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IMMUNOLOGICALLY ACTIVE POLYSACCHARIDES OF *ECHINACEA PURPUREA* CELL CULTURES

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Key Word Index—*Echinacea purpurea*; Asteraceae; tissue culture; fucogalactoxyloglucan; acidic arabinogalactan; structure analysis; immunological activity.

Abstract—From the medium of *Echinacea purpurea* cell cultures three homogeneous polysaccharides, two neutral fucogalactoxyloglucans with mean M_r of 10000 and 25000 and an acidic arabinogalactan with a mean M_r of 75000, have been isolated by DEAE-Sepharose CL-6B, DEAE-Trisacryl M and Sephacryl S400 column chromatography. Their structures were elucidated mainly by methylation analysis, partial acidic and enzymatic hydrolysis and ^{13}C NMR spectroscopy. The fucogalactoxyloglucan of mean M_r 25000 enhances phagocytosis *in vitro* and *in vivo*. The arabinogalactan specifically stimulates macrophages to excrete the tumour necrosis factor (TNF).

INTRODUCTION

In a previous publication [1] we have described the isolation of some polysaccharides and the structure determination of one immunostimulating 4-*O*-methylglucuronoarabinoxylan from the herbal plant *Echinacea purpurea* (L.) Moench, which might partly account for the pharmacological activity of *Echinacea* plant preparations. Because of standardization problems concerning the chemical composition and biological activities of *Echinacea* extracts, due to varying plant origin, we tried to produce the polysaccharides by cell cultures of *Echinacea purpurea*. The present paper describes the isolation and structure determination of three immunologically active polysaccharides from the culture medium.

RESULTS

Production of polysaccharides from cell cultures

Echinacea purpurea cells were cultivated in a suspension culture at 24°C in a Linsmaier/Skoog-medium [2] containing 2,4-dichlorophenoxyacetic acid as the only growth hormone. After a growth period of 14–21 days, the cultures were filtered and the medium extracted.

Isolation and purification of the polysaccharides

Three volumes of ethanol to the medium were added to obtain a precipitate which was freed from protein by treatment with tri-chloroacetic acid and then divided in two main polysaccharide fractions by precipitation with increasing concentrations of ethanol (1:1, 1:4). Both polysaccharide fractions were subjected to anion exchange chromatography on DEAE-Sepharose CL 6B (acetate form). The 1:1 fraction could be resolved into a neutral fraction A and three acidic polysaccharides (B, C, D). The first fraction was purified by further DEAE-Sepharose CL B and DEAE-Trisacryl M chromatography, followed by Sephacryl S 400 gel-chromatography to remove low M_r sugar components. The 1:4 precipitation product was separated in a similar manner into a neutral fraction E and an acidic fraction F. Both fractions were further purified by ultragel AcA 202 and DEAE-Trisacryl M (acetate form) respectively. The symmetrical elution profiles obtained for the polysaccharides A, E and F in the gel chromatography and high pressure gel permeation chromatography (HP-GPC) [3] revealed their homogeneity. The mean M_r of the neutral polysaccharides A and E were determined as 25000 and 10000 respectively, while polysaccharide F was found to have a M_r of 75000 or 110000 depending on the buffer system used.

Structure determination of polysaccharides A and E

Both polysaccharides yielded glucose, xylose, galactose and fucose, in the same molar ratio of 1.5:1.0:0.4:0.1. The GC/MS-identification of the partially methylated alditol-acetates obtained by the Hakomori methylation [4], hydrolysis and reduction with $NaBH_4/NaBD_4$ and acetylation showed $1 \rightarrow 4$ and 1,4,6-linked glucose, xylose and galactose in $1 \rightarrow 2$ linkage and as terminal sugar, and fucose as a terminal sugar exclusively (Table 1). All sugars were present in a pyranoid form. Accordingly, polysaccharides A and E are concluded to be fucogalactoxyloglucans with a $1 \rightarrow 4$ glucan backbone and substituent chains or units on the 0-6 position of some of the glucose residues accounting for about 65% of the polysaccharides. Since both polysaccharides only differed in M_r (10000 and 25000), further analyses were only carried out with polysaccharide E. Partial hydrolysis of the fully methylated polysaccharide E with 90% formic acid resulted in a loss of all methylated fucose residues. After methylation of any hydroxyl then exposed with deuterated methyl iodide, a total hydrolysis of the degradation product was

Table 1. Methylation analysis of polysaccharides A, E, F, of partially hydrolysed polysaccharide F (G) and of carboxyl-reduced partially hydrolysed polysaccharide F (H)

Polysaccharide	A	E	F	F*	G	H	
Partially methylated alditolacetates‡	Molar composition						Characteristic mass fragments *m/z* (C-1-H) (C-1-D)
2,3,5-Me_3-arabinitol			1.5	3.7			101, 117, 129, 145, 161
2,3,4-Me_3-xylitol	1.55	1.66					89, 101, 115, 117, 131, 161, 175
2,3,4-Me_3-fucitol							
3,4-Me_2-rhamnitol						†	88, 99, 101, 129, 131, 189, 253
2,3-Me_2-arabinitol			1.4	2.2			101, 117, 129, 161, 189
2,3,4,6-Me_4-galactitol	0.25	0.34	0.6	0.8	0.37	0.37	101, 117, 129, 145, 161, 205
3,4-Me_2-xylitol	0.4	0.5					87 (88), 101, 117, 129 (130), 161, 189 (190)
3-Me-rhamnitol						†	87, 101, 129, 143, 189, 203
2-Me-arabinitol			0.7	0.9			99, 111, 117, 127, 141, 159, 172
2,4,6-Me_3-galactitol			0.97	0.7	0.41	0.41	101, 117, 129, 161, 173, 233, 277
3, 4, 6-Me_3-galactitol	0.35	0.41					87, 99, 101, 129, 145, 161, 189
2,3,6-Me_3-glucitol	1.0	1.0					87, 99, 101, 113, 117, 129, 131, 161, 173, 233
2,3,4-Me_3-galactitol			1.0	1.0	1.0	1.0	99, 101, 117, 129, 159, 161, 173, 189, 233
Xylitolpentaacetate	†	†					85, 103, 115, 127, 145, 158, 175, 187, 200, 217
2,3-Me_2-glucitol	1.97	2.0					85, 87, 99, 101, 117, 127, 159, 161, 187, 201, 261
2,3-Me_2-galactitol						0.13	87, 101, 117, 129, 161, 203, 263
2,4-Me_2-galactitol			4.9	4.0	0.37	0.37	87, 101, 117, 129, 189, 233

* Acetylated before methylation.
† No integration value.
‡ Analysed by GC/MS.

performed followed by reduction and GC/MS analysis of the obtained *o*-methylated alditol acetates. Contrary to the native polysaccharide, the partially degraded polysaccharide showed a markedly lower proportion amount of 1 → 2 linked galactose units, whereas the terminal galactose residues had about 60% of the methyl groups at the 0-2 positions (*m/z* 121, 164) deuterated. Thus the terminal fucose is 1 → 2 linked to galactose. Further information about the branching chains could be obtained by analysing the degradation products of the enzymatic hydrolysis. After hydrolysis with cellulase 'Onozuka R^{10}' from *Trichoderma viride* [5], the hydrolysis mixture was reduced with $NaBD_4$ and the methylated disaccharide alditols analysed by GC/MS. Five peaks were observed. The component of peak 1a (R_t = 0.87, retention time referred to methylated cellobiitol) eluted in the disaccharide alditol range, showed characteristic *m/z* ions of the aA (*m/z* 175, 143 and 111) and bA series (*m/z* 236, 204 and 172) (according to the nomenclature of Kochetkov and Chizov [6, 7] (Table 2). These ions can be attributed to a pentosylhexitol derivative consisting of a reducing hexose (*m/z* 236) and a non-reducing pentose (*m/z* 175), the second of which being linked to position 1 of the pentosyl moiety. In addition characteristic ions at *m/z* 134, 178 and 222 indicated that the reducing hexose was substituted in position 6 (Fig. 1) Further mass-fragments at *m/z* 296 and 261 confirmed this interpretation. Since xylose was the only pentose detected and the cellulase hydrolysis mainly splits β-D-1 → 4-linkages, the methylated disaccharide alditol which gave rise to peak 1 could be assigned to a 1 → 6 linked xylosylglucose disaccharide (Fig. 1). The component of peak 2a had the same R_t as methylated cellobiitol and showed ions at *m/z* 236 and 219 of the A-series which are characteristic for a hexosyl-hexitol disaccharide. This was confirmed by mass fragments at *m/z* 187 (219 − 32) and *m/z* 172 (236 − 64) as well as by ions at *m/z* 296 (236 + 60) of the abJ$_1$-series and an ion at *m/z* 381 (471 − 90) (Fig. 2). Since fragments at *m/z* 222 and *m/z* 178 indicative for a 1 → 6 linkage are missing and an intensive ion at *m/z* 134 could be observed, the component of peak 2a must be identical with methylated cellobiitol (glucosyl (1 → 4) glucose). The other peaks could not be assigned.

Since polysaccharides E and A according to the methylation analysis contain a relatively high percentage of 1 → 6 linkages, polysaccharide E was subjected to partial acetolysis, followed by desacetylation, reduction with $NaBD_4$ and successive methylation of the resulting product. Five peaks could be detected in the disaccharide range by GC/MS. The components of peaks 1b (R_t = 0.89) and 3b (R_t = 0.95) were identified as equally fragmenting hexosyl (1 → 2) pentitol derivates (Table 2). Since xylose represents the only (1 → 2) linked pentose, and galactose the only terminal hexose, peak 1b and 3b could be assigned to permethylated alditol-derivates of galactosyl (1 → 2) xylose. Since the ^{13}C NMR indicated β-configuration for all hexose units in the polysaccharide it is likely that one disaccharide derivative was an artefact, formed by an acid catalysed anomerisation during acetolysis, as described by Aspinall [8]. Peak 4b (R_t = 1.01) should be identical with the anomeric linked form. At least peak 2b (R_t = 0.9) could be assigned to a permethylated fucosyl (1 → 2) galactose-moiety (Table 2).

In order to obtain larger oligosaccharide fragments, we incubated polysaccharide E with partially purified endo-(1 → 4)-β-D-glucanase [9]. Fractionation of this hydrolysis product on Bio-Gel P-2 afforded five fractions. Three anthrone-positive [10] fractions (2, 3, 4) were subjected to methylation analysis.

A hydrolysate of fraction 4 contained xylose and glucose, the two others (2, 3) glucose, galactose, xylose and

Table 2. *m/z* values of relative intensities in the mass spectra of permethylated oligosaccharide alditols from (a) cellulase treated and (b) partially acetolysed polysaccharide E

m/z of diagnostic ions	Relative intensity				
	Peak 1a	Peak 2a	Peak 1 + 3b	Peak 2b	Peak 3 + 4b
45	90	78	94	40	72
46	38	24	20	13	18
71	66	44	48	31	40
72			62	22	
75	62	44	32	26	47
88	98	100	100	100	100
89	33	40	24	24	40
90	42	12			12
101	100	90	70	62	87
102	32				
111	30	42	26		38
115	34	24			28
127	10	10	12		12
129				18	
133			14		
134	12	10			10
143	60				
145				18	
146	32				
155		6,5			11
157				12	
160			18		
172	8	18		12	10
175	42				
177				6	
187	9	50	14		50
189				26	
192			90		
204	2	1,0			
207				4	
219	6	20	6		12
235					
236	22	58		20	48
250	6				
252			18		
261	2				
294			0,5		
296	10	18		16	18
362				3	
381		0.6			6

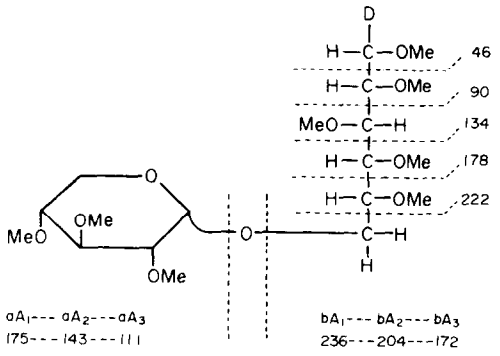

Fig. 1. Mass spectral fragmentation of methylated xylosyl-glucose moiety from degraded polysaccharide E after reduction with $NaBD_4$.

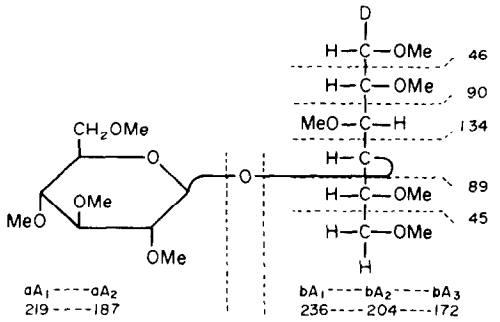

Fig. 2. Mass spectral fragmentation of methylated cellobiose from degraded polysaccharide E after reduction with $NaBD_4$.

Table 3. Methylation analyses of the Bio-Gel P-2 oligosaccharide fractions

Partially methylated sugar	Molar sugar composition					
	Bio-gel P-2 peak					
	2		3		4	
2,3,4-Me_3-fucitol	4.7	1.0*	2	0.7*	–	
2,3,4-Me_3-xylitol		3.7*		1.3*		
2,3,4,6-Me_4-galactitol	1.3		0.5		—	
3,4-Me_2-xylitol	1.9		0.5		—	
3,4,6-Me-galactitol	1.5		0.7		—	
2,3,4-Me_3-glucitol	1.0		1.0		1.0	
2,3,6-Me_3-glucitol	2.3		1.09		1.0	
2,3-Me_2-glucitol	4.8		2		2.0	

*Calculated values.

fucose. Methylation analysis of fraction 4 revealed for glucose a molar ratio of 1:1:2 for (1 → 4)-, (1 → 6)- and 1,4,6-linkages, whereas xylose was found as a terminal sugar only (Table 3). This is compatible with the structural features shown in Fig. 3. In rechromatographed fraction 3, apart from the terminal xylose, (1 → 2)-linked xylose and galactose as well as terminal galactose and fucose in a molar ratio of 2:1:1:1 could be detected leading to the partial structure of a decasaccharide (see structure II of Fig. 3). This structure proposal is supported by the reported elution behaviour of nonsaccharides on Biogel P-2 [9]. The reduced percentage of side chains and the increased percentage of terminal xylose in proportion to the glucan backbone may be due to a contamination by the heptasaccharide I. For fraction 2 methylation analysis revealed a molar ratio of 1:2:5 for 1 → 6 linked, 1 → 4 linked, and 1,4,6-linked glucose. The proportion of 1 → 2-linked xylose to terminal xylose was 2:4. Besides we found about 1.5 parts of 1 → 2 linked and terminal galactose. Based on these data fraction 2 could be assigned to a heptadecasaccharide (structure III), in which the

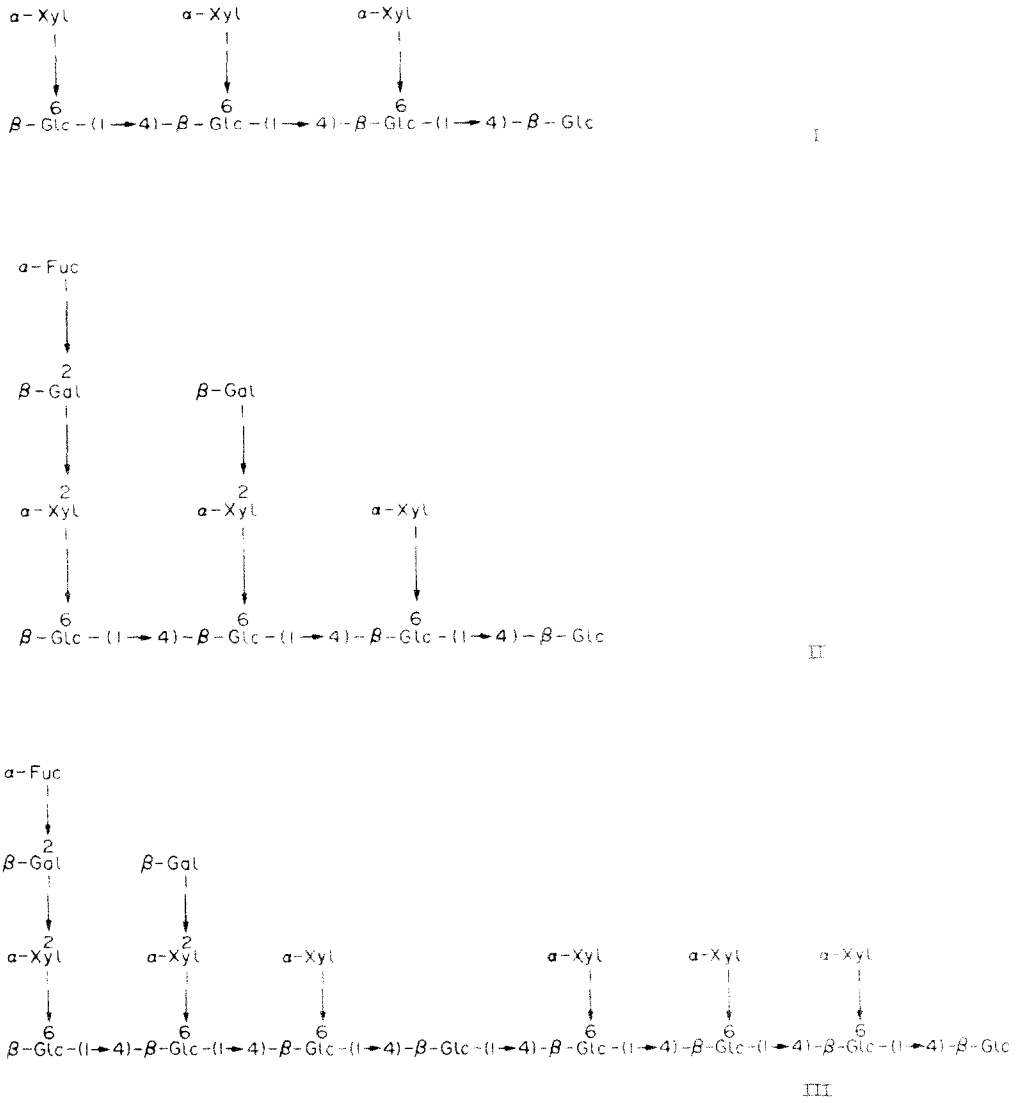

Fig. 3.

heptasaccharide and decasaccharide are (1 → 4)-linked together. Since the galactose content as determined is higher than calculated for structure III (Table 4), it is likely that a second oligosaccharide unit of a similar structure and about the same M_r is present. It can be assumed that instead of a terminal galactose and a fucosyl-(1 → 2) galactose moiety two galactosyl (1 → 2) galactose units [11] are attached to the xyloglucan skeleton. According to the ^{13}C NMR spectrum xylose and fucose must possess the α-L-, glucose and galactose the β-D-configuration. The α-configuration of fucose could be confirmed also by enzymatic hydrolysis of the corresponding oligosaccharide fractions with a α-L-fucosidase [12]. Polysaccharide E contains about 8% acetyl groups as determined by ^{13}C and ^{1}H NMR spectra. Their positions could not be elucidated.

Structure determination of polysaccharide F

Polysaccharide F contains arabinose and galactose in a molar ratio of 1:1 and about 16% galacturonic acid, as determined by GC of the alditolacetates and the carbazol test respectively [13]. According to the methylation analysis (Table 1) arabinose is found mainly as terminal sugar besides in 1 → 5 and 1,3,5-linkages in a molar ratio of 2:1. The main percentages of galactose (about 75% of the hexose content) must be linked 1,3,6. In addition 1 → 6 and 1 → 3 linked galactose occurs in a molar ratio of 2:1, whereas terminal galactose could be detected only in a low proportion. These findings were supported by a detailed analysis of the ^{13}C NMR spectrum. Two low field shifted signals at 109.5 and 107.8 ppm could be assigned to the anomeric atoms of terminal and 1 → 5 linked arabinose respectively. The C-1 signal for the 1,3,5 linked arabinose could not be detected due to its low intensity.

By comparing the chemical shifts of the anomeric C-atoms with the ^{13}C NMR data reported in the literature for methyl-α-L-arabinofuranoside [14] and arabinans [15], it can be concluded that the terminal arabinosyl residues are directly bound to the galactosyl residues. The 1 → 5 and 1,3,5 linked arabinosyl residues however might be part of an arabinan chain. Two signals at 103.4 and

Table 4. Molar sugar composition of the Bio-Gel P-2 oligosaccharide fractions

Sugar	Bio-Gel P-2 peaks		
	2	3	4
Fucose	—	1*	0.9
Xylose	2.5	2.7	5.4
Galaktose	traces	1.1	2.5
Glucose	4	4	8
Peak ratio	1	1	1.5

*Not integrated exactly.

103.7 could be assigned to the anomeric C-atoms of 1,3,6- and 1 → 3-, 1 → 6 and terminal linked pyranosidic galactose respectively. The 2:1 ratio of the two signals for the branched galactose chain and the otherwise linked galactosyl residues is in good agreement with the results of the methylation analysis.

Partial hydrolysis of the polysaccharide with 0.05 N trifluoracetic acid resulted in total removal of all arabinosyl residues. Galactose and oligosaccharides of low M_r were detectable only in low concentration. The methylation analysis of the degradation product revealed the following alteration in the molar ratio of the single components: increase of 1 → 6 linked galactose and decrease of 1,3,6-linked galactose, increase of terminal galactose and an increase of the 1 → 6 to 1 → 3 linked galactose ratio from 1:1 in native polysaccharide to 2.5:1 in the degraded polysaccharide. This result can be explained by a removal of terminal arabinose in position 0-3.

The ^{13}C NMR data of this partial hydrolysis product are shown in Table 5. The assignment of the signals was achieved by comparison with the ^{13}C NMR data of oligosaccharides published by Collins [16] and Kovac [17]. The three signals between 104.18 and 104.57 ppm could be assigned to the C-1 atoms of galactose. The broad signals at 82.66 and 82.47 ppm might be attributed to the C-3 atoms of 1 → 3 linked galactose and one signal shifted about 0.2 ppm to higher field to the same C-atom of 1,3,6-substituted galactose.

This broadening of the C-3 resonance of 3-glycosidated galactosyl residues has been observed by Karacsony [18] and is explained by a partially hindered mobility of the galactosyl residues in oligosaccharide or polysaccharide of different chain length. From this result it can be deduced that the backbone of the polysaccharide consists of 1 → 3 linked galactose, whereas the side chains contain 1 → 6 linked galactose with 1 → 3 linked terminal arabinosyl residues. To examine the bonding mode of the galacturonic acid the partially hydrolysed polysaccharide was methylated, reduced with $LiAlD_4$ and the prepared partially methylated alditolacetate investigated in the usual manner [19]. Besides the known components the presence of 1 → 2 and 1,2,4-linked rhamnose could be deduced from the alditol derivatives. Another component, identified as deuterated 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methylgalactitol showed characteristic mass fragments at *m/z* 203 and 263, indicating a dideuteration in position C-6 and suggesting the presence of a 1 → 4 linked galacturonic acid. This linkage was confirmed also by pectinase degradation [20]. Since after reduction with $LiAlD_4$ 1 → 2 and 1,2,4-linked rhamnose could be detected, it is likely that rhamnose is part of a galacturonic acid chain which can be split off only after reduction [21] of the galacturonic acid. The 1,2,4-linked rhamnose could be branching point to the arabinan or the arabinogalactan skeleton. The analysis indicates that polysaccharide F is a complex arabino-3,6-galactan composed of three different structural elements: (i) a 1 → 3 linked galactose backbone with 1 → 6 linked 7-or 8-membered galactose side chains on every second galactose unit. These side chains carry terminal arabinose in 1 → 3 linkage (structure a). (ii) A galacturonic acid- and rhamnose-containing backbone with 1 → 4 and 1 → 2 linkages respectively, which may be attached to the former skeleton via a rhamnose unit (structure b). (iii) A largely branched arabinan with 1 → 5 linkages and about one third 1 → 3 branches (structure c).

Immunological activity

Three different immunological test systems were used to determine the influence of polysaccharides on the human phagocytotic system. In the *in vitro* granulocyte test [22], which has been performed with human granulocyte fractions, polysaccharide A enhanced the phagocytosis at a concentration of 10^{-1}–10^{-2} mg/ml between 20 and 28%.

Correlated results were obtained in the *in vivo* carbon clearance test [23]. The value of RC_{tr}/Rc_c of 1.6 (DSK value 2) shows a significant increase of the serum elimination rate of carbon particles, which were i.v. administered. Polysaccharide E and polysaccharide F stimulated the phagocytosis to a smaller extent. Additionally, polysaccharide F specifically stimulated macrophages to excrete the tumour necrosis factor (TNF).

DISCUSSION

According to Albersheim [9], polysaccharides isolated from tissue cultures represent primary cell wall components. In his proposed cell wall model [24] the isolated fucogalactoxyloglucan can be incorporated next to the cellulose layer, whereas the arabinogalactan can be regarded as a bonding chain between the hemicellulose and the glycoprotein part. The neutral fucogalactoxyloglucan

Table 5. ^{13}C NMR spectral data of polysaccharide F after partial hydrolysis (G)

Sugar linkage	Chemical shifts					
	C-1	C-2	C-3	C-4	C-5	C-6
β-D (1 → 3) Galp	104.4	71.08	82.66	69.48	75.88	61.78
β-D (1 → 6) Galp	104.18	71.58	73.48	69.48	74.55	70.11
Terminal β-D Galp	104.54	71.58	73.48	69.48	75.88	61.78
β-D 1,3,6 Galp	104.18	71.08	82.47	69.48	74.55	79.11

of *Echinacea* cell culture has a different structure than that isolated from *Echinacea purpurea* plants [1], which is a xylan. Albersheim *et al.* [9, 24] reported xyloglucans as the major extracellular compounds from sycamore (*Acer pseudoplatanus*) suspension cultures, and from red kidney bean cell cultures (*Phaseolus vulgaris*). In contrast, the *Echinacea*-fucoxyloglucan shows differences in the side chain structures, as far as the galactose-(1 → 2) xylose moieties are concerned. Related polysaccharides were also isolated from soya bean [25], jojoba seeds [26], and *Phaseolus mungo* [27]. The isolated arabinogalactan (PSF) from *Echinacea* cell culture can be classified, according to Stephen [28], as an arabinogalactan of type II with a (1 → 3)-linked β-D-galactan backbone which is probably covalently connected with a rhamnogalacturonan chain and an arabinan chain. Similar highly complex polysaccharides were also isolated from *Panax ginseng* [29], *Zostera marina* [30, 31], *Hibiscus ficulneus* [32], and *Viscum album* [33]. The arabinogalactan isolated from *Echinacea* plants [34], however, shows only little structural similarity with that obtained from cell culture. It has a backbone with galactose and rhamnose residues in a ratio of 1:1 and is characterized by the presence of glucuronic acid instead of galacturonic acid.

EXPERIMENTAL

Analytical methods. Optical rotations were measured in aq. solution using a Perkin-Elmer 241 polarimeter at 365 nm at 20°, TLC: silica gel 60 F_{254} (Merck). Sugars were detected by spraying with aniline diphenylaminophosphoric acid (100°/10 min). Protein estimation was carried out according to ref. [35], the uronic acid according to ref. [13] and the anthrone test according to ref. [10]. ^{1}H NMR [Bruker WP 200 SY (50 Hz)], medium D_2O; chemical shifts are given in δ values.

GC. Perkin-Elmer 900 and Perkin-Elmer-Sigma 1 B (Argon, FID), for partially methylated alditol-acetates on UV 225-Chrompack 14024-column (25 m, 0.23 mm Ø, HD, 210° isotherm), for alditol acetates on glass column GP 3% SP-2330 on 100/120 supercort (225° isotherm 6H × 2 mm).

GC/MS: Fractorap 2110, Carlo Erba and Kratos MFC 500 for partially methylated alditol-acetates on OV-225-Chromapack 14024-capillary column (25 m, 0.23 mm Ø, 150/210°/5° min on OV-1701 quartz-capillary column (30 m × 0.25 mm Ø, 150/−260°/3° min), for permethylated alditol acetates in OV-1701 quartz-capillary column (150/260°/3° min), coupled with Varian MS CH 7/188 and Kratos MS 80 FRA. CC: Bio-Gel P-2 (100/200 mesh) (Biorad), AcA 202 Ultragel and DEAE-Trisacryl M (LKB), Sephacryl S 400 and DEAE-Sepharose CL 6 B (Pharmacia). Dextrans T 2000, T 500, T 110, T 70, T 40, T 10 (Pharmacia). The detection of fractions at gel filtration and anion-exchange chromatography was performed with a differential refractometer R 403 (Waters Assoc. Inc.), UV-detector LKB 2238 Uvicord S II (206 nm) and a polarimeter. HPLC gel permeation chromatography was accomplished by connecting two columns in series from the following set of four columns: 11-Bonda-gel columns (E 125, E 500, E 1000) and 11-Porasil GPC 60-column. The main column was equipped with a Bischoff precolumn (1%, 10 cm, packed with 1-125-protein material from Waters Assoc. Inc. Solvent for elution: 0.2 or 0.5 N phosphate buffer pH 6.0; pump: Lewa, flow rate 16 ml/hr; pressure: 5-10 bar; detector: differential refractometer R-401 and UV-detector LKB 215-Uvicord SD (206 nm); injection 5-15 μl of 1% buffer-soln. Enzymes: α-L-fucosidase from bovine epididymis (EC 3.2.1.51); polygalacturonase from *Aspergillus niger* (EC 3.2.15, Sigma); cellulase Onuzuka R-10 from *Trichoderma viride* (EC 3.2.1.4, Sigma); partially purified endo (1 → 4)-β-glucanase was isolated from cellulase Onuzuka R-10 on DEAE-Sepharose CL6B (column: A = 2 cm², l = 30 cm, solvent: 20 mM phosphate buffer, pH = 7.0; flow rate: 28 ml/hr; substance amount: 200 mg/2 ml; NaCl gradient: 0/0.5 N, 250/250 ml; the fractions at NaCl conc. *ca* 0.25 N were eluted as a symmetric peak (206 nm).

Cell cultures. The suspension cultures were prepared from leaves and stems of cultivated *Echinacea purpurea* plant material in our laboratory. The cultures were grown on Linsmaier Skoog-medium [2] with addition of 2,4-dichlorophenoxyacetic acid at 24°. After a growth period of about 14 days the cell material was filtered and the nutrient medium worked up as described below. The cell residue was lyophilized.

Isolation of crude polysaccharides. To the filtered medium (20 l) 95% EtOH (9 l/3 l medium) was added while stirring. The ppt. were kept overnight and then separated by centrifugation (10000 Upm). The combined residues were dissolved in 4 l H_2O and 15% TCA added slowly under ice cooling. After 1 hr the ppt. was centrifuged. The clear supernatant was divided into 4 portions of 2 l and each ppitd with 8 l EtOH. After 12 hr the ppt. were again centrifuged, combined and dissolved in 2 l 2% aq. NaOAc. The solute was stirred for 8 hr at 4° and then filtered. To the filtrate (1.8 l) 18 l EtOH were added, and the residue centrifuged after 12 hr. The residue was dissolved in 200 ml H_2O and then filtered. To the supernatant of the 1:1 pptn. 1.5 fold vol. EtOH were added and the ppt. kept for 48 hr. After centrifugation the residue was dissolved in 50 ml H_2O, dialysed 48 hr and then lyophilized. Yields: 1:1 ppt. *ca* 2 g (= 1%), 1:4 ppt. *ca* 1 g (= 0.5%) of the lyophilized cell material.

Isolation of neutral polysaccharide A and fraction B, C and D. The 1:1 ppt. was dissolved in H_2O (0.3 g/30 ml) and subjected to DEAE-Sepharose CL-6B-(A = 5.3 cm², l = 58 cm) chromatography. The water eluate, containing polysaccharide A with + opt. rot., was dialysed and lyophilized. By elution with 0.2 M NaCl gradient polysaccharide B with − opt. rot. (15 mg) and with 0.4 M NaCl two polysaccharide fractions with + opt. rot. (C = 15 mg, D = 63 mg) were obtained. The fractions were collected, dialysed and lyophilized. Polysaccharide fraction A was purified from acidic polysaccharides residues by chromatography on DEAE-Sepharose CL-6B and DEAE-Trisacryl M, both in the acetate form (A = 5.3 cm², l = 50 cm, 70 mg/5 ml H_2O, eluent: dist. H_2O). After dialysis and lyophilization 45 mg neutral polysaccharide was obtained. This polysaccharide was subjected to a further purification step on Sephacryl S 400 (A = 2.0 cm², l = 60 cm, eluent: dist. H_2O). The polysaccharide with + rotation (Ve 50/110 ml) was separated from polysaccharide components of low M_r (Ve 110/130 ml, no optical activity). After dialysis and lyophilization 36 mg polysaccharide was obtained (yield 0.07%/100 g lyoph. cell material).

Isolation of polysaccharides E and F from 1:4 precipitation. A water solution of 1:4 pptn (250 mg/10 ml) was subjected to DEAE-Sepharose CL 6 B (A = 5.3 cm², l = 50 cm). With H_2O polysaccharide E (+ opt. rot.), with 0.2 N NaCl soln. the acidic polysaccharide F (+ opt. rot.) were obtained. Both polysaccharides were dialysed and lyophilized to obtain 0.125 g polysaccharide E and 85 mg polysaccharide F. Purification of polysaccharide E (30 mg) on Ultrogel AcA 202 (A = 0.5 cm², l = 45 cm, eluent: 0.2 N NaCl soln) resulted in the compound in the fractions Ve = 10/20 ml. Polysaccharide F (85 mg) was purified on a second DEAE-Trisacryl column M (A = 5 cm², l = 14 cm, eluent: H_2O and then 0/1 N NaCl, 250/250). The pure compound was obtained with 0.2 M NaCl, dialysed and lyophilized (yield: 65 mg). At least both polysaccharides were subjected to a gel filtration on Sephacryl S 400 (eluent: 0.2 N NaCl soln) to yield 0.18% polysaccharide E and 0.14% polysaccharide F.

Methylation analysis. (i) The methylation analysis of polysaccharides was carried out according to the modified Hakomori method [36]. The sugar components of the methylated polysaccharides were analysed as their partially methylated alditol acetates by GC/MS and identified by comparing their retention times and mass spectra with those of authentic samples or with literature values [36]. (ii) The oligosaccharides were reduced with $NaBD_4$ in aq. solution at room temperature for 2 hr. After removal of excess $NaBD_4$ by adding HOAc, as described previously, the freeze-dried residue was once more dried for 12 hr over P_2O_5 *in vacuo*, and then subjected to GC/MS analysis.

Hydrolysis methods. (i) Complete hydrolysis of the native and methylated polysaccharides was carried out with 2 N trifluoroacetic acid at 100° for 3 hr, or at 121° for 1.5 hr [37]. The quantitative determination of the neutral sugars was performed by GC analysis of the prepared alditol acetates (int. standard = inositol) [38]. (ii) The partial hydrolysis of 90 mg polysaccharide F was carried out in 5 ml 0.05 N trifluoroacetic acid in a steam bath. After 2 hr the acid was evapd, the degradation products redissolved in H_2O for 24 hr at room temp., dialysed and the nondialysable fraction lyophilized. (iii) The permethylated polysaccharide E (7 mg) was hydrolysed in 5 ml 90% HCO_2H for 40 min at 70°. The hydrolysis product was freeze-dried and then subjected to methylation with CD_3I.

Preparation of carboxyl reduced methylated alditol acetates. 5 mg of methylated polysaccharide F was dialysed, lyophilized and dried for 24 hr over P_2O_5 at 40° in vacuum. The residue was dissolved in 3 ml dried tetrahydrofuran, 35 mg $LiAlD_4$ was added to the solution and the mixture stirred for 24 hr at room temp. After removing the excess of $LiAlD_4$ by addition of H_2O, the solution was filtered, the residue washed several times with $CHCl_3$ and the combined filtrates evapd. After hydrolysis the partially methylated alditol acetates were analysed by GC/MS.

Acetylation of polysaccharide F [36]. 5 mg polysaccharide F was dissolved in 0.4 ml formamide, and after addition of 0.2 ml pyridine and 0.15 ml Ac_2O stirred for 3 hr at room temp. After addition of 10 ml ice-cooled H_2O, the pptd acetylation product was centrifuged, the residue washed twice with 1 ml H_2O and then freeze-dried.

Acetolysis of polysaccharide E [39]. 15 mg polysaccharide E was dried for 12 hr over P_2O_5 at 40°, and then kept in Ac_2O–pyridine (1:1, 1 ml) for 12 hrs at room temp. and then heated for 8 hr at 100°. The cooled reaction mixture was evapd, the residue dissolved in a mixture of Ac_2O, HOAc and conc H_2SO_4 (1:1:1.1 ml) and kept for 12 hr at room temp. The mixture was neutralized with 5 ml pyridine and then evapd under vacuum. The viscous residue was suspended in 5 ml H_2O and the mixture extracted with 4×5 ml $CHCl_3$. The combined $CHCl_3$ phases were evapd after drying. The residue was dissolved in 1 ml MeOH after heating to 50°, 1 ml 1 M ethanolic NaOMe added and kept for 8 hr at room temp. The reaction mixture was purified on a cation exchange column [Dowex AG W-X8(H^+)], filtered and then evapd to dryness. The residue was dissoved in 1 ml H_2O, centrifuged and the supernatant freeze dried. The oligosaccharide mixture was dissolved in 5 ml H_2O and a small amount of $NaBD_4$ added until alkaline. After 2 hr the excess of $NaBD_4$ was destroyed by addition of HOAc, the solution evapd and the borate removed by distillation twice with a mix. of 3 ml MeOH and 2 ml HOAc. The reduced oligosaccharide were dissolved in H_2O, the solution freeze-dried and the residue methylated after drying 12 hr over P_2O_5 at 40° under vacuum. The methylated oligosaccharide was identified by GC/MS.

Enzymatic degradation. (i) With cellulase: 15 mg polysaccharide E were dissolved in 5 ml 0.1 M NaOAc buffer pH 4.7 and incubated with 20 mg cellulase for 60 hr at 37°. After incubation the higher M_r polysaccharide components were pptd by addition of 95% EtOH and the ppt. centrifuged. The supernatant was evapd, the residue dissolved in 2 ml dist. H_2O and chromatographed on a small cation exchange column (Dowest AG 50 W-X8(H^+), H = 0.12 cm^2, l = 6 cm). The Na^+ ion-free eluate containing mono- and oligosaccharides, was freeze dried, reduced with $NaBD_4$ and then methylated. The identification of the reduced, permethylated oligosaccharides was achieved by GC/MS. (ii) With pectinase: 1 mg polysaccharide F was dissolved in 200 μl dist. H_2O and incubated with 5 μl pectinase for 3 hr at room temp. The liberated galacturonic acid was identified by TLC. (iii) With α-L-fucosidase: 1 mg fucose containing oligosaccharide was dissolved in 100 μl 0.1 M NaOAc buffer, and after addition of 100 μl α-L-fucosidase incubated for 6 hr at 37°. α-Fucose was identified by TLC. (iv) With partially purified endo(1 → 4)-β-glucanase: 70 mg polysaccharide E were dissolved in 7 ml 50 mM NaOAc buffer pH 5.2 and incubated with 3.5 ml enzyme solution for 48 hr at 40°. After 10 min heating on the steambath the oligosaccharide mixture was lyophilized. The mixture was dissolved in 1 ml dist. H_2O and chromatographed on a Bio-Gel P-2 column($A = 2$ cm^2, $l = 90$ cm, eluant: dist. H_2O, flow rate 9 ml/hr, at a constant temp. of 50°). By using a refractometer 5 fractions were detected (Ve: 50–60 ml; Ve: 64–75 ml; Ve: 76–90 ml; Ve: 91–99 ml). The corresponding fractions were combined and freeze-dried. Some fractions were purified by gel filtration chromatography on Biogel P-2 under identical conditions.

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