthol positive fractions were pooled, exhaustively dialyzed against H_2O, freeze-dried, and weighed. This material was used for all analytical work.

Colorimetric Assay. Carbohydrate content was performed according to the method of Dubois,[8] reading absorbance at 490 nm and using glucose as a standard. Total protein content was estimated by using the Bradford reagent (Bio-Rad) and bovine serum albumin as a standard. Nucleic acid content was tested spectrophotometrically reading the absorbance at 260 nm. Pyruvate was detected after polysaccharide hydrolysis (100 °C, 3 h) using a solution of 0.5% w/v of 2,4-dinitrophenylhydrazine in 2 M HCl.[3] Sulfate presence was identified by the method of Silvestri.[9] Uronic acid was identified as reported by Jansson.[10]

Molecular Weight. Molecular weight was estimated by (1) gel filtration on a Sepharose CL-6B column (1 × 80 cm) using H_2O−pyridine−HOAc (500:5:2) as eluant (fractions were collected at 3.7 mL h^{-1} and tested by a spot test on TLC sprayed with α-naphthol) and (2) density gradient centrifugation using a sucrose gradient from 0 to 50% w/v at 130000*g* for 16 h. Centrifuge tubes were fractionated in 0.2 mL fractions diluted with H_2O, dialyzed against water for 72 h, and tested for carbohydrate presence as reported above. In both experiments 10 mg of EPS and a mixture of dextrans for calibration curves (10 mg each of T-700, mol wt 670 000; T-400, 410 000; and T-150, 154 000) were used.

Sugar Analysis. Hydrolysis of EPSs was performed with 2 M TFA at 120 °C for 2 h. Sugar components were identified by TLC and high-pressure anion exchange-pulsed amperometric detector (HPAE-PAD) using sugar standards for identification and calibration curves. TLC was developed with the following solvent systems: (a) Me_2CO−*n*-BuOH−H_2O (4:1:1) for neutral sugars; (b) *n*-BuOH−H_2O−HOAc (3:1:1) for acidic sugars; (c) *n*-BuOH−EtOH−H_2O (5:3:2) for oligosaccharides. Sugars were visualized by spraying the plates with α-naphthol. HPAE-PAD Dionex equipped with a Carbopac PA1 column was eluted isocratically with (a) 15 mM NaOH for neutral sugars and (b) 100 mM NaOH and 150 mM NaOAc for acidic sugars.[11]

Methylation of the polysaccharides was carried out according to the method described earlier.[12] The methylated material (0.5 mg) was hydrolyzed with 2 M TFA at 120 °C for 2 h and then transformed in partially methylated alditol acetates by reduction with $NaBH_4$, followed by acetylation with Ac_2O−pyridine (1:1) at 120 °C for 3 h. Unambiguous identification of sugars was obtained by gas-liquid chromatography (GLC) and GC-MS using sugar standards. GLC was performed on a Hewlett-Packard 5890A instrument equipped with an HP-5-V column and N_2 flow of 100 mL min^{-1} and a flame ionization detector (FID). The program temperature was 170 °C (1 min), from 170 to 180 °C at 1 °C min^{-1}, 180 °C (1 min), and from 180 to 210 °C at 4 °C min^{-1}. GC-MS were performed on a Hewlett-Packard 5890-5970 instrument equipped with an HP-5-MS column and with an N_2 flow of 50 mL min^{-1}; the program temperature was 170 °C (1 min) and from 170 to

250 °C at 3 °C min^{-1}. The reduction of the carboxyl group of the fractions was performed following the method of Taylor.[12]

The absolute configuration of the sugars was performed as described by Leontein,[13] using optically active (+)-2-butanol by GLC of their acetylated (+)-2-butyl glycosides. For GLC runs the same instrument and conditions described in the methylation analysis were used. Retention times were determined by comparison of the sample with authentic standards.

Spectroscopic Studies. Optical rotations were obtained on a Perkin-Elmer 243-B polarimeter at 25 °C in H_2O. NMR spectra were performed on a Bruker AMX-500 (500 and 125 MHz for ^1H and ^{13}C, respectively) at 70 °C. Samples were exchanged twice with D_2O with intermediate lyophilization and then dissolved in 500 μL of D_2O to a final concentration of 40 mg/mL. Chemical shifts were reported in ppm relative to sodium 2,2,3,3-d_4-(trimethylsilyl)propanoate for ^1H and $CDCl_3$ for ^{13}C NMR spectra. The $J_{H-1,C-1}$ values were determined by HMQC inverse detected experiments.[14]

Biological Assay. The brine shrimp (*Artemia salina*) assay was performed in triplicate with appropriate amounts of polysaccharides dissolved in DMSO (1% final volume) at concentrations of 500, 50, 5, and 0 ppm, in the presence of avarol at 10 ppm, using 10 freshly hatched larvae suspended in 5 mL of artificial seawater.[15] Briefly, for each dose tested, surviving shrimp were counted after 24 h, and the data statistically analyzed by the Finney program,[16] which affords IC_{50} values with 95% confidence intervals.

Acknowledgment. This work was supported by Regione Campania Lg 5. The authors thank V. Calandrelli, I. Romano, E. Pagnotta, and E. Esposito for technical assistance; O. De Luca for GC-MS; and V. Mirra for NMR service.

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NP058043M