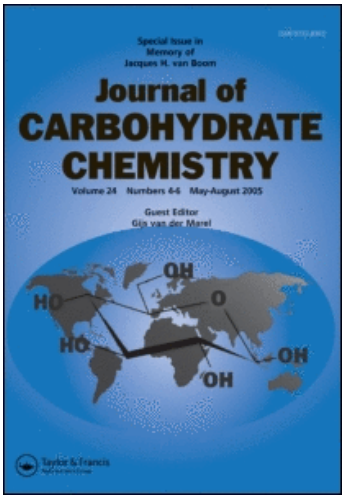

Journal of Carbohydrate Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713617200

Arabinogalactan from Western Larch, Part II; A Reversible Order-Disorder Transition

Glenn R. Ponder[a]; Geoffrey N. Richards[a]
[a] Shafizadeh Center for Wood and Carbohydrate Chemistry, University of Montana, Missoula, MT, USA

To cite this Article Ponder, Glenn R. and Richards, Geoffrey N.(1997) 'Arabinogalactan from Western Larch, Part II; A Reversible Order-Disorder Transition', Journal of Carbohydrate Chemistry, 16: 2, 195 — 211
To link to this Article: DOI: 10.1080/07328309708006521
URL: http://dx.doi.org/10.1080/07328309708006521

J. CARBOHYDRATE CHEMISTRY, 16(2), 195-211 (1997)

ARABINOGALACTAN FROM WESTERN LARCH, PART II; A REVERSIBLE ORDER-DISORDER TRANSITION

Glenn R. Ponder* and Geoffrey N. Richards

Shafizadeh Center for Wood and Carbohydrate Chemistry
University of Montana, Missoula, MT 59812, USA

Received October 2, 1996 - Final Form January 10, 1997

ABSTRACT

At the molecular level, larch arabinogalactan (AG) fragments into subunits when exposed to mild alkali or heat. This process is accompanied by a large increase in NMR spectral resolution, especially for NMR signals associated with main-chain units, and by the disappearance of ^{1}H NMR signals probably due to persistent hydrogen bonds. Analysis by size-exclusion chromatography (SEC) shows that smaller AG species undergo the transition in preference to larger ones, and the transition can be reversed by drying from or freezing an aqueous solution of the "fragments". These observations indicate that the fragmentation is a dissociation of a molecular assembly accompanied by an order-disorder transition, analogous to the known triple helix → single-chain random coil transition of (1→6)-branched (1→3)-β-glucans. The small amount of uronic acid units in AG and the consequent ion-exclusion effect in SEC provide a probe to study this transition, and results provide evidence in support of the concept that the transition involves dissociation of a triplex.

INTRODUCTION

The "molecules" of arabinogalactan (AG) from Western larch (*Larix occidentalis*) have recently been reported by Prescott, *et al*[1] to fragment into subunits when treated with alkaline borohydride or heat. This report appeared when we were studying the same phenomenon, which we first observed as a dramatic shift in LC retention time for AG in the early stages of alkaline degradation. Prescott, *et al.* speculated that this fragmentation

involves scission of covalent bonds. A more likely possibility is that the "fragments" are AG molecules, and that the fragmentation is caused by disruption of hydrogen bonds which hold the molecules together in an ordered structure. Ordered tertiary structures occur frequently among natural polysaccharides, often as multiple helices maintained by hydrogen bonding and susceptible to disruption by alkali or heat.[2] Of particular interest are certain (1→6)-branched (1→3)-β-D-glucopyranans such as scleroglucan, which exists in a triple helical conformation, both in the solid state and in aqueous solution, and which is susceptible to an alkali-induced order-disorder transition from triple helix to single-chain random coil.[3] In this paper we present evidence in support of the view that fagmentation of AG is a similar transition.

Regarding terminology, the acronym "AG" is used to denote the ordered multiplex, as it exists in the larch tree and in aqueous solution prior to disordering, and "DAG" is used to denote disordered AG, *i.e.*, the molecules freed by disruption of the multiplex. In addition, only the high weight fraction of larch AG, known as AG-A, will be considered. The low molecular weight fraction, AG-B, is not susceptible to this transition, nor is it associated with DAG. (AG-B is a special case, and it will be discussed in a subsequent paper.) The AG used in this study is referred to in the preceding paper (Part I) as "AG-A".

RESULTS AND DISCUSSION

This work began with an observation of the LC behavior of AG after brief contact with saturated, aqueous $Ca(OH)_2$ (lime water). The aim of that work was to achieve alkaline "peeling" for structural analysis. (Results of such "alkaline degradation analysis" will be discussed in Part III of this series.) Using the HPLC system described in the preceding paper (Part I) with 50 mM $NaNO_3$ eluent, the oligomeric products of the peeling reaction coelute at 9.8 min retention time. However, before the reaction which generates these products gets underway, but soon after dissolution in alkali, the retention time for the AG peak shifts from 8.30 to 8.74 min. Pullulan calibration suggests that this means a MW change from 17.0 to 9.3 kDa. Of course neither of these numbers can be regarded as accurate absolute weight determinations due to structural and conformational differences between AG and pullulan. (Light scattering, equilibrium sedimentation and osmometry indicate that the average "molecular" weight of AG is about 37 kDa.)[1, 4] Neither can we regard these numbers as accurate relative weight determinations, since conformational differences between AG and DAG are also possible.

To study this transition, we changed the base from $Ca(OH)_2$ to NaOH, the latter being less likely to induce the peeling reaction.[5] Experiments which measured dependence on time, NaOH and AG concentrations were then conducted. Using SEC to follow the transition, it was found that a minimum of 0.5 M NaOH was needed to completely effect the transition of a 5% AG solution in 30 min. Lesser NaOH concentrations resulted in slower change, such that peeling began before the transition was complete. Figure 1 illustrates a typical case. These chromatograms were obtained by treating AG (5%) with 0.3 M NaOH for the times indicated, stopping the transition in each case with an equivalent amount of HCl. The half-way point is illustrated by the "t = 5 min" chromatogram which shows a single broad peak between the peaks for AG (t = 0) and for DAG. The chromatogram for DAG is added for reference only in Figure 1, since the transition with 0.3 M NaOH never reached completion, and after 5 h the effects of peeling (*i.e.*, peak at 9.8 min) were apparent. This experiment was repeated using 1% and 10% AG concentrations. The half-way point was reached after about 30 s for the transition with 1% AG and after about 2 h with 10% AG. In both cases, the peak for the halfway point had the same appearance as the "t = 5 min" peak in Figure 1. In all cases, the first hint of a peak at 9.8 min appeared after 2 h, when the transition was still incomplete.

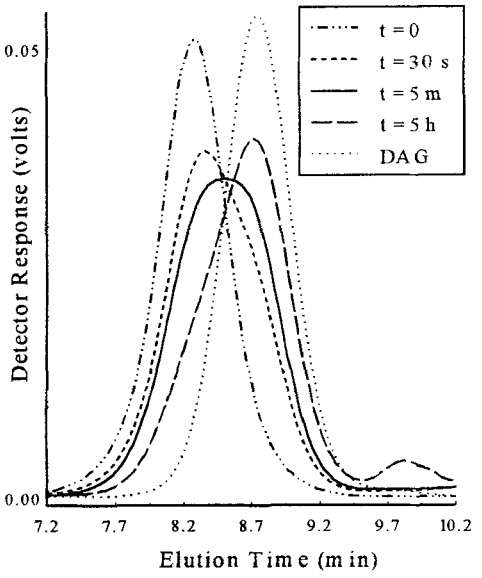

Figure 1. AG (5%) in 0.3 M NaOH, 25 °C.

Similar experiments were also conducted with 0.1 M $NaBH_4$ included in the mixture, and the SEC observations were the same except that the peak at 9.8 min did not appear. These same results were obtained again by conducting these experiments without $NaBH_4$ in the mixture but using AG that had been previously reduced with $NaBH_4$. As expected, the borohydride, whether present in the mixture or used beforehand to reduce the AG, protects against the peeling reaction, which can only proceed if there is an aldehyde group at the reducing end of the main-chain.[6] However, the need for borohydride is obviated by the fact that complete disordering can be achieved by using sufficiently strong alkali at room

Table 1: Methylation Analysis of AG

Derivative[a]	Retention time[b]	Deduced Linkage	Mol%[c]
2,3,5-Me_3-Ara	0.76	Ara*f*-(1→	6.9 ± 0.3
2,3,4-Me_3-Ara	0.80	Ara*p*-(1→	3.9 ± 0.1
2,5-Me_2-Ara	0.86	→3)-Ara*f*-(1→	4.1 ± 0.3
2,3,4,6-Me_4-Gal	1.00	Gal*p*-(1→	26.0 ± 0.6
2,4,6-Me_3-Gal	1.12	→3)-Gal*p*-(1→	2.6 ± 0.8
2,3,4-Me_3-Gal	1.20	→6)-Gal*p*-(1→	21.4 ± 1.0
2,6-Me_2-Gal	1.21	→3,4)-Gal*p*-(1→	2.9 ± 0.4
2,4-Me_2-Gal	1.35	→3,6)-Gal*p*-(1→	30.5 ± 0.8
2-Me-Gal	1.40	→3,4,6)-Gal*p*-(1→	1.7 ± 0.3

a. 2,3,5-Me_3-Ara = 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methyl-L-arabinitol, *etc.*

b. On HP Ultra-2 capillary column, relative to 2,3,4,6-Gal.

c. $n = 5$, values ± 1 standard deviation, corrected by use of effective carbon response factor.[23]

temperature and by neutralizing the mixture within a few minutes. We have found that treatment of AG (10%) with 1.0 M NaOH for 5 min is sufficient to effect complete disordering, as evidenced by SEC; *i.e.*, increased NaOH and/or time had no effect on the shape or elution time of the DAG peak. In addition, conditions as extreme as 2 M NaOH for 30 min at 25 °C caused no peeling, as evidenced by lack of an SEC peak at 9.8 min. Standard conditions were 10% AG in 1.0 M NaOH at 25 °C for 15 min followed by neutralization. These conditions were used to produce the DAG chromatogram in Figure 1.

Compositional analyses of AG and DAG revealed no significant differences between them; each were found to exhibit a molar Gal/Ara ratio of 5.7. Methylation analyses also revealed no significant differences between the two forms, although it was subsequently found that solvent conditions used in methylation (NaOH in DMSO) could disorder AG; *i.e.*, AG probably became DAG during methylation. Table 1 lists results for AG (these are averaged from a selection of optimized analyses, as described in Experimental).

NMR analyses of AG and of DAG revealed several significant differences between them. Figure 2 shows the ^{13}C NMR spectrum of AG in D_2O, and Figure 3 shows the corresponding spectrum for DAG acquired under the same conditions. The improvement in resolution seen in Figure 3 cannot be attributed solely to a smaller molecular size for DAG, as has been previously suggested.[1] We have used extensive partial acid hydrolysis of AG to produce a polymeric product in 20% yield, which therefore presumably has a much smaller average molecular weight than the original material, but the NMR spectra of this product

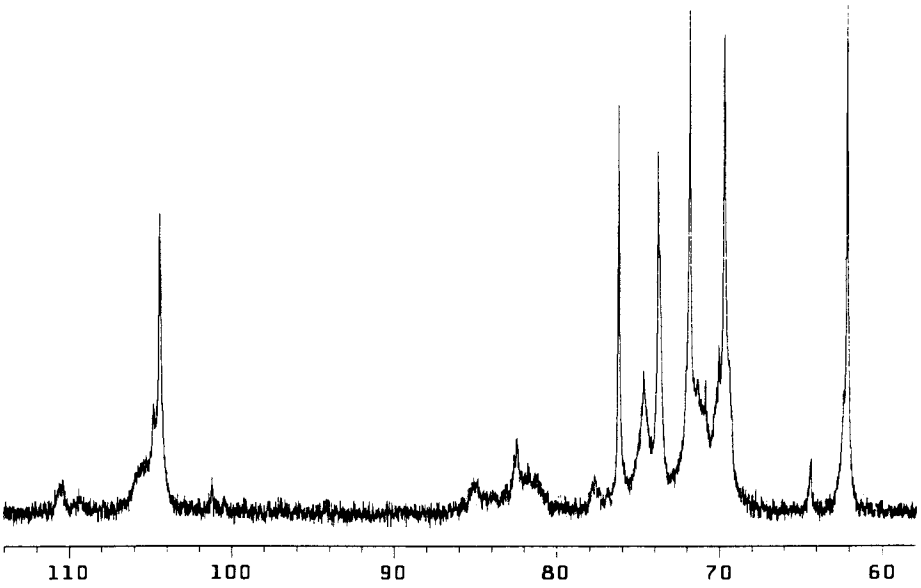

Figure 2. ^{13}C NMR Spectrum of AG in D_2O at 25 °C.

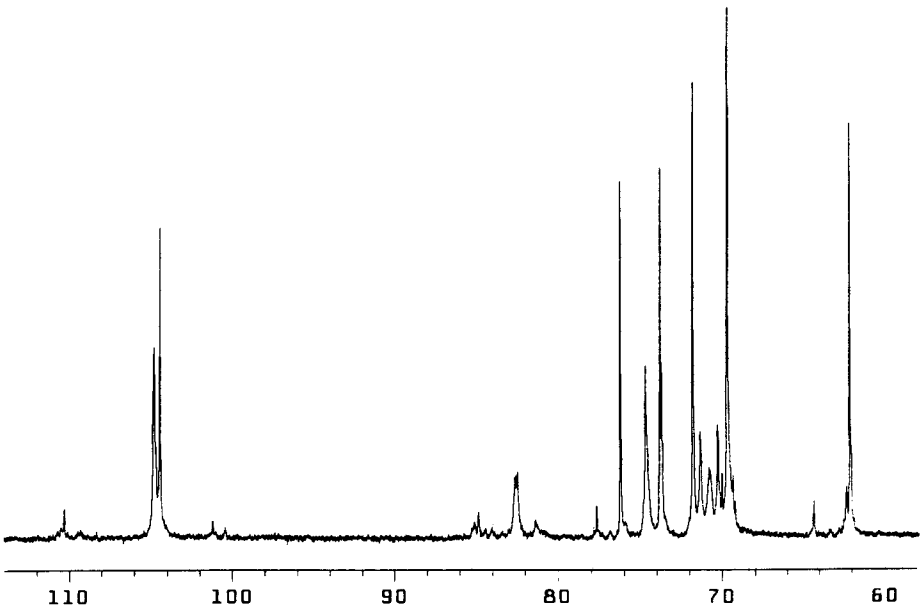

Figure 3. ^{13}C NMR Spectrum of DAG in D_2O at 25 °C.

Table 2: Summary of Major ^{13}C Chemical Shifts for DAG (or for AG)

Glycosidic linkage	Chemical shift (ppm)[a]					
	C-1	C-2	C-3	C-4	C-5	C-6
β-D-Gal*p*-(1→	104.4	71.8	73.8	69.7	76.2	62.1
→6)-β-D-Gal*p*-(1→	104.4	71.8	73.7	69.7	74.7	70.3
→3,6)-β-D-Gal*p*-(1→	104.8	71.3	82.5	69.7	74.7	70.8
α-L-Ara*f*-(1→	110.3	81.3	77.6	84.8	62.4	
β-L-Ara*p*-(1→	101.2	69.4	69.8[b]	70.0	64.4	
→3)-α-L-Ara*f*-(1→	109.3	81.0	85.1	84.1	62.4	

a. Chemical shifts measured relative to 2-methyl-2-propanol (30.695 ppm) in D_2O at 25 °C. Some values given for Ara linkages are approximate shifts of weak, diffuse signals.

b. Obscured by Gal*p* C-4 resonance, and tentatively assigned by reference to NMR study[12] of arabinobiose [L-Ara*p*-β-(1→3)-L-Ara] derived from AG by acid hydrolysis.[7]

nevertheless exhibit no resolution improvement over the spectrum of Figure 2.[7] The improvement observed in Figure 3 can however be attributed to increased conformational freedom resulting from disruption of an ordered tertiary structure. Numerous examples of this phenomenon exist in the polysaccharide literature; *e.g.*, the higher-order helical structures of some (1→3)-β-glucans lead to very poor solution spectra, and the practice of disrupting such ordered structures with alkali or heat in order to improve NMR spectral resolution is a common one.[2, 8] Using the spectrum of Figure 3, together with an APT spectrum to aid methylene carbon assignments, we have refined our interpretation of the ^{13}C NMR spectrum of AG, which appears to be identical to that of DAG in most respects apart from resolution. Table 2 lists ^{13}C NMR chemical shifts (ppm) and peak assignments made by reference to model compounds,[9] to NMR studies of arabinogalactan-proteins,[10] and of arabinogalactans from other species of larch,[11] and to our own NMR studies of the acid, alkaline and Smith degradation products of AG.[7, 12] These assignments differ significantly from carbon NMR assignments recently reported for a similar sample of AG from Western larch.[13]

Figure 4 shows the ^1H NMR spectrum of AG in D_2O, and Figure 5 shows the corresponding spectrum for DAG acquired under the same conditions. Once again, the greater resolution of the disordered form is immediately evident. Interpretation of the spectrum in Figure 5, by comparison with published data[10] and by using results from 2D correlations (COSY and HMQC),[12] leads to the assignments given in Table 3. Only shifts for the major Gal residues are given, as there are many uncertainties for Ara. Ara H-1 signals occur between 4.9 and 5.7 ppm; their complexity suggests a variety of linkage environments.

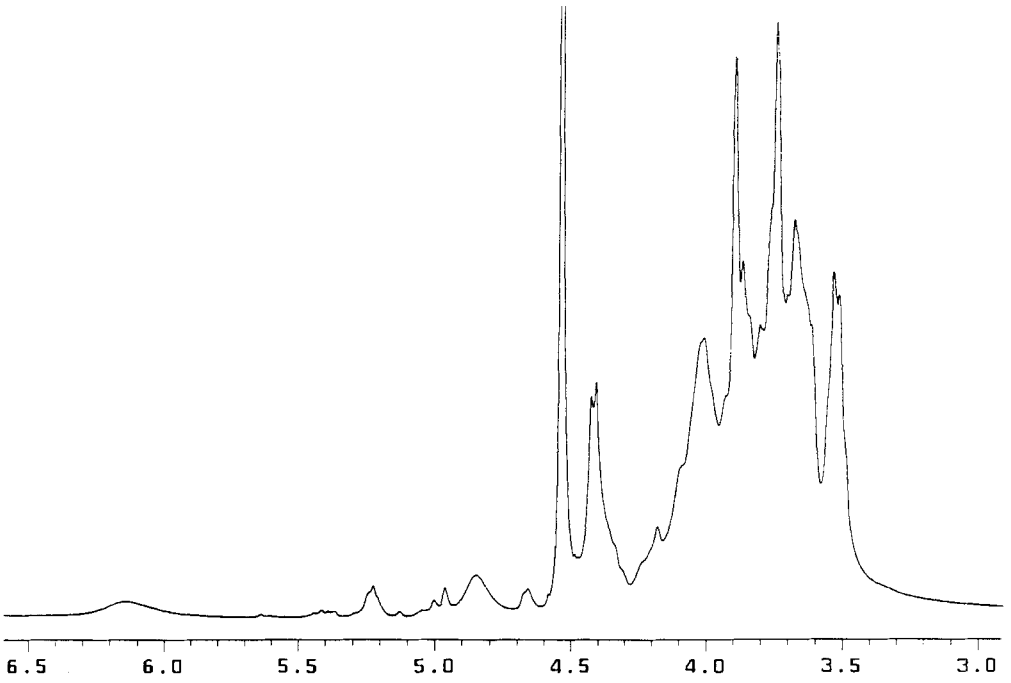

Figure 4. ^{1}H NMR Spectrum of AG in D_2O at 45 °C.

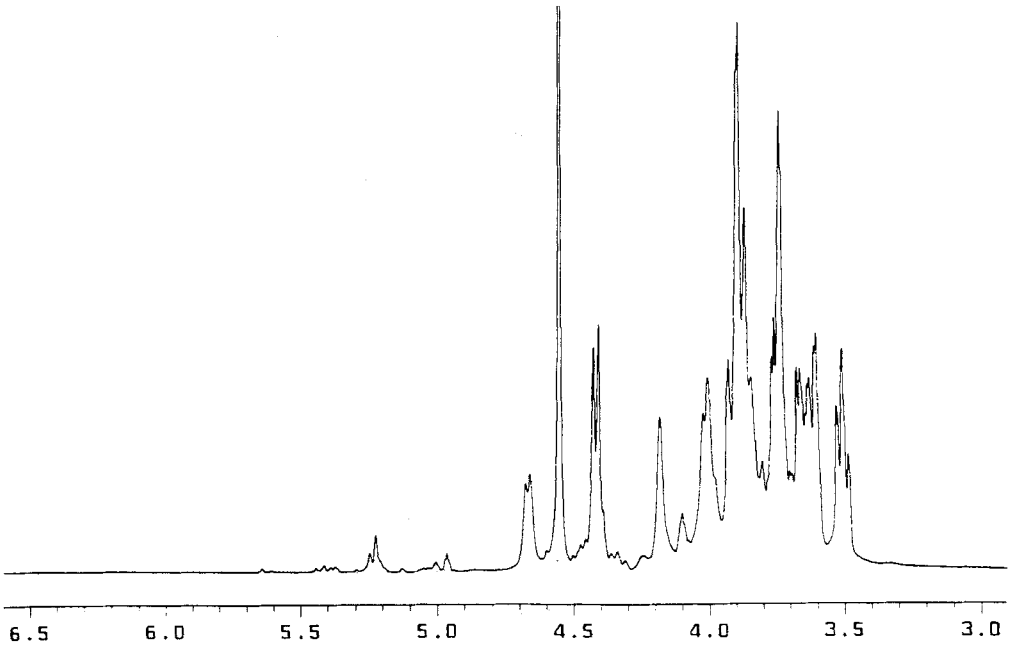

Figure 5. ^{1}H NMR Spectrum of DAG in D_2O at 45 °C.

Table 3: Summary of Major ^{1}H Chemical Shifts for DAG (or for AG)

Glycosidic linkage	Chemical shift (ppm)[a]					
	H-1[b]	H-2	H-3	H-4	H-5	H-6
β-D-Gal*p*-(1→	4.42 d	3.51	3.62	3.89	3.67	3.73
→6)-β-D-Gal*p*-(1→	4.42 d	3.51	3.62	3.92	3.89	3.89/4.01
→3,6)-β-D-Gal*p*-(1→	4.66 d	3.76	3.86	4.18	3.89	3.89/4.01

a. Chemical shifts measured relative to 2-methyl-2-propanol (1.203 ppm) in D_2O at 45 °C.
b. $J_{1,2} \approx 7.5$ Hz

After resolution, the next most conspicuous difference between the spectra of Figures 4 and 5 is the presence of two broad signals at 4.86 ppm and 6.18 ppm in the spectrum of AG, which are absent in the spectrum of DAG. These signals do not correspond to any expected anomeric signals, nor do they correlate with any of the other proton signals in a COSY spectrum of AG. Their lack of fine structure is typical of hydroxyl proton signals, and we have tentatively assigned them as hydroxyl protons involved in persistent hydrogen bonds and thus resistant to deuterium exchange. The signal at 6.18 ppm disappears completely at a very early stage of the disordering process, while the signal at 4.86 ppm disappears gradually during the course of the transition. The former observation may reflect a weaker type of hydrogen bond, analogous to the "loose cross-links" which are "readily broken in the lower range of concentration of alkali"during the helix→coil transition of some 6-branched (1→3)-β-D-glucans.[14] An observed temperature dependence for the chemical shift of this signal,[12] from 6.20 ppm at 30 °C to 6.10 ppm at 70 °C, supports this interpretation.

Another difference between Figures 4 and 5 is the intensities of the signals at 4.18 and 4.67 ppm. These are due to the H-4 and H-1 protons, respectively, of →3,6)-Gal*p*-(1→ units, found primarily in the main-chain. The selective enhancement of these signals in the DAG spectrum brings their relative strength to the expected level in view of the known abundance of such residues in AG, and it indicates that the transition involves a change in the main-chain specifically. The main-chain units are the ones which we would expect to be the most conformationally restrained in a multiple helix structure; *e.g.*, in the triple helix of scleroglucan, the main-chains are nested helices hydrogen bonded to each other, and the side-chains protrude outside the helix core, where they possess relative conformational freedom.[15] Such restraint could confine otherwise equivalent atoms to different chemical environments. This concept is reinforced by Figures 2 and 3, where we see the relative strength of the C-1

signal for →3,6)-Gal*p*-(1→ units (104.8 ppm) enhanced on going from AG to DAG. Again, this suggests that the main-chain in particular has gained conformational freedom.

In addition to alkali, heat is also known to disrupt higher-order polysaccharide structures.[2] AG solutions were heated at 120 °C for various lengths of time and analyzed by SEC to give the chromatograms of Figure 6. The figure shows that after 1 h, about 50% of the AG had become disordered. After 8 h, the chromatogram (not shown) indicated that 66% of the AG had become DAG. By 24 h this situation had changed little; the DAG peak in the "t = 24 h" chromatogram contains 67% of total area. After 2 days, hydrolytic degradation was becoming apparent, and the relative proportions of the AG and DAG peaks continued unchanged. A few milligrams of the ordered material were isolated from an aliquot of the 24 h heated sample by gel chromatography (Sephadex G-75) and then heated for an additional 24 h.

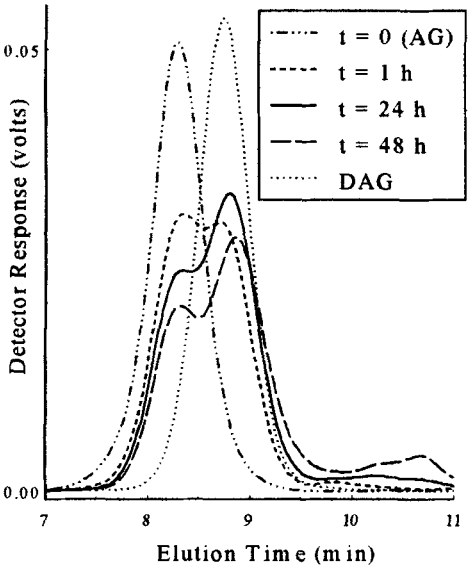

Figure 6. AG (1%) heated at 120 °C.

SEC analysis showed that this additional heating caused no significant disordering; thus, the two-peak chromatograms of Figure 6 do not illustrate equilibria. In addition, the SEC retention time of this persistently ordered AG corresponded to a MW which was 15% greater than that of the original AG, indicating that the low end of the weight distribution of AG is preferentially disordered. An AG solution was also boiled under reflux and sampled periodically for SEC analysis. Results showed that the point at which the AG/DAG ratio no longer changed was reached after 5 days and corresponded to 43% conversion to DAG.

High temperature therefore induces a proportion of multiplexes at the low end of the weight distribution to disrupt, and this proportion increases with temperature, *i.e.*, multiplexes at the high end of the distribution are more thermally stable than those at the low end, and persist as ordered structures long after the smaller multiplexes have dissociated. This is consistent with what is known of the stability of helical conformations of polysaccharides. Because of long range cooperative interactions, longer helices are more stable than shorter ones.[16] To test this concept, we heated three different AG fractions obtained by fractional

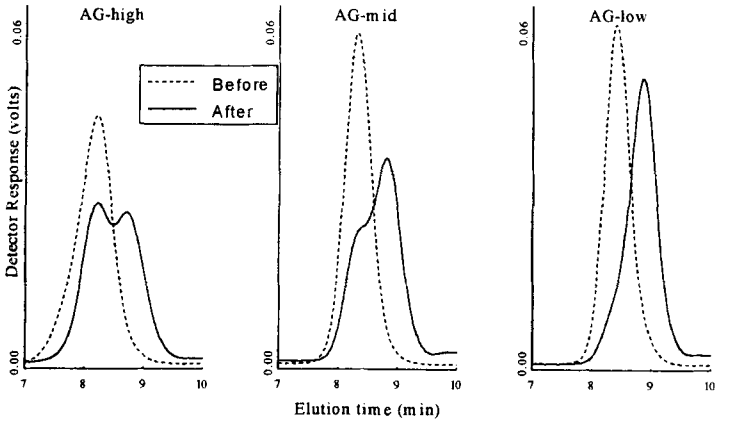

Figure 7. Effect of multiplex size on disordering by heat.

precipitation. These are referred to as "AG-high", "AG-mid" and "AG-low" in Figure 7, and they exhibited SEC-apparent MW's of 19.0, 16.4 and 14.4 kDa, respectively. They were heated as 1% aqueous solutions at 120 °C for 18 h, and results are shown in Figure 7, which shows a trend of increasing susceptibility to disorder with decreasing multiplex weight.

Compelling evidence that the transition does not involve scission of covalent bonds consists of its reversibility by simple evaporation or freezing of an aqueous solution of DAG.

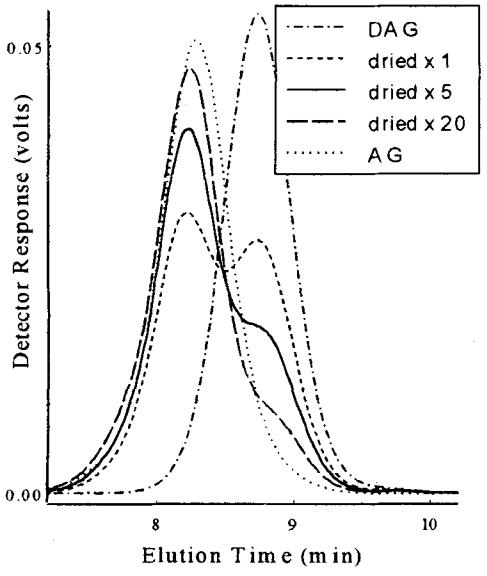

Figure 8. DAG repeatedly dried from and redissolved in water.

The intermediate chromatograms of Figure 8 were made by successive rotary evaporations and redissolutions in water, starting with a solution of DAG. The figure shows that this procedure causes initially rapid reordering, but repeated drying cycles give increasingly limited returns, such that even after 20 cycles a small portion of DAG remains (the chromatogram of original AG is added for reference only to Figure 8). A ^{1}H NMR spectrum of reordered AG resembles that of original AG, including the broad signals at 4.86 and 6.18 ppm.

Freezing has the same effect as drying, and freezing-induced formation of

higher-order polysaccharide structures has been previously reported.[17] Samples which give chromatograms like those of Figure 8 can be generated by repeatedly freezing (at -20 °C) and thawing a solution of what was originally all DAG. As with drying, repeated cycles give limited returns, and after 20 freeze-thaw cycles a small amount of DAG remains. In addition, it was found that if the DAG solution is frozen slowly, more reordering occurs than if frozen quickly. Thus, freeze-drying DAG solutions requires rapid freezing to minimize reordering.

We have also studied these transitions by a technique which relies on the SEC effects of trace amounts of uronic acid groups in AG. As described in the preceding paper (Part I), this effect is a consequence of the partial exclusion of negatively charged species from the stationary phase when low ion strength eluent is used, and it results in charged AG species eluting from our HPLC column well before neutral ones when 0.2 mM $NaNO_3$ eluent is used. Figure 9 includes chromatograms of AG and of DAG obtained using these conditions (the intermediate chromatogram in Figure 9 is discussed below). Maintaining the convention established in the preceding paper, and ignoring a probable but small A3 component, we may refer to the three AG peaks (left to right) as A2, A1 and A0, the numbers denoting number of acid units per multiplex. Extending this convention to the DAG chromatogram, we may refer to its three peaks as D2, D1 and D0, the numbers referring to acid units per molecule.

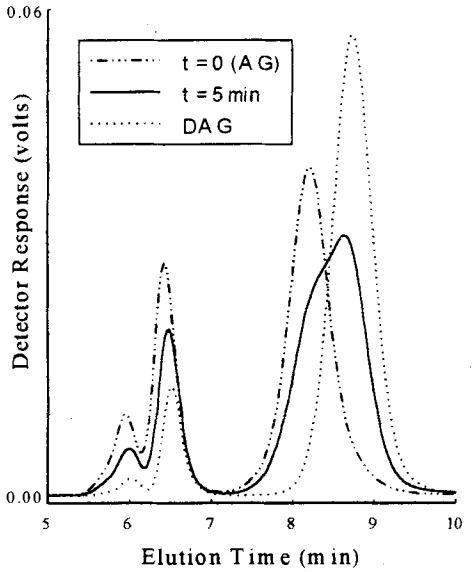

Figure 9. Disordering by alkali. Eluent: 0.2 mM $NaNO_3$

The relation between these two chromatograms can be understood in the context of the transition as a fragmentation. In the AG multiplexes which comprise the ion-excluded peaks (A2 and A1), we assume that usually only one of the constituent molecules contains the uronic acid residue(s). As these multiplexes disorder, the neutral molecules become no longer part of assemblies subject to ion-exclusion. These freed neutral molecules therefore elute with a retention time corresponding only to their size and so contribute to the area of the D0 peak, which grows during the transition at the expense of all three of the original peaks. Presumably when

Table 4: Triplex Interpretation of Low-Ion Strength Chromatograms

DAG	D2	D1	D0
Relative peak area (%)	2.2	10.5	87.3
Molecule	X	Y	Z
AG	A2	A1	A0
Relative peak area (%)	8.9	24.6	66.5
Triplex	XZ_2 & Y_2Z	YZ_2	Z_3

disordering is complete, molecules with one uronic acid residue comprise the D1 peak, and molecules with two uronic acid residues comprise the D2 peak.

Comparison of the DAG and AG chromatograms provides insight into the number of molecules per multiplex. Table 4 lists the observed relative areas in the peaks of these chromatograms. If we begin by assuming a triplex model,[18] and designate the molecules in peaks D2, D1 and D0 as X, Y and Z, respectively, then we can write formulae for possible triplexes in peaks A2, A1 and A0 as shown in the table. In the formation of these triplexes we may assume, to a first approximation, that all three types of molecules (X, Y and Z) will each combine with two Z type molecules to form three major types of triplexes (XZ_2, YZ_2 and Z_3). Use of this assumption and the observed DAG peak areas leads to the prediction that the proportions for A2:A1:A0 should be 7:31:62. This prediction can be made closer to the observed proportions by correcting for the fact that some Y molecules are likely to end up together in the same triplex (Y_2Z); this correction will increase the predicted size of the A2 and A0 peaks at the expense of the A1 peak. If we arbitrarily assume that this is the fate of 20% of Y molecules, then the prediction for A2:A1:A0 becomes 10:25:65, much closer to the observed proportions (Table 4). Still better agreement between predicted and observed peak areas can be achieved by correcting for the likely presence of small amounts of XYZ and Y_3 triplexes (*i.e.*, triply charged triplexes in an A3 shoulder of the A2 peak, see Part I). When we begin the above type of analysis with the assumption that the multiplex is a duplex or a quadraplex, agreement between predicted and observed areas for the AG chromatogram cannot be arrived at by any set of plausible assumptions. The duplex and quadraplex models are untenable simply because there is either too much area or too little area, respectively, in the ion-excluded peaks of the AG chromatogram.

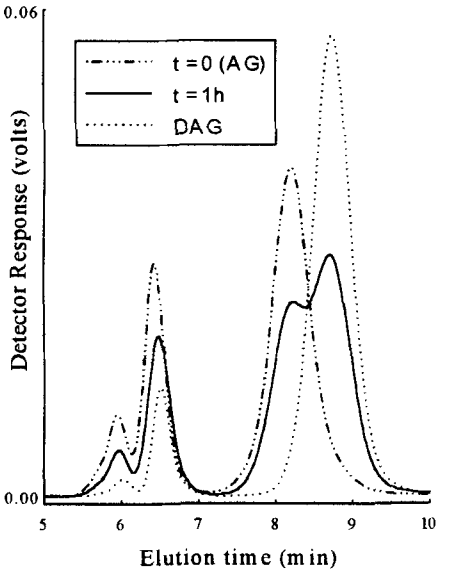

Figure 10. Disordering by heat. Eluent: 0.2 mM $NaNO_3$

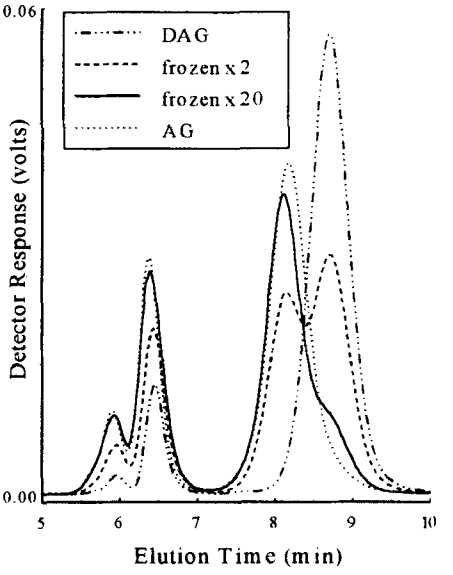

Figure 11. Reordering by freezing. Eluent: 0.2 mM $NaNO_3$

In addition to these analyses of AG and of DAG, we have also used low ion strength eluent in SEC analyses of intermediate states for the forward transition (both alkali- and heat-induced) and for the reverse transition (both drying- and freezing-induced). Key results from these analyses are shown in Figures 9-11. Figure 9 includes the chromatogram for the half-way point of the transition of 5% AG with 0.3 M NaOH; *i.e.*, this is the same sample that elutes as a single broad peak in Figure 1. Figure 10 shows the chromatogram of the 1 h sample for the heating of a 1% AG solution at 120 °C; *i.e.*, this is the same sample that elutes as two slightly resolved peaks in Figure 6. These differences in intermediate chromatograms may reveal different mechanisms for the alkali- and heat-induced transitions. The former appears to involve partly disordered assemblies which survive as stable forms when the transition is interrupted, but the latter appears to involve no such intermediates.

Turning now to Figure 11 and the freezing-induced reverse transition, it appears that this is a reversal of the heat-induced forward transition, *i.e.*, the multiplex reforms all at once. Figure 8 shows that the same is true for the drying-induced reverse transition, and analysis of the Figure 8 samples with low ion strength eluent gives chromatograms like those in Figure

11. It is also apparent from these analyses that the small amount of DAG that resists reordering even after 20 cycles of drying or freezing consists mostly of neutral molecules (right shoulder on reformed A0 peak, Figure 11). As a result, the area in the A0 peak is not quite restored to its original proportion, whereas A2 and A1 are restored almost completely.

EXPERIMENTAL

Materials and General Methods. AG was supplied by Larex, Inc. (St. Paul, MN), and was the same purified material described in Part I. Intermediate states for alkali-induced transitions were generated by combining AG with aqueous NaOH, then periodically removing aliquots and neutralizing by addition of 5 M HCl. For samples analyzed by SEC using 50 mM $NaNO_3$ eluent, the NaCl eluted at 10.8 min, well past analytes of interest. For SEC analyses in 0.2 mM $NaNO_3$, salts interfere with ion-excluded carbohydrates, so these analysis were preceded by dialysis of the samples (MWCO 3.5k tubing) for 2 days against running water, followed by concentration by rotary evaporation to 1%. This was also our standard method of purifying batches of DAG, for which freeze-drying was avoided prior to LC analysis.

Samples for Figure 6 were prepared by heating aqueous AG solutions (2 mL, 1%) in glass vials with teflon-lined screw caps, in a thermostatically controlled heating block at 120 °C. The "AG" samples used to make Figure 7 were obtained by a fractional precipitation of AG, which involved incremental addition of MeOH (200 mL) to AG (10 g) in water (80 mL). The AG was collected as 10 fractions which began to precipitate when the solution reached 53% MeOH (v/v), and which contained 2, 11, 10, 13, 23, 7, 9, 16, 6, and 3 %, respectively, of the total AG. Fractions were isolated by centrifugation and dried at 40 °C/1mm Hg. The third ("high"), seventh ("mid") and tenth ("low") fractions were used to make Figure 7.

Samples for the drying-induced reverse transition were made by repeated rotary vacuum evaporation to dryness at 40 °C of 1% aqueous solutions of DAG with intermediate redissolution in water at room temperature. The freezing-induced reverse transition was carried out in a conical flask originally containing aqueous DAG (1%) at −20 °C, thawing the next day under ambient conditions, then removing a sample before returning to the freezer.

Compositional Analysis. Compositional analyses were performed using the method of Blakeney *et al.*[19], and the resulting alditol acetates were analyzed by GC (see below). Erythritol was used as internal standard, and hydrolysis was achieved in 2 M TFA at 110 °C for 1 h. Five analyses of AG gave an average Gal/Ara molar ratio of 5.74 ± 0.16.

Methylation Analysis. Methylation was carried out using the method of Ciucanu and Kerek.[20] Careful control of the amount of powdered NaOH was necessary to minimize a degradation which caused diminished 2,4-Me-Gal derivatives in the final mixture, thus leading to terminal/branch ratios greater than one. (This amount varied from 60 to 90 mg, depending apparently on fineness of the NaOH powder.) For this reason also, contact time between NaOH and sample (4-6 mg) in DMSO (2 mL) prior to addition of MeI (250 μL) was minimized. Less NaOH than the optimum resulted in undermethylation, and little advantage was gained by performing more than one methylation (stirring for 1 h at room temperature).

The approximately equimolar relation between →3)-Ara*f*-(1→ and Ara*p*-(1→ linkages (Table 1) is easily obscured if conditions are not optimal; thus, overly harsh hydrolysis conditions will preferentially destroy the 2,5-Me_2-Ara derivative, while undermethylation will inflate the amount of this derivative at the expense of the 2,3,5-Me_3-Ara derivative. Optimum hydrolysis conditions for methylated AG involved heating in 90% TFA (330 μL) for 10 min at 110 °C, then dilution with water (1.25 mL) and heating for 1 h at 120 °C.

The procedure for reduction and acetylation was the same as that used in the compositional analyses, $NaBH_4$ replaced by $NaBD_4$. Analysis of partially methylated alditol acetates was performed using GC (see below). The five sets of results used to compile the data in Table 1 were selected from results of 12 separate methylation analyses of AG performed under optimized conditions. The selection was based on minimizing standard deviations and maximizing internal consistency and agreement with compositional analysis.

A significant derivative (ca. 1 mole%) detected in the analysis but not listed in Table 1 was 4-Gal. We regard this as a product of undermethylation, and its GC peak area is included with that of 2,4-Gal. Methylation of the 2-position of a main-chain unit in AG is apparently hindered by substitution at the 3-position. This problem has been observed in the analyses of other (1→3)-glycans; *e.g.*, the 2-position is a frequent site of undermethylation in (1→3)-β-glucans[21] and in Smith degraded AG.[7]

For disordering in DMSO/NaOH, AG (20 mg) was dissolved in DMSO (2 mL) over powdered NaOH (100 mg) and stirred for 1 h. HCl (0.5 M, 5 mL) was added, the solution dialyzed for 2 days (MWCO 1,000 tubing), concentrated to 2 mL and analyzed by SEC, which showed that nearly all AG was disordered. (DMSO alone did not effect this change.)

Chromatography. The SEC system is described in the preceding paper (Part I). GC analyses were performed using a Hewlett-Packard (HP) 5890A gas chromatograph fitted with

two columns and detectors: (1) HP Ultra 1 (25 m x 0.2 mm) cross-linked phenyl methyl silicone-fused-silica capillary column connected to HP 5970 series mass-selective detector (75 eV ionizing potential), and (2) HP Ultra 2 (25 m x 0.33 mm) capillary column connected to a flame ionization detector (FID). Column head pressure (He) was maintained at 40 kPa.

For GC analysis of alditol acetates, the temperature program began at 55 °C isothermal for 1 min followed by a rate of 30 °C/min to 220 °C, then 6 °C/min; products of interest eluted during the final rate. Relative retention times on the Ultra 2 column for peracetylated erythritol, arabinitol and galactitol were 1.00, 1.15 and 1.33, respectively, and relative FID response factors were 1.00, 0.94 and 0.96, respectively. Response factors for Ara and Gal were arrived at by subjecting these aldoses (and erythritol) to all steps of the compositional analysis, thus allowing for acid degradation losses.

For GC analysis of partially methylated alditol acetates, the temperature program began at 55 °C isothermal for 1 min followed by a rate of 30 °C/min to 180 °C, then 4 °C/min; derivatives eluted during the final rate. Relative retention times on the Ultra 2 column are listed in Table 1. Derivatives were identified by their mass spectra[22] and quantified by integrated FID peak areas corrected with effective carbon-response factors.[23]

NMR. NMR spectra were measured in D_2O (sample concentration: 7%) using a Varian Unity*plus* 400 MHz spectrometer. APT, COSY and HMQC spectra were obtained using standard software applications packages.

ACKNOWLEDGMENT

The authors wish to express appreciation to Larex, Inc. for financial support and for supplying the arabinogalactan.

REFERENCES AND NOTES

1. J. H. Prescott, P. Enriques, C. Jung, E. Menz and E. V. Groman, *Carbohydr. Res.*, **278**, 113 (1995).
2. D. A. Rees, E. R. Morris, D. Thom and J. K. Madden in *The Polysaccharides*, Vol 1; G.O. Aspinall, Ed.; Academic Press: Orlando, 1983, pp 195-290.
3. G. Brigand in *Industrial Gums, Polysaccharides and Their Derivatives*, 3rd ed.; R. L. Whistler and J. N. BeMiller, Eds.; Academic Press: San Diego, 1993, pp 461-74.
4. H. A. Swenson, H. M. Kaustinen, J. J. Bachhuber and J. A. Carlson, *Macromolecules*, **2**, 142 (1969).
5. D. O'Meara and G.N. Richards, *J. Chem. Soc.*, 1944 (1960).

6. R.L. Whistler and J.N. BeMiller, *Adv. Carbohydr. Chem.*, **13**, 289 (1958).
7. G.R. Ponder, University of Montana, Missoula, MT, unpublished results.
8. C. Jones, *Adv. Carbohydr. Anal.*, **1**, 145 (1991).
9. a) K. Bock and H. Thøgersen, *Ann. Rep. NMR Spec.*, **13**, 1 (1982).
b) J. G. Collins, J. H. Bradbury, E. Trifonoff and M. Messer, *Carbohydr. Res.*, **92**, 136 (1981).
c) Y. Kawabata, S. Kaneko, I. Kusakabe and Y. Gama, *Carbohydr. Res.*, **267**, 39 (1995).
10. A. M. Gane, D. Craik, S. L. A. Munro, G. J. Howlett, A. E. Clarke and A. Bacic, *Carbohydr. Res.*, **277**, 67 (1995).
11. P. Odonmažig, A. Ebringerová, E. Machová and J. Alföldi, *Carbohydr. Res.*, **252**, 317 (1994).
12. M. Manley-Harris, University of Waikato, New Zealand, personal communication, 1996.
13. E. V. Groman, P. M. Enriques, C. Jung and L. Josephson, *Bioconjugate Chem.*, **5**, 547 (1994).
14. H. Saitô, T. Ohki and T. Sasaki, *Carbohydr. Res.*, **74**, 227 (1979).
15. T. L. Bluhm, Y. Deslandes, R. H. Marchessault, S. Pérez and M. Rinaudo, *Carbohydr. Res.*, **100**, 117 (1982).
16. D.A. Rees, *Polysaccharide Shapes*, Outline Studies in Biology, Chapman and Hall, London, 1977.
17. I. C. M. Dea, E. R. Morris, D. A. Rees, E. J. Welsh, H. A. Barnes and J. Price, *Carbohydr. Res.*, **57**, 249 (1977).
18. Molecular weight determinations by Prescott, *et al.*[1] point to a quadraplex model.
19. A. B. Blakeney, P. J. Harris, R. J. Henry and B. A. Stone, *Carbohydr. Res.*, **113**, 291 (1983).
20. I. Ciucanu and F. Kerek, *Carbohydr. Res.*, **131**, 209 (1984).
21. T.T. Stevenson, Alpha-Beta Technology, Worcester, MA, personal communication, 1997.
22. N. C. Carpita and E. M. Shea, in *Analysis of Carbohydrates by GLC and MS*, C. J. Biermann and G. D. McGinnis, Eds.; CRC Press: Boca Raton, 1989, p 157.
23. D. P. Sweet, R. H. Shapiro and P. Albersheim, *Carbohydr. Res.*, **40**, 217 (1975).