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Larch arabinogalactan for hepatic drug delivery: isolation and characterization of a 9 kDa arabinogalactan fragment ☆

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Abstract

Purified arabinogalactan [AG(37 kDa)] from *Larix occidentalis* is composed of repeating units of similar molecular weight and composition. A 9 kDa arabinogalactan [AG(9 kDa)] has been obtained in high yield from AG(37 kDa) either by autoclaving at 121°C or by exposure to alkaline solution in the presence of sodium borohydride. The weight average molecular weight of AG(37 kDa) was determined to be 37 and 38 kDa by intensity light scattering and sedimentation equilibrium, respectively. The weight average molecular weight of AG(9 kDa) was determined to be 9.1 and 9.5 kDa by intensity light scattering and sedimentation equilibrium, respectively. MALDI-TOF mass spectrometry yielded a molecular weight at the peak of the distribution of 8.3 kDa for AG(9 kDa). Both AG(37 kDa) and AG(9 kDa) exhibited narrow molecular-weight distributions ($M_w/M_n \sim 1.2$). AG(37 kDa) and AG(9 kDa) exhibit nearly identical ^{13}C-NMR spectra, monosaccharide composition, and sugar linkages. It is proposed that AG(37 kDa) is composed of covalently bound subunits of AG(9 kDa). AG(37 kDa) and AG(9 kDa) bind isolated hepatocyte asialoglycoprotein receptor equally well. As a result AG(9 kDa) is a candidate for use in hepatocyte directed drug delivery and may be more desirable for such use than is AG(37 kDa).

Keywords: Arabinogalactan; Hepatic drug delivery

☆ See ref. [1] for the previous report in this series.

Abbreviations: AG(37 kDa), purified arabinogalactan; AG(9 kDa), 9 kDa fragment of arabinogalactan; GFC/LLS, gel filtration chromatography-laser light scattering; MWCO, molecular weight cutoff; kDa, kiloDaltons; MALDI-TOF, matrix-assisted, laser-desorption ionization-time of flight; PB-1, 20 mM potassium phosphate, 150 mM sodium chloride, 0.1% sodium azide (pH 7.2); PB-2, 20 mM potassium phosphate, 0.25% sodium chloride, 0.1% sodium azide (pH 7.2).

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1. Introduction

Purified arabinogalactan from *Larix occidentalis* (larch) is tightly bound in vitro and in vivo by rat liver asialoglycoprotein receptor. In vivo larch arabinogalactan is rapidly and specifically internalized within hepatocytes by receptor-mediated endocytosis. As a consequence of these properties Groman et al. [1] proposed that arabinogalactan can serve as a carrier for the delivery of drugs to the liver. Recently, Enriquez et al. [2] reported that larch arabinogalactan conjugated with the antiviral agent vidarabine monophosphate was effective in suppressing serum viral DNA titers in woodchucks infected with woodchuck hepatitis virus.

We have reported that purified arabinogalactan is a reasonably homogenous polysaccharide with a weight-average molecular weight of 40 kDa [1]. [1] In contrast to our observations, larch arabinogalactan has often been reported to consist of two components of different molecular weights—one component of high molecular weight (37–100 kDa) and a second one of lower molecular weight (7.5–18 kDa) [3].

Structural studies on larch arabinogalactan using Smith degradation led to the proposal by Churms et al. that larch arabinogalactan consists of regularly separated units containing a backbone core of ~ 12 monosaccharides [4]. The presence of such repeating units (subunits) could explain the high and low molecular-weight forms of arabinogalactan if either the forces that hold the subunits together are non-covalent or the subunits are joined covalently but hydrolyze during arabinogalactan isolation.

In this paper we report two processes for obtaining a 9 kDa arabinogalactan fragment in high yield by treatment with 1 M sodium hydroxide or by heating at pH 7. Our results are surprising in that at both of these conditions normal glycosidic bonds are stable. Our results support Churms' proposal that arabinogalactan consists of subunits of similar molecular weight and composition.

2. Materials and methods

All materials were obtained from common commercial sources unless stated otherwise. Gum arabic was obtained from Sigma Chemical Co (Catalog # G-9752, lot #61H0824).

Purified arabinogalactan.—Purified arabinogalactan was prepared as previously described [1]. Crude arabinogalactan (Stractan 2, Champion Corp., Tacoma, WA, 100 g) was dissolved in 2 L of water and purified by ultrafiltration with 100 and 10 kDa membranes. The concentrated solution was filtered through a 0.2-micron filter and freeze-dried to yield 85 g of purified arabinogalactan [AG(37 kDa)].

Preparation of the 9 kDa arabinogalactan fragment [AG(9 kDa)].—Method 1. In a typical preparation AG(37 kDa) (5 g) was dissolved in 25 mL of water and equilibrated

[1] Groman et al. [1] reported that purified arabinogalactan has a weight-average molecular weight (M_w) of 40 kDa. In this paper we revise the weight-average molecular weight estimate to 37 kDa, based upon additional data and improved calibration of our light-scattering method. We refer to this form of arabinogalactan as AG(37 kDa). The purified arabinogalactan described in both papers is the same.

to 37°C. A second solution of 2 M NaOH (25 mL) containing 0.1 M $NaBH_4$ was prepared and equilibrated to 37°C. The reaction was started by the addition of the NaOH solution to the AG(37 kDa) solution. Following this addition, solid $NaBH_4$ was added to bring the final concentration of $NaBH_4$ to 0.1 M. [2] The reaction was allowed to proceed for 1 h and was then terminated by neutralization with 6 M HCl to pH 7–8. The solution was diluted to 200 mL with water and subjected to five ultrafiltration cycles (YM3 membrane, 3 kDa MWCO, Amicon, Beverly, MA). The ultrafiltration cycle consisted of bringing the volume of the ultrafiltrate to 200 mL using water as diluent and reducing the volume to 20 mL. The sample [consisting of a mixture of AG(9 kDa) and AG(37 kDa)] was next purified by passage through an ultrafiltration membrane (YM10 membrane, 10 kDa MWCO, Amicon) to remove higher molecular-weight components. The filtrate was freeze-dried and the final product weighed. The isolated powder was denoted AG(9 kDa).

Method 2. AG(37 kDa) was treated as described in Method 1 except that the reaction product was purified by exhaustive dialysis (Spectropor 3, 3.5 kDa MWCO) against distilled water. The retained fraction was freeze-dried and weighed.

Method 3. AG(37 kDa) (4 g) was dissolved in 25 mL of water and equilibrated at 37°C. A second solution of 2 M NaOH (25 mL) containing 0.1 M $NaBH_4$ was prepared and equilibrated to 37°C. The reaction was started by the addition of the NaOH solution to the AG(37 kDa) solution. Following this addition, solid $NaBH_4$ was added to bring the final concentration of $NaBH_4$ to 0.1 M. The reaction was allowed to proceed for 1 h and was terminated by neutralization with 6 M HCl to pH 7–8. The reaction solution was diluted to a volume of 200 mL with water and ultrafiltered against a 3 kDa MWCO membrane (YM3) to a volume of 20 mL. The cycle was repeated five times. The *retentate* was freeze-dried and weighed. The isolated powder was denoted AG(9 kDa). The yield of the isolated AG(9 kDa) was between 85 and 90% of the AG(37 kDa).

Method 4. Arabinogalactan (20 mg/mL) was dissolved in water, sealed in a 10 mL vial, and autoclaved (121°C) for 0.5 or 6 h.

Molecular-weight determinations by light scattering.—Solutions of AG(37 kDa) and the reaction products obtained from Methods 1, 2, 3, and 4 were dissolved in buffer PB-1 (20 mM potassium phosphate, 150 mM NaCl, 0.1% NaN_3, pH 7.2) to give concentrations between 5 and 30 mg/mL. The samples were introduced to a gel-filtration column (Ultrahydragel 250, Waters) using an automated sample injector with a 20 μL injection loop. In the flowing system the sample passed through a differential refractometer (Waters, model 410) and a flow-cell light-scattering detector (Precision Detectors, Amherst, MA) temperature equilibrated to 35°C. The molecular weight distributions of the samples were determined with software supplied by Precision Detectors using the measured refractive indices and light scattering intensities. The molecular weight was expressed as a weight-averaged value (M_w), a number-averaged value (M_n), and a molecular weight at the peak of the refractive index curve (M_p). For a

[2] The same concentration of $NaBH_4$ (0.1 M) was used in all reactions described in this paper for the preparation of AG(9 kDa) by Method 1, Method 2, and Method 3.

discussion of M_w, M_n, and M_p see Cowie [5]. The instrument was calibrated at the beginning and end of each run with bovine serum albumin (66 kDa) (Sigma Chemical Company, Catalog # A-8022).

Molecular-weight determinations by sedimentation equilibrium.—The weight averaged and *z*-averaged molecular weights of AG(37 kDa) and AG(9 kDa) were determined with an analytical ultracentrifuge (Beckman, model E) under equilibrium conditions [6]. The samples were dissolved in buffer PB-2 (20 mM potassium phosphate, 0.25% NaCl, 0.1% NaN_3, pH 7.2) to concentrations on the order of 15 mg/mL. The samples were exhaustively dialyzed in Spectrapor 3 dialysis tubing against 0.22-μm filtered PB-2. The ultracentrifuge was equipped with a laser light source and a video-based Rayleigh optical system for determining the refractive index as a function of radial position in the cell. The molecular weights were determined from the concentration distribution in the sample cell.

Molecular weight determinations by mass spectroscopy.—Matrix-assisted-laser desorption-ionization/time-of-flight (MALDI-TOF) mass spectra were measured using a VG TofSpec mass spectrometer (VG Instruments, Danvers, MA) equipped with a 337 nm nitrogen laser. Mass spectral data were stored and analyzed using a VAX 4000 data station. Broad peaks were observed for AG(9 kDa) using a matrix of *trans*-3-indoleacrylic acid in 50 mM sodium chloride. No peaks were observed with AG(37 kDa). Baseline correction of the MALDI-TOF mass spectra of AG(9 kDa) were performed using Kaleidagraph (Macintosh format; Synergy Software) and Peakfit (MS-DOS format; Jandel Scientific) software. The baselines were fitted to the equation $y = ax^b$, where a and b are empirically determined constants.

Monosaccharide composition.—Monosaccharide composition was determined at the University of Georgia Center for Carbohydrate Research (Athens, GA) using the alditol acetate method as previously described by Groman et al. and the references cited therein [1]. Quantitative analysis of the composition of the samples was determined by GC/MS with *myo*-inositol as an internal standard.

Linkage analysis.—Linkage analysis was performed at the University of Georgia Center for Carbohydrate Research (Athens, GA) using a modification of York [7] described in detail in Groman et al. [1].

^{13}C-NMR spectroscopy.—Fourier transform ^{13}C-[^{1}H]-NMR (75 MHz) spectra were obtained for samples in D_2O. Digital resolution was 0.50 Hz (0.909 s acquisition time with 1 Hz digital line broadening). Sample temperatures were maintained within 19–22°C ($\pm$0.3°C during a single spectral acquisition). Chemical shifts were referenced to internal 1% DSS (sodium 4,4-dimethyl-4-silapentanesulfonate).

Aldehyde measurement.—Relative aldehyde contents of AG(37 kDa) and AG(9 kDa) were quantitated by the production of a colored soluble formazan salt from the dye Tetrazolium Blue as described by White and Kennedy [8] and were expressed as the ratio obtained from a comparison of test samples with a standard preparation of AG(37 kDa).

Receptor assay.—Asialoglycoprotein receptor from rat liver was isolated following the method of Hudgin et al. [9]. The assay results are reported as the concentration of AG(37 kDa) and AG(9 kDa) necessary to inhibit 50% of binding of ^{125}I-labeled arabinogalactan (IC_{50}); the assay was performed as previously described [1].

3. Results

Effect of alkali on purified arabinogalactan [AG(37 kDa)].—Fig. 1 shows the molecular-weight distributions of crude arabinogalactan, AG(37 kDa), and AG(9 kDa) obtained by Methods 1 and 3. Crude arabinogalactan presented a single, nonsymmetrical peak, with low molecular-weight contaminants. Purification of crude arabinogalactan by ultrafiltration (10 kDa MWCO membrane) eliminated low molecular-weight impurities and resulted in a more symmetrical peak. The molecular-weight distribution for both crude arabinogalactan and AG(37 kDa) was unimodal with no indication of a 9 kDa peak. The molecular weights at the peaks of crude arabinogalactan and AG(37 kDa) were 35.1 and 35.9 kDa, respectively. AG(9 kDa) obtained by Method 1 using ultrafiltration purification to remove high molecular-weight impurities or obtained by Method 3 directly showed nearly identical molecular-weight distributions and both preparations were free of AG(37 kDa) contamination. The molecular weights at the peaks for AG(9 kDa) prepared by Method 1 and Method 3 were 8.1 and 8.4 kDa, respectively. AG(37 kDa) and AG(9 kDa) (obtained by Method 1 or Method 3) had a high degree of molecular-weight homogeneity with values of M_w/M_n of 1.17, 1.08, and 1.15, respectively.

The observation that AG(37 kDa) broke down into a 9 kDa fragment in the presence of 1 M sodium hydroxide led to an investigation of reaction conditions that might produce this fragment with the aim of defining conditions that could quantitatively convert AG(37 kDa) to AG(9 kDa). The effects of temperature, incubation time, and base concentration on the conversion of AG(37 kDa) to AG(9 kDa) are illustrated in

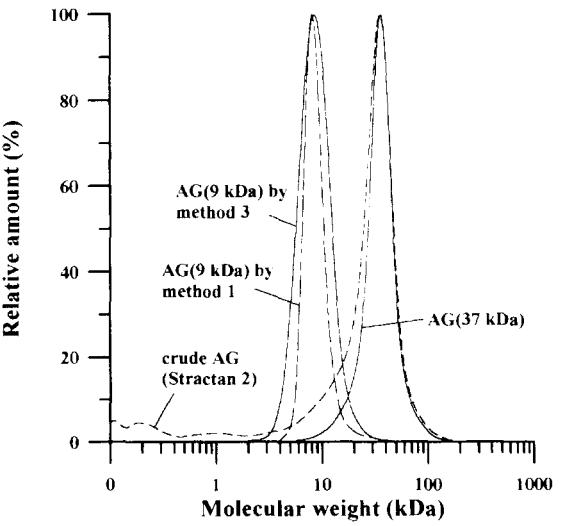

Fig. 1. Molecular-weight distributions for Stractan 2, AG(37 kDa), and AG(9 kDa) prepared by Methods 1 and 3. The molecular weights at the maxima of the distributions are 35.0, 35.9, and 8.1, and 8.4 kDa for the four respective preparations.

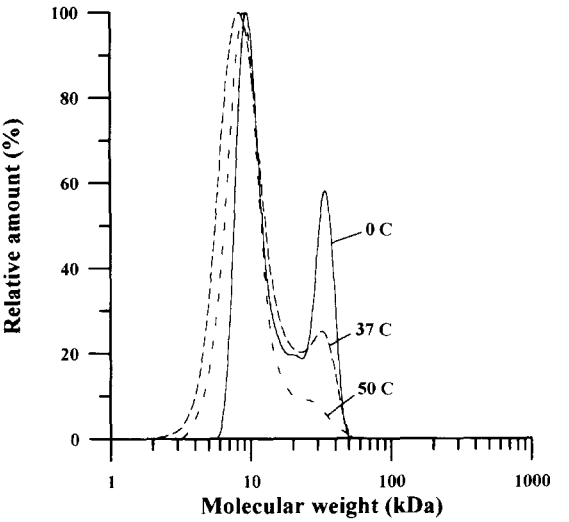

Fig. 2. Molecular-weight distributions for arabinogalactan (AG(37 kDa), 100 mg/mL) subjected to 1 M sodium hydroxide/0.1 M sodium borohydride for 1 h at 0, 37, or 50°C and isolated as described in Method 2. The molecular weights at the maxima of the distributions are 9.3, 9.2, and 8.9 kDa for the reactions at 0, 37, and 50°C, respectively.

Figs. 2–4, respectively. Conversion of AG(37 kDa) to AG(9 kDa) in the presence of 0.1 M sodium borohydride showed a temperature dependence between 0 and 50°C. At 50°C the conversion to AG(9 kDa) was nearly complete after 1 h in 1 M sodium hydroxide (Fig. 2). Fig. 3 shows the progressive conversion to AG(9 kDa) with time at 37°C when AG(37 kDa) was incubated in 0.5 M sodium hydroxide/0.1 M sodium borohydride. Fig. 4 shows the result of treating AG(37 kDa) in the presence of 0.1 M sodium borohydride with 0.5 M and 0.1 M sodium hydroxide for 1 h at 50°C. Conversion to the 9 kDa fragment occurred at base concentrations as low as 0.5 M but did not occur at 0.1 M sodium hydroxide within 1 h. The reactions represented by Figs. 2–4 were conducted at an arabinogalactan concentration of 100 mg/mL, the same concentration used in Methods 1 and 2.

Conversion to the 9 kDa arabinogalactan occurred at pH 7 at sufficiently high temperatures (Method 4, Fig. 5). After 6 h at 121°C approximately 70% of AG(37 kDa) was converted to AG(9 kDa). Sodium borohydride was not included in this experiment.

None of the conditions above using an AG(37 kDa) concentration of 100 mg/mL effected the complete conversion of AG(37 kDa) to AG(9 kDa). When the concentration of AG(37 kDa) was lowered to 80 mg/mL in 1 M sodium hydroxide/0.1 M sodium borohydride and incubated at 37°C for 1 h, the AG(9 kDa) preparation showed no residual AG(37 kDa) (Fig. 1).

A series of experiments were performed to determine the role of borohydride, hydroxide, and oxygen during alkaline treatment of AG(37 kDa). Samples were prepared as described in Method 2. The addition of sodium borohydride to AG(37 kDa) in the absence of sodium hydroxide had no effect on the molecular weight and molecular

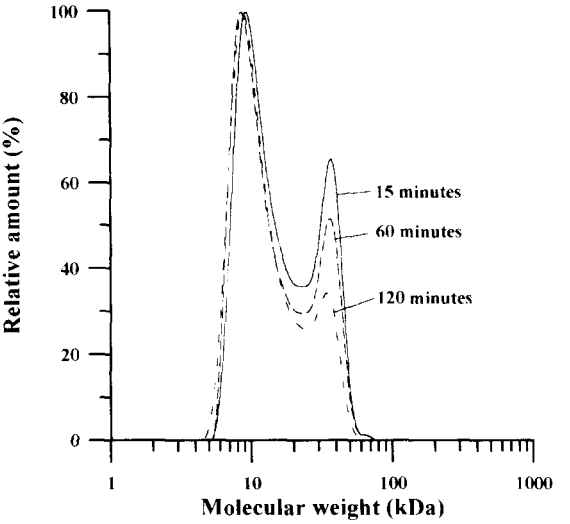

Fig. 3. Molecular-weight distributions for arabinogalactan (AG(37 kDa), 100 mg/mL) subjected to 0.5 M sodium hydroxide/0.1 M sodium borohydride at 37°C for different times and isolated as described in Method 2. The molecular weights at the maxima of the distributions are 9.1, 8.5, and 8.8 kDa for the reactions run for 15 min, 60 min, and 2 h, respectively.

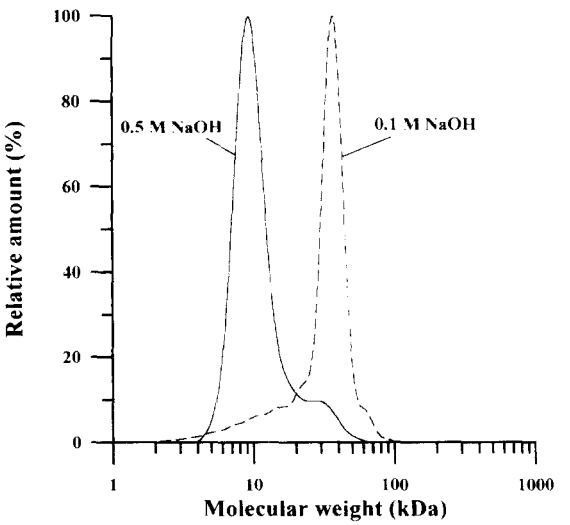

Fig. 4. Comparison of the molecular-weight distributions of arabinogalactan (AG(37 kDa), 100 mg/mL) subjected to alkaline treatment in the presence of 0.1 M sodium borohydride at one of two base concentrations for 1 h at 50°C and isolated as described in Method 2. The molecular weights at the maxima of the distributions are 9.1 kDa for the reaction run in 0.5 M sodium hydroxide and 36.3 kDa for the reaction run in 0.1 M sodium hydroxide.

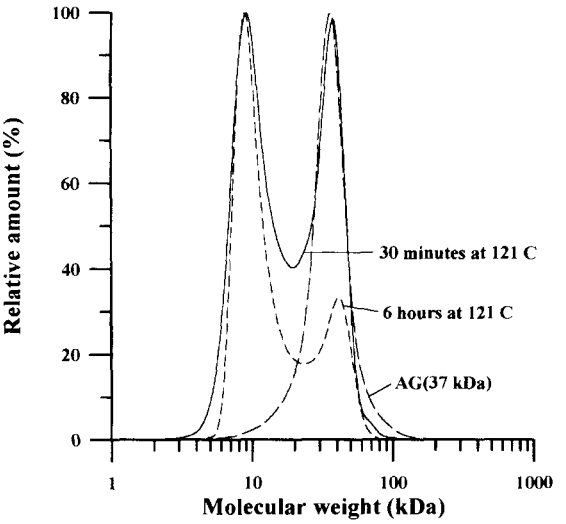

Fig. 5. Molecular-weight distributions for arabinogalactan (AG(37 kDa), 20 mg/mL) heated to 121°C for either 30 min or 6 h (Method 4). The molecular weights of the peak maxima of the sample which was heated for 30 min are 9.1 and 36.3 kDa; for the sample which was heated for 60 min the maxima are 8.8 and 39.9 kDa.

weight distribution of AG(37 kDa), but caused a 50% decrease in the relative aldehyde content reflecting incomplete reduction of the reducing terminus. When borohydride was omitted from the reaction of AG(37 kDa) with sodium hydroxide, variable and lower yields of arabinogalactan were obtained, particularly at elevated temperatures and hydroxide concentrations (Table 1). Omission of borohydride also resulted in substantial browning of the reaction solution and the isolated product. Treatment of AG(37 kDa) under a nitrogen atmosphere following Method 3 resulted in a similar yield and molecular weight distribution of AG(9 kDa) as was found when the reaction was run in the presence of oxygen.

Effect of ionic strength, pH, and urea on the molecular weight of AG(37 kDa).—High ionic strength, extremes of pH, and urea can often cause the dissociation of macromolecules into subunits. AG(37 kDa) (20 mg/mL) was incubated for 1 h at 25°C at pH 4 (0.1 M potassium hydrogenphthalate/hydrochloric acid), pH 10 (0.05 M sodium carbonate buffer), or 1 M sodium chloride (pH 7). Following the incubation, the molecular-weight distribution of the AG(37 kDa) was determined by GFC/LLS. The mobile phase in the GFC/LLS system was identical to the incubation system. In a second experiment AG(37 kDa) and AG(9 kDa) were incubated in 4 M urea (pH 7) for 24 h and then examined by GFC/LLS using PB-1 as eluent. No change in size distribution occurred with any incubation conditions for AG(37 kDa) or AG(9 kDa).

Characterization of AG(9 kDa).—AG(9 kDa) had a relative aldehyde content of between 1 and 5% of that obtained with AG(37 kDa) indicating that greater than 95% of the reducing termini of AG(9 kDa) were reduced. The molecular-weight values for AG(37 kDa) and AG(9 kDa) measured by GFC/LLS, sedimentation equilibrium, and

Table 1
Effect of sodium borohydride (0.1 M) on the recovery of AG(37 kDa) after exposure to varying temperatures, base concentrations, and incubation times

Sodium hydroxide	Temperature (°C)	Time (h)	Mass recovery (%)	
			Reaction with borohydride	Reaction without borohydride
1 M	37	1	98	88
1 M	37	4	100	71
1 M	50	1	98	61
1 M	50	4	93	30
4 M	37	1	99	91
4 M	37	4	92	61
4 M	50	1	82	38
4 M	50	4	55	22

AG(37 kDa) (100 mg/mL) was treated as indicated in the table, neutralized with 6 M hydrochloric acid and purified by dialysis against a 3500 MWCO membrane. The freeze-dried samples were weighed and the recovery calculated.

mass spectrometry are summarized in Table 2. The mass spectrum of AG(9 kDa) is shown in Fig. 6. The mass distribution was found to be lognormal with a mean molar mass of 8.3 ± 2.6 kDa. This mass distribution is almost identical to that determined by GFC/LLS measurements (mean molar mass of 8.9 ± 3.1 kDa). The molecular weights determined by GFC/LLS, sedimentation equilibrium, and mass spectrometry are in close agreement.

The alditol acetate method to determine monosaccharide composition and the compositional estimation from linkage analysis yielded a ratio for galactose to arabinose for AG(9 kDa) of 6.3 ± 1.1 ($n = 6$) and 5.8 ± 0.8 ($n = 6$), respectively. This ratio determined for AG(37 kDa) by the alditol acetate and Me_3Si methods yielded an average value of 5.5 ± 0.5 ($n = 6$). Within the variation in the methods, the compositions of AG(37 kDa) and AG(9 kDa) were identical. Galactose and arabinose constituted greater than 99% of the total monosaccharides of AG(9 kDa). The linkage analyses indicated

Table 2
Comparison of molecular weights determined for AG(37 kDa) and AG(9 kDa) by GFC/LLS, sedimentation equilibrium and mass spectrometry techniques

Sample	Technique					
	GFC/LLS			Sedimentation equilibrium		Mass spectrometry
	M_w	M_n	M_w / M_n	M_w	M_z	
AG(37 kDa)	37.1	31.6	1.17	38.0	32.0	—
AG(9 kDa) [a]	9.1	8.4	1.08	9.5	8.7	8.4
AG(9 kDa) [b]	9.2	8.0	1.15	—	—	—

Measurements were made as described under Methods. Molecular weights are expressed in units of kDa.
[a] AG(9 kDa) prepared by Method 1.
[b] AG(9 kDa) prepared by Method 3.

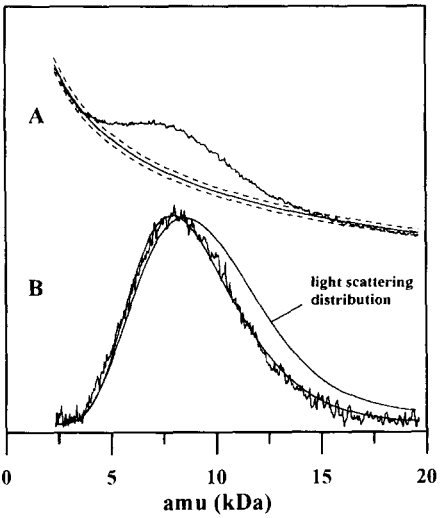

Fig. 6. MALDI-TOF mass spectrum of AG(9 kDa) (Method 1) using *trans*-3-indoleacrylic acid/50 mM sodium chloride matrix. **A**, Raw data. Solid curve is the calculated baseline. Dotted curves are the 95% confidence intervals. **B**, Baseline-corrected mass spectrum. Solid curve is the calculated lognormal distribution curve. The molecular weight distribution of AG(9 kDa) from laser light-scattering measurements is shown by the labeled curve.

that the most abundant glycosyl residues were 3,6-linked galactosyl (25.1%), terminal galactosyl (22.5%), and 6-linked galactosyl (19.2%) residues. The results of AG(37 kDa) linkage analyses are presented for comparison (Table 3).

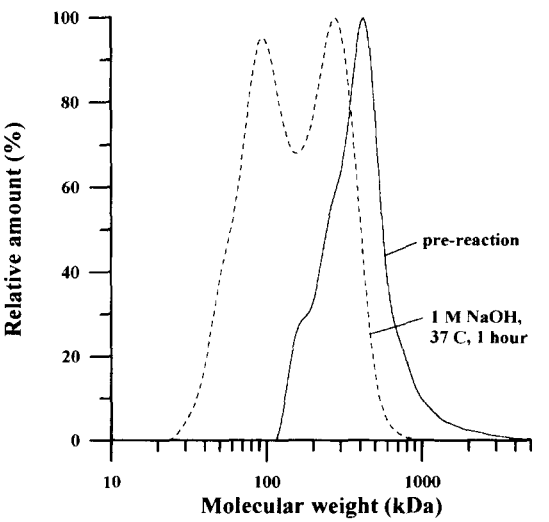

Fig. 7. Molecular-weight distributions of gum arabic before and after incubation in 1 M sodium hydroxide/0.1 M sodium borohydride at 37°C for 1 h.

Table 3
Linkage analysis of AG(37 kDa) and AG(9 kDa)

Linkage	Mole percent	
	AG(37 kDa) [a]	AG(9 kDa) [b]
Galactose		
3,4,6-	2.2 ± 0.3	3.6 ± 1.2
2,3,6-	1.6 ± 0.1	1.1 ± 0.5
3,6-	30.7 ± 0.8	25.1 ± 1.6
3,4-	2.3 ± 0.3	5.9 ± 0.71
6-	19.4 ± 0.6	19.2 ± 1.0
3-	1.6 ± 0.1	5.1 ± 1.2
Terminal pyranose	26.8 ± 0.9	22.5 ± 3.3
4-	trace	2.0 ± 0.4
2-	trace	0
2,6-	trace	0.5 ± 0.9 (trace)
2,3,4,6-	trace	0
% of total galactose	84.5 ± 1.3	85.1 ± 1.8
Arabinose		
3,4-	trace	0
4-	trace	0
Furanose 3-	3.9 ± 0.1	5.9 ± 0.7
Terminal furanose	7.4 ± 1.0	5.0 ± 0.6
Terminal pyranose	4.2 ± 0.3	4.0 ± 0.8
% Total of arabinose	15.5 ± 1.4	14.9 ± 1.8

AG(9 kDa) was prepared by Method 1.
[a] $n = 3$; values ± 1 standard deviation.
[b] $n = 6$; values ± 1 standard deviation.

The C-13 spectra of AG(37 kDa) and AG(9 kDa) are identical with respect to peak position and intensity suggesting preservation of the basic structural architecture during the conversion of AG(37 kDa) to AG(9 kDa). The only difference in the two spectra is the wider line widths of AG(37 kDa), attributable to the increased translation and rotational correlation times of the larger polysaccharide in solution [10,11]. Peak assignments and spectrum for AG(37 kDa) have been previously published [1].

Receptor interaction.—The binding of AG(37 kDa) and AG(9 kDa) with the asialoglycoprotein receptor was compared by an in vitro assay. Comparison of their abilities to inhibit binding of ^{125}I-tyramine to the receptor showed that AG(9 kDa) and AG(37 kDa) exhibited equal IC_{50} values, expressed as the weight concentration necessary to displace 50% of the tracer.

Effect of alkali on other polysaccharides.—The effect of alkaline/borohydride reduction (50 mg/mL polysaccharide, 1 M sodium hydroxide, 0.1 M borohydride, 37°C, 1 h) on the molecular weight distributions of gum arabic, dextran T-10, dextran T-40, p-HES and galactan was investigated. Only the arabinogalactan moiety of the gum arabic arabinogalactan–protein, which is also a Type II arabinogalactan, exhibited a change in molecular weight distribution (Fig. 7). Under the conditions examined ~ 50% of the gum arabic shifted to a lower molecular-weight form.

4. Discussion

Larch arabinogalactan consists of regularly separated units of similar molecular weight.—In this paper we describe the effect of dilute alkali on the structure and molecular weight distribution of purified arabinogalactan, denoted AG(37 kDa), a polysaccharide of interest because it binds the asialoglycoprotein receptor found in hepatocytes. AG(37 kDa) is obtained from crude arabinogalactan by two ultrafiltration steps. The filtration through the 100 kDa membrane removes large marcromolecules and aggregates left from the processing of the crude arabinogalactan. The effluent is then ultrafiltered against a 10 kDa membrane and AG(37 kDa) is obtained by lyophilization of the retained solution. As shown in Fig. 1, the principal difference between the molecular weight distribution of crude arabinogalactan and AG(37 kDa) is the fraction of low molecular weight material in crude arabinogalactan. This material is removed by the ultrafiltration process. Both crude arabinogalactan and AG(37 kDa) appear to be free of AG(9 kDa). Using a curve fitting algorithm consisting of the summation of different proportions of AG(37 kDa) and AG(9 kDa), we estimate that the mass contribution of AG(9 kDa) to crude arabinogalactan is less than 2%. Integration of the molecular weight distribution curve for crude arabinogalactan demonstrated that 14.1% of the mass of crude arabinogalactan has a molecular weight less than 10 kDa, and less than 1% of its mass has a molecular weight greater than 100 kDa. This is consistent with the observation that the recovered mass percentage of AG(37 kDa) relative to crude arabinogalactan is 85%.

When AG(37 kDa) is treated with sodium hydroxide solutions of 0.5 M or greater and 0.1 M sodium borohydride, the weight average molecular weight of the resulting arabinogalactan falls approximately four-fold. The inclusion of borohydride prevents further degradation of the resulting low molecular weight arabinogalactan fragment through the so called peeling reaction [12,13]. The resulting AG(9 kDa) is obtained in high yield (85–90%) and with high molecular-weight homogeneity ($M_w/M_n = 1.15$). For comparison pharmaceutical grade dextran T-40 has a M_w/M_n ratio of 1.4 (data not shown). The high yield of AG(9 kDa) shows that very little degradation of arabinogalactan to monosaccharides and low molecular weight oligosaccharides (< 1 kDa) occurs during this treatment. The high degree of homogeneity of the resulting arabinogalactan fragment is consistent with a structure for AG(37 kDa) that can be cleaved to release subunits of similar size and composition.

Our observation of alkali-labile linkages in AG(37 kDa) suggests three possible models which might explain the covalent bond cleavage yielding AG(9 kDa). In the first model the monosaccharide composition of AG(37 kDa) would include arabinose, galactose, and carboxyl-containing monosaccharides such as galacturonic acid. Four AG(9 kDa) fragments are linked through three galacturonic acid residues by ester bonds to form AG(37 kDa). Ester linkages are alkali-labile [14] consistent with our observations and provide a mechanism for generating AG(9 kDa). In the second model AG(37 kDa) contains esterified galacturonic acids with four side chains of AG(9 kDa) attached at C-4 of the esterified galacturonic acid. These side chains are released in a base catalyzed β-elimination in the presence of sodium borohydride in a manner similar to that found with pectic polysaccharides [15]. In the third model AG(9 kDa) fragments are

linked to a protein or peptide through the hydroxyl groups of four serine (threonine) residues. Such linkages are also alkali-labile [16]. The presence of protein or uronic acid residues have been reported in arabinogalactans isolated from trees closely related to the western larch [3,17].

We believe that none of these models can explain the observed alkali-lability of AG(37 kDa). First, we have determined the monosaccharide composition of AG(37 kDa) by three methods (alditol acetate [1], Me_3Si [1], and TFA hydrolysis [18]). The results of the TFA hydrolysis are unpublished. All three methods detected only arabinose and galactose as constituents of AG(37 kDa). Monosaccharide composition determinations of AG(9 kDa) (alditol acetate method) also only detected arabinose and galactose. Furthermore, FTIR measurements (carbonyl) and base titration [19] of both AG(37 kDa) and AG(9 kDa) failed to reveal the presence of any acidic group (unpublished results). These five methods rule out the presence of carboxyl containing monosaccharides as constituents of AG(37 kDa). This conclusion is consistent with earlier reports of larch arabinogalactan composition [3,20]. Secondly, AG(37 kDa) contains less than 0.01% nitrogen [1]. This level of nitrogen corresponds to less than 1 amino acid residue per mole of AG(37 kDa). Amino acid analysis of AG(37 kDa) failed to detect any amino acids [1]. We conclude that AG(37 kDa) is protein free consistent with earlier published reports for arabinogalactan isolated from the western larch [3].

While the chemical mechanism of AG(9 kDa) formation has not been determined, our observations are consistent with the hypothesis that AG(37 kDa) breaks down to a 9 kDa fragment [AG(9 kDa)] through covalent bond-cleavage. In the presence of alkali the formation of AG(9 kDa) increases with longer reaction time, higher temperature, and higher hydroxide concentration. High temperature at neutral pH is also sufficient to cause substantial conversion of AG(37 kDa) to AG(9 kDa). Finally, agents used to break weak associations between subunits of macromolecules (pH 4, pH 10, 1 M sodium chloride, and 4 M urea) fail to affect the size distribution of AG(37 kDa); therefore, support could not be obtained for the alternate explanation of our results, namely that AG(37 kDa) is comprised of non-covalently associated subunits.

With the exception of their molecular weight, AG(37 kDa) and AG(9 kDa) have nearly identical structures and compositions. The mean molar percentages of the sugar linkages are similar, with small differences observed in the fraction of 3-linked galactose, 3,4-linked galactose, and 3,6-linked galactose. With the possible exception of the just mentioned linkages, the composition and linkage of AG(37 kDa) is unchanged by alkaline borohydride treatment to form the 9 kDa arabinogalactan fragment. The ^{13}C-NMR spectra of AG(37 kDa) and AG(9 kDa) are identical except that broader spectral lines are observed in the AG(37 kDa) spectrum due to its greater molecular weight. Finally, in vitro comparison of AG(37 kDa) and AG(9 kDa) using isolated asialoglycoprotein receptor shows equivalent bioactivity.

Churms et al. [4] previously reported, based upon Smith degradation studies, that larch arabinogalactan consists mainly of (1 → 3)-linked blocks of β-D-galactopyranosyl residues. These blocks, which consist of the galactose backbone of arabinogalactan, contain about 12 residues and are separated at regular intervals by sugar units vulnerable to periodate treatment. Based on the linkage data reported in our paper, we conclude that each galactose residue in the backbone of arabinogalactan is modified with a single

branch consisting of an average of 3 sugar residues. Assuming this degree of branching for Churms' arabinogalactan model, one calculates that each of Churms' blocks would consist of about 48 sugar residues having an approximate molecular weight of 7.8 kDa, a value in reasonable agreement with the number average molecular weight (8.4 kDa) measured for AG(9 kDa). Based on the evidence presented above together with Churms' proposal, we propose that larch arabinogalactan consists of a series of subunits joined through an as yet undetermined linkage which is susceptible to cleavage at low alkali concentrations and moderate temperatures.

Previously reported bimodal distributions of larch arabinogalactan.—Larch arabinogalactan has been often reported to consist of two components of different molecular weights, one high molecular weight component with values recorded in the range of 37–100 kDa (70–95%) and a second component of lower molecular weight with values in the range 7.5–18 kDa (5–30%) [3,21]. A notable exception to these reported distributions is that of Churms et al. [4], who reported a larch arabinogalactan preparation having only 20% of the high molecular-weight form. Some of the variation in the values ascribed within each range may be attributable to analytical methodology [22,23].

Adams and Ettling [23] have further suggested that isolation methodology may play a role in apparent physical/chemical differences of arabinogalactan seen between various investigators. The possibility of introducing a second component (low molecular-weight form) during the isolation of arabinogalactan must be considered in view of our observations concerning arabinogalactan's lability in mild alkali. Conditions used to isolate arabinogalactan from *L. occidentalis* include extraction at 70°C for several days [24,25] and use of magnesium oxide (an alkali) [23,26]. It is noteworthy that the approximate ratios of the upper and lower ranges of the previously reported two molecular weight forms of arabinogalactan have a ratio of 5.5 and 4.9, respectively, which is similar to the weight average molecular weight ratio of AG(37 kDa) and AG(9 kDa) of 4.

Alkaline cleavage of larch arabinogalactan.—Although a considerable literature exists concerning the use of alkaline degradation for the elucidation of the structure of polysaccharides, the exact nature of the degradative reactions has not been determined [27,28]. The exclusion of oxygen during alkaline degradation reduces product variability [28] and borohydride reduction of the terminal sugar residue prevents degradation by alkali [13]. In general, conditions cited in the literature to effect alkaline degradation of polysaccharides use a combination of hydroxide concentrations in excess of 1 M, temperatures above 50°C and hydrolysis times greater than 10 h. Whistler and BeMiller [12], in a review of alkaline degradation of polysaccharides, cite conditions for alkaline degradation of polysaccharides using saturated lime water between 25 and 37°C for several months, treatment of amylose with 1 N sodium hydroxide at 100°C for 24 h, and use of 15–30% aq potassium hydroxide at 100°C for 1 h in the degradation of glycogen [12]. In another example Young and Sarkanen extensively degraded arabinogalactan using 0.1 M sodium hydroxide, at 78°C for 24 h [29]. In a procedure to reduce polysaccharide aldehydic end groups with borohydride, Richards and Whelan [30] recommended conditions using 0.25 M sodium hydroxide at 30°C for 1 h, expecting no glycosidic hydrolysis to occur during this treatment.

The susceptibility of AG(37 kDa) to specific cleavage by 0.5 M hydroxide at 25°C

within a time as short as 15 min is surprising when compared to alkali degradative methods reported in the literature. The alkaline cleavage of AG(37 kDa) is unrelated to the peeling reaction which occurs sequentially from the reducing end of the polysaccharide. Borohydride-reduced AG(37 kDa) with a M_w of 36.5 kDa should be protected from degradation by the peeling reaction and inert to mild alkali treatment. Nevertheless, when borohydride reduced AG(37 kDa) is treated simultaneously with alkali and borohydride it undergoes fragmentation to AG(9 kDa) in a yield similar to that obtained by method 1 (data not shown). Similarly the fragmentation cannot be attributed to base treatment in the presence of oxygen as a similar product is obtained from the reaction run under a nitrogen atmosphere. While the cleavage of AG(37 kDa) is unexpected it is not unique. We have observed a similar effect with gum arabic, a polysaccharide related to arabinogalactan (Fig. 7).

Suitability of AG(9 kDa) for targeted drug delivery.—We have proposed that AG(37 kDa) is an economically feasible, highly efficient carrier for conjugation with drugs for targeting to hepatocytes via the asialoglycoprotein receptor [1]. In this paper we demonstrate the isolation and characterization of an arabinogalactan fragment with a three to four fold lower molecular weight than our starting purified arabinogalactan without any loss in potency in binding the asialoglycoprotein receptor. A lower molecular weight form of arabinogalactan offers several potential advantages with respect to its use in targeted drug delivery. A 9 kDa arabinogalactan would have selected advantages over AG(37 kDa) in both subcutaneous and intramuscular administration routes due to enhanced diffusion resulting from its lower molecular weight [31]. With a lower molecular weight the possibility of host generation of antibodies to an arabinogalactan–drug conjugate should decrease [6]. Finally, a lower molecular weight arabinogalactan may have a better adverse (clinical) reaction profile than higher molecular-weight forms. This has been seen with dextrans where higher adverse reaction rates are associated with higher molecular-weight, as described by Mishler, and Hedin, and references cited therein [32,33].

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