ELSEVIER

Carbohydrate Research 275 (1995) 295–307

CARBOHYDRATE RESEARCH

Characterization of a monoclonal antibody that recognizes an arabinosylated (1 → 6)-β-D-galactan epitope in plant complex carbohydrates

Wolfram Steffan [a,1], Pavol Kováč [b], Peter Albersheim [a,c], Alan G. Darvill [a,c], Michael G. Hahn [a,d,*]

[a] *Complex Carbohydrate Research Center, The University of Georgia, Athens, GA 30602-4712, USA*
[b] *National Institutes of Health, NIDDK, Bethesda, MD 20892, USA*
[c] *Department of Biochemistry and Molecular Biology, The University of Georgia, Athens, GA 30602-7229, USA*
[d] *Department of Botany, The University of Georgia, Athens, GA 30602-7271, USA*

Received 12 December 1994; accepted 20 April 1995

Abstract

Monoclonal antibody CCRC-M7 is a representative of a group of antibodies with similar binding specificity that were generated using the plant cell-wall pectic polysaccharide, rhamnogalacturonan I, as immunogen. The epitope recognized by CCRC-M7 is present in several plant polysaccharides and membrane glycoproteins. Selective enzymatic or chemical removal of arabinosyl residues from rhamnogalacturonan I reduced, but did not abolish, the ability of CCRC-M7 to bind to the polysaccharide. In contrast, enzymatic removal of both arabinosyl and galactosyl residues from rhamnogalacturonan I completely abolished binding of CCRC-M7 to the resulting polysaccharide. Competitive ELISAs using chemically defined oligosaccharides to compete for the CCRC-M7 binding site showed that oligosaccharides containing (1 → 6)-linked β-D-galactosyl residues were the best competitors among those tested, with the tri-, penta-, and hexa-saccharides being equally effective. The combined results from indirect and competitive ELISAs suggest that the minimal epitope recognized by CCRC-M7 encompasses a (1 → 6)-linked β-galactan containing at least three galactosyl residues with at least one arabinosyl residue attached.

Keywords: (1 → 6)-β-D-Galactan; Arabinogalactan; Antibody; Monoclonal; Epitope

* Corresponding author.
[1] Present address: Kraft-Jacobs-Suchard, Research and Development, Banbury, OX16 7QU, UK.

SSDI 0008-6215(95)00174-3

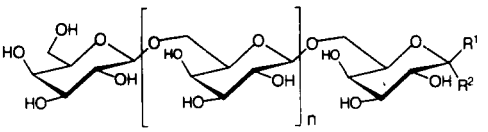
1. Introduction

Monoclonal antibodies have been of great value in studies of the function of cell-surface glycoconjugates in mammalian systems [1,2] and have recently been applied to the study of the complex carbohydrates at the plant cell surface [3,4]. The usefulness of monoclonal antibodies for these studies is significantly enhanced by characterization of the epitope recognized by the antibodies. However, knowledge of the epitopes recognized by most of the monoclonal antibodies directed against plant cell-wall complex carbohydrates is limited. Several of these monoclonal antibodies are directed against arabinogalactan and/or arabinogalactan glycoprotein epitopes [5–10]. Although six of these antibodies bind to gum arabic [7,8,10,11], the epitope structures have not been determined, limiting the usefulness of these antibodies as probes for the localization of specific complex carbohydrates. We have recently generated several monoclonal antibodies against plant cell-wall polysaccharides using, as the immunogen, the pectic polysaccharide rhamnogalacturonan I (RG-I) [12]. The RG-I used in these studies was released and isolated from suspension-cultured sycamore maple (*Acer pseudoplatanus*) cell walls. Nine of these antibodies, of which CCRC-M7 is a representative, recognize an epitope that is widely distributed among plant complex carbohydrates, including RG-I, several arabinogalactans and membrane glycoproteins [12]. In contrast to anti-arabinogalactan monoclonal antibodies that have been previously described, CCRC-M7 does not bind to gum arabic. The results presented here further define the epitope recognized by CCRC-M7.

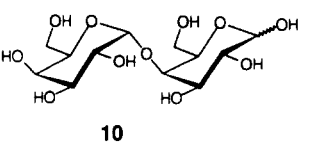
2. Experimental

Materials.—Oligo- and poly-saccharides. 6-*O*-β-D-Galactopyranosyl-D-galactose, 4-*O*-α-D-galactopyranosyl-D-galactopyranose, and exudate gums [ghatti (from *Anogeissus latifolia*), arabic (from *Acacia senegal*), tragacanth (from *Astragalus gummifer*), karaya (from *Sterculia urens*)] were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Gum mesquite (from *Prosopis juliflora*) was from stocks of the CCRC. Glycosyl-residue and -linkage compositions of the exudate gums used in this study were consistent with those reported [13]. (1 → 5)-α-L-Arabinobiose, (1 → 5)-α-L-arabinotetraose, and (1 → 4)-β-D-galactohexaose were obtained from Megazyme (Warriewood, Sydney, N.S.W., Australia). Oligosaccharides **1**, **2**, **3**, **4**, **6**, **7**, **8**, and **9** (for structures see Fig. 1) were synthesized as previously described [14–17]. An arabinogalactan preparation from purple coneflower (*Echinacea purpurea*) (E-AG) [18,19] was donated by Diversa GmbH (Hamburg, Germany). RG-I, purified as described [20,21] from the mixture of pectic polysaccharides released from the walls of suspension-cultured sycamore maple (*Acer pseudoplatanus*) cells by endopolygalacturonase, was provided by A. Whitcombe of this laboratory.

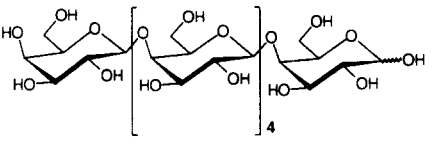
Enzymes. Endo-(1 → 5)-α-L-arabinanase (from *Aspergillus niger*; EC 3.2.1.99) and (1 → 5)-α-L-arabinofuranosidase (from *A. niger*; EC 3.2.1.55), obtained from Megazyme, were purified on a Mono-Q column as described [22]. Endo-(1 → 4)-β-D-galactanase MI (from *A. niger*; EC 3.2.1.89), purchased from Megazyme, and β-D-galactosidase (from *A. niger*; EC 3.2.1.23), purchased from Sigma, were dialyzed against 50 mM NH_4OAc, pH 4.0, and used without further purification.



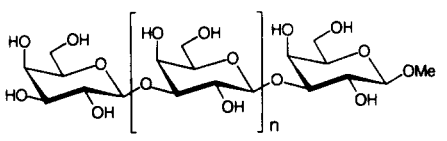
	n	R^1	R^2
1	0	OMe	H
2	1	OMe	H
3	3	OMe	H
4	4	OMe	H
5	0	H, OH	



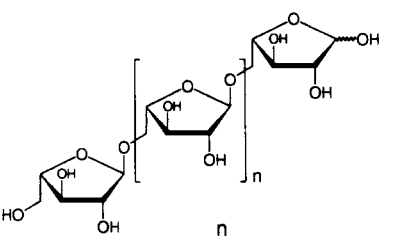
10



11



	n
6	0
7	1
8	2
9	3



	n
12	0
13	2

Fig. 1. Structures of the oligosaccharides used in competitive ELISAs. **1–5** are (1 → 6)-linked β-D-galacto-oligosaccharides, **6–9** are (1 → 3)-linked β-D-galactosides, **10** is (1 → 4)-linked α-D-galactobiose, **11** is (1 → 4)-linked β-D-galactohexaose, and **12** and **13** are (1 → 5)-linked α-L-arabino-oligosaccharides.

Antibodies. Goat anti-mouse IgG-horseradish peroxidase conjugate (GAM-HRP) was purchased from Pierce Chemical Co. (Rockford, IL, USA). The generation of the hybridoma line secreting monoclonal antibody CCRC-M7 is described elsewhere [12].

Chemicals and other supplies. Trifluoroacetic acid (TFA), anthrone, ovalbumin, and Dowex-50W were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 3,3′,5,5′-Tetramethylbenzidine (TMB) was from Pierce Chemical Co. Bio-Gel P-2 and P-10 were from BioRad Laboratories (Richmond, CA, USA). Spectra/Por dialysis membrane was from Spectrum Medical Industries, Inc. (Houston, TX, USA). Polystyrene flat bottom microtiter plates (Immunolon 2) were purchased from Dynatech (Alexandria, VA, USA).

Partial enzymatic hydrolysis of RG-I.—The arabinose and/or galactose content of RG-I was reduced by partial enzymatic hydrolysis as previously described [21] with the following modifications.

RG-I(5% Ara)$_{enz}$ [2]. RG-I (60 mg) was dissolved in 50 mM NH_4OAc, pH 4.0, (3 mL,

[2] The numbers in parentheses refer to the mol% of RG-I accounted for by arabinose and/or galactose after enzymatic or acid hydrolysis of RG-I. The subscripts refer to the method used to obtain these hydrolysis products: enz = enzymatic hydrolysis, ac = acid hydrolysis.

0.02% NaN_3) and treated, for 6 h at 40°C, with a mixture of the purified preparations of endo-(1 → 5)-α-L-arabinanase (600 μg) and (1 → 5)-α-L-arabinofuranosidase (600 μg). The reaction mixture was then neutralized with NH_4OH, dialyzed (MWCO: 3500) against water, and lyophilized. This procedure was repeated twice, yielding RG-I(5%Ara)$_{enz}$ (40 mg).

RG-I(2%Ara)$_{enz}$. RG-I (5% Ara)$_{enz}$ (20 mg) was digested again with endo-(1 → 5)-α-L-arabinanase (500 μg) and (1 → 5)-α-L-arabinofuranosidase (500 μg) as described above, except that the reaction mixture was placed inside a dialysis bag (MWCO: 12000–14000) that was then kept in 50 mM NH_4OAc, pH 4.0 (3 × 1.5 L, 0.02% NaN_3), for 7 h at 40°C. The reaction mixture was neutralized with NH_4OH and lyophilized. Gel-permeation chromatography of the residue using a Bio-Gel P-10 column (85 × 1.5 cm) eluted with water showed a single carbohydrate peak in the excluded volume (detection: anthrone assay [23]), the fractions of which were combined and lyophilized. The enzymes used in the digestions were removed by $(NH_4)_2SO_4$ (3.7 M) precipitation, leaving the polysaccharides in solution. RG-I(2%Ara)$_{enz}$ (10 mg) was obtained after dialysis against water and lyophilization.

RG-I(6% Ara, 9% Gal)$_{enz}$. A solution (4 mL) of RG-I (36 mg) in 50 mM NH_4OAc, pH 4.0, 0.02% NaN_3, was treated with a mixture of endo-(1 → 4)-β-D-galactananase, (1 → 4)-β-D-galactosidase, endo-(1 → 5)-α-L-arabinanase, and (1 → 5)-α-L-arabinofuranosidase (500 μg each) for 4 h at 30°C. The reaction mixture was neutralized with NH_4OH, dialyzed against water, and lyophilized. This procedure was repeated twice, and an additional treatment (same amount of enzymes) was carried out inside a dialysis bag for 20 h at 30°C as described above. RG-I(6%Ara, 9%Gal)$_{enz}$ (3.8 mg) was obtained after gel-permeation chromatography and $(NH_4)_2SO_4$ precipitation, which were performed as described above for RG-I(2% Ara)$_{enz}$.

Partial acid hydrolysis of RG-I.—RG-I (58 mg) was dissolved in 0.5 M TFA (4.7 mL) and kept for 23 h at 60°C. The solution was dialyzed (MWCO: 3500) against water and lyophilized to give RG-I(0.4%Ara)$_{ac}$ (28.3 mg). Another experiment using the same procedure yielded RG-I(0.09% Ara)$_{ac}$. Sugar composition analysis (Table 1) of the treated samples suggested < 2% hydrolysis of the glycosidic linkages of the sugars in RG-I except for arabinose and fucose.

Table 1
Glycosyl-residue composition analyses of RG-I and various partial hydrolysis products of RG-I obtained by treatment with enzymes or trifluoroacetic acid (TFA) (values expressed as mol%)

Polysaccharide	L-Ara	L-Rha	L-Fuc	D-GalA	D-Gal	D-Glc
RG-I	30	22	1	21	25	1
RG-I(5%Ara)$_{enz}$	5	30	2	29	32	2
RG-I(2%Ara)$_{enz}$	2	27	2	30	37	2
RG-I(6%Ara,9%Gal)$_{enz}$	6	37	4	40	9	4
RG-I(0.4%Ara)$_{ac}$	0.4	31	0	29	37	3
RG-I(0.09%Ara)$_{ac}$	0.09	31	0	29	37	3

Partial acid hydrolysis of arabinogalactan from Echinacea purpurea *(E-AG).*—A solution of E-AG (103 mg) in 50 mM TFA (2.5 mL) was kept at 95°C for 4.5 h, neutralized, and lyophilized. The residue was dissolved in a minimum of water and applied to a Bio-Gel P-2 column (1 × 100 cm), and the column was eluted with deionized water. Fractions of 1 mL were collected and analyzed for hexose content and ability to competitively inhibit binding of CCRC-M7 (see below) to immobilized RG-I. Fractions containing oligosaccharides having dp's between 2 and 15 were pooled and lyophilized, yielding 68 mg. A part of that product (21.6 mg) was futher purified by semipreparative HPAE–PAD (see below).

Semipreparative HPAE–PAD.—Semipreparative HPAE–PAD was performed with a Dionex metal-free BioLC interfaced to an Autolon Series 400 computer. Carbohydrates were separated in a CarboPac PA1 column (9 × 250 mm) and detected with a pulsed amperometric detector. Carbohydrates were eluted at 5 mL/min with NaOAc (80 mM for 5 min, 100 mM for 10 min, 150 mM for 10 min, and 600 mM for another 15 min) in 100 mM NaOH. To facilitate the detection of carbohydrates, NaOH (400 mM) was added post-column at a flow rate of 0.6 mL/min with a pressurized reagent delivery system. Eluent fractions of interest were pooled, deionized by passing through a Dowex 50 column (40 mL), and lyophilized.

Carbohydrate analysis.—Glycosyl-residue composition analysis was performed by formation of trimethylsilyl methylglycosides (TMS-MG) and analysis by GLC as described [24]. The derivatives were separated on a DB-1 column (0.25 mm × 30 m) using an HP 5890 gas chromatograph. Small quantities of arabinosyl residues (< 1 mol%) were quantified by comparison with a mixture of standard sugars containing 1 mol%, 0.1 mol%, or 0.01 mol% arabinose. Glycosyl-linkage compositions were determined by formation of the partially methylated alditol acetates (PMAAs) as described [24], except that potassium rather than sodium methanesulfinylmethanide was used. The PMAAs were separated and analyzed by GLC–MS on a DB-1 column (0.25 mm × 15 m) using an HP-5890 gas chromatograph interfaced with an HP-5970 mass-selective detector.

Electrospray mass spectrometry (ES–MS).—ES–MS was performed with a PE-Sciex API III biomolecular analyzer operating in the positive-ion mode. Solutions (100 μL) of oligosaccharides (1 mg/mL) in CH_3CN were infused into the electrospray source at 4 μL/min using a Harvard 22 syringe pump. The ionspray was operated at 5000 V with an orifice potential of 35 V. Ten scans (100–1500 amu) were collected and averaged.

Enzyme-linked immunosorbent assay (ELISA).—Indirect ELISAs, which measure binding of antibody to immobilized polysaccharides, and competitive ELISAs, which measure the ability of carbohydrates to inhibit binding of antibody to immobilized RG-I, were carried out as previously described [12] except for the following modifications. Antigens, in 20 mM sodium phosphate (Na_2HPO_4–NaH_2PO_4), pH 7.8, were immobilized to the untreated microtiter plates by drying the solutions in the wells overnight at 37°C. CCRC-M7 hybridoma culture supernatant was diluted 1:2 for indirect ELISAs and 1:16 for competitive ELISAs in 50 mM Tris–HCl, pH 7.6, containing 500 mM NaCl and 0.2% (w/v) ovalbumin. Goat anti-mouse IgG–horseradish peroxidase conjugate was used as secondary antibody and 3,3′,5,5′-tetramethylbenzidine as the peroxidase substrate.

3. Results

Interaction of monoclonal antibody CCRC-M7 with dearabinosylated / degalactosylated RG-I.—Initial characterization [12] suggested that CCRC-M7 recognizes an arabinogalactan epitope in RG-I. Arabinose and galactose are the predominant sugars of the side chains of RG-I [21,25]. In order to obtain additional evidence that CCRC-M7 binds to arabinogalactan side chains, RG-I was partially selectively hydrolyzed, enzymatically or with TFA, in order to reduce the content of arabinosyl and/or galactosyl residues, and the resulting products tested for their interaction with CCRC-M7. Five hydrolysis products were obtained, and their glycosyl residue compositions were determined (Table 1). The ability of CCRC-M7 to bind to partially dearabinosylated RG-I was reduced, but not abolished, by decreasing the arabinose content of the polysaccharides (Fig. 2); CCRC-M7 was still able to bind to RG-I preparations containing as little as 0.09% arabinose. In contrast, RG-I that contains 6% arabinosyl, but only 9% galactosyl residues, is no longer recognized by CCRC-M7. These results provide evidence that galactosyl residues are essential for CCRC-M7 binding and suggest that arabinosyl residues are part of the recognized epitope.

Interaction of CCRC-M7 with various plant gums.—Several plant exudate gums known to be rich in arabinosyl and galactosyl residues were tested for their ability to bind to CCRC-M7 in both indirect and competive ELISAs. None of the gums tested were recognized as effectively as RG-I (IC_{50} = 0.067 mg/mL). Only gum ghatti and gum mesquite showed significant reactivity with CCRC-M7. In competitive ELISAs (Fig. 3), the effectiveness of gum ghatti (IC_{50} = 0.8 mg/mL) was about 10-fold less, and that of gum mesquite (IC_{50} = 3.7 mg/mL) about 50-fold less than the effectiveness of RG-I in competing for the CCRC-M7 binding site. Gum tragacanth, gum karaya, and gum arabic showed little or no ability to compete with immobilized RG-I for the CCRC-M7 binding site.

Various attempts to generate arabinogalactan oligosaccharides for epitope identifica-

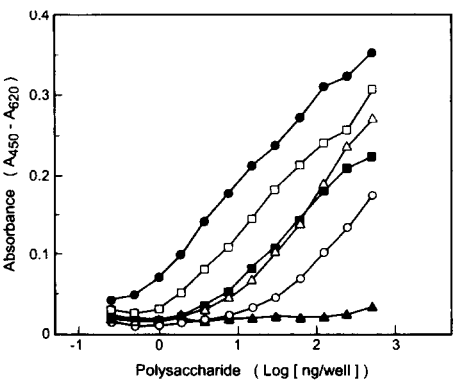


Fig. 2. Indirect ELISAs measuring binding of monoclonal antibody CCRC-M7 to sycamore maple RG-I and to the various products obtained by partial enzymatic and acid hydrolysis of RG-I. Polysaccharides tested were: RG-I (●), RG-I(5%Ara)$_{enz}$ (□), RG-I(2%Ara)$_{enz}$ (○), RG-I(0.4%Ara)$_{ac}$ (△), RG-I(0.09%Ara)$_{ac}$ (■), and RG-I(6%Ara,9%Gal)$_{enz}$ (▲). See Table 1 and Experimental for descriptions of these polysaccharides.

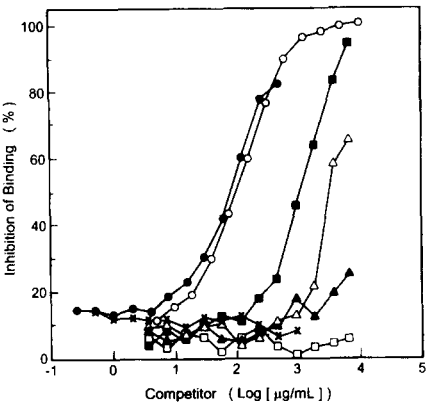


Fig. 3. Competitive inhibition of binding of monoclonal antibody CCRC-M7 to sycamore maple RG-I (0.5 μg; 2.5 pmol/well) by exudate gums. Competitors were: gum tragacanth (▲), gum arabic (□), gum mesquite (△), gum ghatti (■), and gum karaya (X). RG-I (●) and E-AG (○) were used for reference.

tion from gums reactive with CCRC-M7 failed. Neither treatment of gum ghatti with purified endo-(1 → 5)-α-L-arabinanase [22], nor fragmentation of gum mesquite by partial acid hydrolysis or treatment with lithium [25,26] yielded sufficient amounts of oligosaccharides to permit purification, characterization, and ligand competition studies.

Generation of arabinogalactan oligosaccharides by partial acid hydrolysis of an arabinogalactan from purple coneflower.—Arabinogalactan oligosaccharides derived from RG-I would be useful reagents to further characterize the ligand-binding specificity of CCRC-M7. However, attempts to obtain homogeneous arabinogalactan oligosaccharides by partial enzymatic digestion of RG-I [22] were unsuccessful. On the other hand, oligosaccharides that do interact with CCRC-M7 were obtained from a commercially available arabinogalactan (E-AG) prepared from purple coneflower (*Echinacea purpurea*). E-AG (IC_{50} = 0.1 mg/mL) was almost as effective as sycamore maple RG-I in inhibiting binding of CCRC-M7 to immobilized RG-I (Fig. 3). Thus, E-AG was used as a starting material for the generation of arabinogalactan oligosaccharides. The oligosaccharides released from E-AG by partial acid hydrolysis were separated by gel-permeation chromatography on Bio-Gel P-2. Further fractionation of the dp 2–15 oligosaccharides by semipreparative HPAE–PAD (Fig. 4) yielded many components. Six fractions were recovered in sufficient amounts for further analysis to determine their glycosyl-residue and glycosyl-linkage compositions, and in three cases, to determine the size(s) of the oligosaccharides present in the fractions (Table 2). Oligosaccharides present in each of the six fractions were competitors for the binding of CCRC-M7 to RG-I as determined by competitive ELISA (Table 2).

The results of glycosyl-residue composition analyses established that the major monosaccharide constituent present in each of these fractions was galactose and, in the case of the best competitor (B), galactose was 97% of the sugars present. Other monosaccharide constituents present were galacturonic acid, rhamnose and arabinose. A fraction with 0.1% arabinose was the most potent competitor. The least effective competitor among those tested was the fraction with the highest arabinose content (2%).

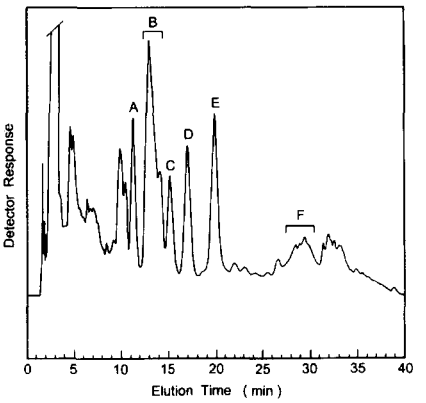


Fig. 4. HPAE–PAD profile of the semipreparative separation of the E-AG hydrolysis products. Technical details are given in Experimental.

Table 2
IC_{50} [a] values and glycosyl-residue [b] and -linkage compositions [c] of the oligosaccharides obtained by partial acid hydrolysis of coneflower arabinogalactan (E-AG)

	E-AG	A	B	C	D	E	F
~ IC_{50} (μM)	0.5	1400	18	1100	200	170,000	200
Glycosyl residues (TMS-MG)	29% Ara 1% Rha 7% GalA 63% Gal	1% Ara 99% Gal	0.1% Ara 1% Rha 97% Gal	100% Gal	n.d. [d]	2% Ara 2% Rha 16% GalA 80% Gal	2% Rha 10% GalA 88% Gal
Glycosyl linkages (PMAA)	n.d.	T-Gal*p* 6-Gal*p*	T-Gal*p* 3-Gal*p* 6-Gal*p* 3,6-Gal*p* 4,6-Gal*p* 3,4,6-Gal*p*	T-Gal*p* 6-Gal*p*	T-Gal*p* 6-Gal*p*	T-Gal*p* 3-Gal*p* 6-Gal*p* 4,6-Gal*p*	T-Gal*p* 3-Gal*p* 6-Gal*p* 3,6-Gal*p* 2,3,4,6-Hex
ES-MS	n.d.	n.d.	$(Hex)_6$ $(Hex)_5$ $(Hex)_4$	$(Hex)_4$	n.d.	n.d.	$(Hex)_6$ $(Hex)_5$ $(Hex)_4$

[a] The IC_{50} is defined as the concentration of a saccharide required to give 50% inhibition of the binding of monoclonal antibody CCRC-M7 to immobilized sycamore maple RG-I. A molecular weight estimate of 900 was used for the calculation of the IC_{50} values for the oligosaccharides. The IC_{50} value for E-AG was calculated using a molecular weight estimate of 100,000.

[b] Glycosyl-residue compositions were determined by GLC analysis of the per-*O*-trimethylsilyl methyl glycosides (TMS-MG).

[c] Glycosyl-linkage compositions were determined by GLC–MS analysis of the partially methylated alditol acetates (PMAA); data were not quantified. Galactosyluronic acid linkages were not determined.

[d] Not determined.

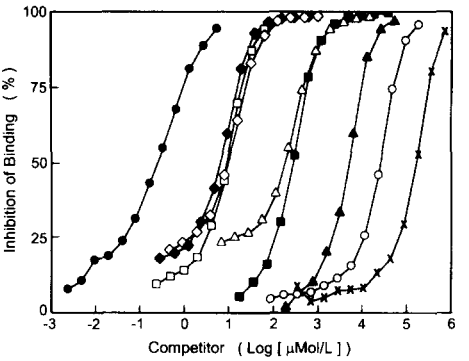


Fig. 5. Competitive inhibition of binding of monoclonal antibody CCRC-M7 to sycamore maple RG-I (0.5 µg; 2.5 pmol/well) by various oligosaccharides in ELISAs. Oligosaccharide competitors were: **1** (△), **2** (◇), **3** (◆), **4** (□), **11** (▲), **5** (■), **13** (○), and **12** (×); structures shown in Fig. 1. RG-I (●) was used for reference.

The only homogeneous fraction (C) apparently contained exclusively 6-linked galactotetraose (Table 2). These data provide further support that galactose is an essential part of the epitope recognized by CCRC-M7.

Reaction of CCRC-M7 with selected oligosaccharides.—Commercially available oligosaccharides containing galactosyl or arabinosyl residues (**5**, **10**, **11**, **12**, and **13**), as well as chemically synthesized methyl β-glycosides of (1 → 6)-and (1 → 3)-linked β-D-oligogalactosides (**1**, **2**, **3**, **4**, **6**, **7**, **8**, and **9**), were assayed for their ability to compete for the binding of monoclonal antibody CCRC-M7 to RG-I (for oligosaccharide structures see Fig. 1). The result of competitive ELISAs for those oligosaccharides that were effective competitors of RG-I are shown in Fig. 5. The IC_{50} values of all oligosaccharides tested are listed in Table 3. The most effective competitors were the (1 → 6)-linked β-D-galactosides **2**, **3**, and **4**, which were equally potent, followed by the (1 → 6)-linked β-D-galactobioside **1** and -biose **5**, which were about 25 times less effective than the larger oligosaccharides. All β-(1 → 3)-linked galactosides (**6**, **7**, **8** and **9**) tested, as well as the (1 → 4)-linked α-D-galactobiose (**10**), showed no inhibitory effect at concentrations $< 10^3$ µM. However, competition was observed with (1 → 5)-α-L-arabinobiose (**12**) and (1 → 5)-α-L-arabinotetraose (**13**) at concentrations between 10^4 and 10^6 µM, making these two oligosaccharides at least 1000 times less potent as competitors than the oligogalactosides **2**, **3**, and **4**.

4. Discussion

The results presented here indicate that the epitope recognized by the murine monoclonal antibody CCRC-M7 consists principally of a (1 → 6)-linked β-D-galactan containing at least three galactosyl residues with one or more arabinosyl residues attached, probably at O-3 of the galactosyl residues (Fig. 6). The evidence supporting this conclusion is summarized as follows.

Several lines of evidence indicate that at least three (1 → 6)-linked β-D-galactosyl

Table 3
The abilities of various oligosaccharides to inhibit the binding of CCRC-M7 to sycamore maple RG-I

Competitor	IC_{50} (μM) [a]
Sycamore maple RG-I	0.22
(1 → 6)-β-D-Galactopentaoside (**3**) [b]	6.0
(1 → 6)-β-D-Galactohexaoside (**4**)	8.2
(1 → 6)-β-D-Galactotrioside (**2**)	8.5
(1 → 6)-β-D-Galactobioside (**1**)	160
(1 → 6)-β-D-Galactobiose (**5**)	260
(1 → 4)-β-D-Galactohexaose (**11**)	4200
(1 → 5)-α-L-Arabinotetraose (**13**)	23 000
(1 → 5)-α-L-Arabinobiose (**12**)	150 000
(1 → 3)-β-D-Galactotrioside (**7**)	> 9700
(1 → 3)-β-D-Galactotetraoside (**8**)	> 15 000
(1 → 3)-β-D-Galactopentaoside (**9**)	> 24 000
(1 → 3)-β-D-Galactobioside (**6**)	> 28 000
(1 → 4)-α-D-Galactobiose (**10**)	> 37 000

[a] The IC_{50} is defined as the concentration of the saccharide required to give 50% inhibition of the binding of monoclonal antibody CCRC-M7 to immobilized sycamore maple RG-I (see Fig. 5).
[b] The numbers refer to the oligosaccharides whose structures are given in Fig. 1.

residues constitute an essential structural feature of the epitope recognized by CCRC-M7. First, significant reduction in the galactosyl content of RG-I [RG-I(6%Ara, 9%Gal)$_{enz}$] resulted in the complete loss of the ability of CCRC-M7 to bind the polysaccharide, while reduction of the arabinosyl content alone reduced but did not abolish binding (Fig. 2). Secondly, competitive ELISAs using various arabinogalactan-containing exudate gums showed that gum ghatti, known to be rich in (1 → 6)-linked β-D-galactosyl units

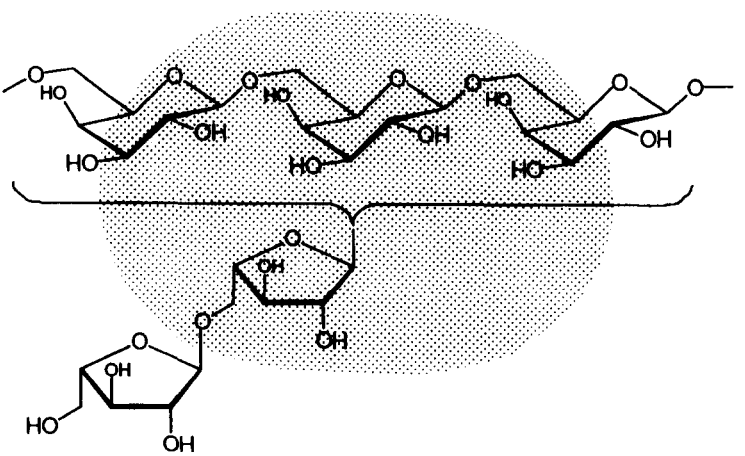


Fig. 6. Diagram of the proposed structure of the carbohydrate epitope recognized by monoclonal antibody CCRC-M7. The number of arabinosyl residues and the site of their attachment to the galactan backbone are not yet known.

[27], is recognized by CCRC-M7. In contrast, CCRC-M7 did not react with gum arabic known to contain (1 → 3)-linked β-D-galactosyl residues as the predominant type of linkage [28,29]. Finally, competitive ELISAs carried out with either arabinose-poor oligosaccharides prepared from coneflower arabinogalactan (Table 2) or with chemically synthesized oligogalactosides (Fig. 5, Table 3) demonstrated that (1 → 6)-linked β-D-galactosides of dp ≥ 3 were the the most effective competitors tested, having an IC_{50} only 10-fold lower than that of intact RG-I. The equal effectiveness in the competitive ELISAs of the trimer, pentamer and hexamer suggests that three galactosyl residues are required for maximum affinity to the CCRC-M7 binding site.

The results of the immunoassays indicate that arabinosyl residues constitute a part of the epitope recognized by CCRC-M7. Progressive reduction in the arabinosyl content of RG-I resulted in a corresponding reduction in the ability of CCRC-M7 to bind to the treated RG-I (Fig. 2). In addition, (1 → 5)-linked α-L-arabino-tetraose and -biose were able to competitively inhibit binding of CCRC-M7 to RG-I, albeit at concentrations > 1000-fold higher than the oligogalactosides (Fig. 5, Table 3). However, the number of arabinosyl residues in the epitope and the site of their attachment to the galactan backbone remain to be established. The ability of CCRC-M7 to bind to gum ghatti, E-AG, and RG-I, in which α-L-arabinofuranosyl residues are found linked only to O-3 of galactosyl residues [19,25–27], suggests that the arabinosyl residues present in the epitope will be linked to this position.

The epitope recognized by CCRC-M7 is different from the galactan epitopes recognized by other mono- and poly-clonal antibodies available. Two other antibodies have been generated that recognize linear, unsubstituted (1 → 6)-linked β-D-galactans. One such antibody is an IgA that was recovered from a mouse myeloma line (J539) and recognizes a (1 → 6)-linked β-D-galactan from the unicellular alga, *Prototheca zopfii* [30]; the epitope encompasses four galactosyl residues [31]. The other antigalactan antibody preparation is a polyclonal rabbit antiserum that was generated against (1 → 6)-linked β-D-galactotetraose coupled to bovine serum albumin [32]. The reactivity of the latter antiserum with radish arabinogalactan glycoproteins increased with enzymatic removal of arabinosyl residues. The fact that the reactivity of CCRC-M7 with RG-I decreased upon removal of arabinosyl residues (Fig. 2) indicates that CCRC-M7 recognizes a different epitope than do the anti-galactan antibodies mentioned above.

CCRC-M7 also recognizes a different arabinogalactan epitope than anti-arabinogalactan antibodies generated by other laboratories. Several such antibodies (e.g., MAC 207 [7,33], PN 16.4B4 [6,34], JIM 4 [8,9], JIM 8 [9,10,35], JIM 13, JIM 15, JIM 16 [9,11]) have been shown to bind to gum arabic, an arabinogalactan not recognized by CCRC-M7 (Fig. 3). However, in contrast to the extent of epitope characterization described here for CCRC-M7, the epitope(s) recognized by the gum arabic-reactive antibodies have not been characterized in detail [7,10]. Very interesting spatial and developmental patterns of arabinogalactan epitope localizations have been observed in different plant tissues using several of the anti-arabinogalactan antibodies [8–10,35,36]. Interpretation of these localization patterns at the molecular level is hampered by the lack of information about the epitopes recognized by the antibodies used in these studies. The type of epitope characterization reported here for CCRC-M7, and previously for CCRC-M1 [12], will enhance the value of these antibodies for studies of the

structure and function of the plant extracellular matrix. To this end, these and other antibodies are being used to localize complex carbohydrate epitopes in *Arabidopsis* roots at various developmental stages (manuscript in preparation).

Acknowledgements

This research was supported by a grant from the US Department of Energy (DE-FG05-93ER20115) to A.D. Also supported in part by the US Department of Energy-funded Center for Plant and Microbial Complex Carbohydrates (DE-FG05-93ER20097). The authors thank M.A. O'Neill for assistance in performing HPAE–PAD and ES-MS, and C.L. Gubbins Hahn for preparation of the figures. W.S. was a recipient of a postdoctoral fellowship from the Deutsche Forschungsgemeinschaft (Bonn, Germany).

References

[1] S.-I. Hakomori, in K.H. Kennett, K.B. Bechtol, and T.J. McKearn (Eds.), *Monoclonal Antibodies and Functional Cell Lines*, Plenum Press, Inc. New York, 1984, pp 67–100.
[2] T. Feizi and R.A. Childs, *Biochem. J.*, 245 (1987) 1–11.
[3] T. Hoson, *Int. Rev. Cytol.*, 130 (1991) 233–268.
[4] J.P. Knox, *Protoplasma*, 167 (1992) 1–9.
[5] M.A. Anderson, M.S. Sandrin, and A.E. Clarke, *Plant Physiol.*, 75 (1984) 1013–1016.
[6] P.M. Norman, P. Kjellbom, D.J. Bradley, M.G. Hahn, and C.J. Lamb, *Planta*, 181 (1990) 365–373.
[7] R.I. Pennell, J.P. Knox, G.N. Scofield, R. Selvendran, and K. Roberts, *J. Cell Biol.*, 108 (1989) 1967–1977.
[8] J.P. Knox, S. Day, and K. Roberts, *Development*, 106 (1989) 47–56.
[9] J.P. Knox, P.J. Linstead, J. Peart, C. Cooper, and K. Roberts, *Plant J.*, 1 (1991) 317–326.
[10] R.I. Pennell, L. Janniche, P. Kjellbom, G.N. Scofield, J.M. Peart, and K. Roberts, *Plant Cell*, 3 (1991) 1317–1326.
[11] M.E. Osman, A.R. Menzies, P.A. Williams, G.O. Phillips, and T.C. Baldwin, *Carbohydr. Res.*, 246 (1993) 303–318.
[12] J. Puhlmann, E. Bucheli, M.J. Swain, N. Dunning, P. Albersheim, A.G. Darvill, and M.G. Hahn, *Plant Physiol.*, 104 (1994) 699–710.
[13] A.M. Stephen, S.C. Churms, and D.C. Vogt, in P.M. Dey (Ed.), *Methods in Plant Biochemistry*, Vol. 2, *Carbohydrates*, Academic Press, London, 1990, pp 483–522.
[14] P. Kováč, E.A. Sokoloski, and C.P.J. Glaudemans, *Carbohydr. Res.*, 128 (1984) 101–109.
[15] P. Kováč, *Carbohydr. Res.*, 153 (1986) 237–251.
[16] P. Kováč, C.P.J. Glaudemans, and R.B. Taylor, *Carbohydr. Res.*, 142 (1985) 158–164.
[17] P. Kováč, R.B. Taylor, and C.P.J. Glaudemans, *J. Org. Chem.*, 50 (1985) 5323–5333.
[18] H. Stuppner, Ph.D. dissertation, Ludwig-Maximilians-Universität, München, 1985.
[19] H. Wagner, H. Stuppner, W. Schäfer, and M. Zenk, *Phytochemistry*, 27 (1988) 119–126.
[20] J.M. Lau, M. McNeil, A.G. Darvill, and P. Albersheim, *Carbohydr. Res.*, 137 (1985) 111–125.
[21] P. Lerouge, M.A. O'Neill, A.G. Darvill, and P. Albersheim, *Carbohydr. Res.*, 243 (1993) 359–371.
[22] P. Lerouge, M.A. O'Neill, A.G. Darvill, and P. Albersheim, *Carbohydr. Res.*, 243 (1993) 373–378.
[23] Z. Dische, *Methods Carbohydr. Chem.*, 1 (1962) 478–481.
[24] W.S. York, A.G. Darvill, M. McNeil, T.T. Stevenson, and P. Albersheim, *Methods Enzymol.*, 118 (1985) 3–40.
[25] J.M. Lau, M. McNeil, A.G. Darvill, and P. Albersheim, *Carbohydr. Res.*, 168 (1987) 245–274.

[26] J.M. Lau, M. McNeil, A.G. Darvill, and P. Albersheim, *Carbohydr. Res.*, 168 (1987) 219–243.
[27] A.M. Stephen, in G.O. Aspinall (Ed.), *The Polysaccharides*, Vol. 2, Academic Press, New York, 1983, pp 97–193.
[28] S.C. Churms, E.H. Merrifield, and A.M. Stephen, *Carbohydr. Res.*, 123 (1983) 267–279.
[29] J. Defaye and E. Wong, *Carbohydr. Res.*, 150 (1986) 221–231.
[30] A. Roy, B.N. Manjula, and C.P.J. Glaudemans, *Mol. Immunol.*, 18 (1981) 79–84.
[31] C.P.J. Glaudemans, P. Kováč, and K. Rasmussen, *Biochemistry*, 23 (1984) 6732–6736.
[32] S. Kikuchi, A. Ohinata, Y. Tsumuraya, Y. Hashimoto, Y. Kaneko, and H. Matsushima, *Planta*, 190 (1993) 525–535.
[33] D.J. Bradley, E.A. Wood, A.P. Larkins, G. Galfre, G.W. Butcher, and N.J. Brewin, *Planta*, 173 (1988) 149–160.
[34] P.M. Norman, V.P.M. Wingate, M.S. Fitter, and C.J. Lamb, *Planta*, 167 (1986) 452–459.
[35] R.I. Pennell, L. Janniche, G.N. Scofield, H. Booij, S.C. de Vries, and K. Roberts, *J. Cell Biol.*, 119 (1992) 1371–1380.
[36] N.J. Stacey, K. Roberts, and J.P. Knox, *Planta*, 180 (1990) 285–292.