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Arabinogalactan from Western larch, Part III: alkaline degradation revisited, with novel conclusions on molecular structure

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Alkaline degradation of larch arabinogalactan (AG) involves rapid peeling of the (1→3)-galactan main chain from the reducing end. The products are the original side chains attached to galactometasaccharinic (GalMS) acids derived from main-chain residues. These products have been separated by GPC and studied by compositional, methylation and NMR analyses. Results confirm that in a typical AG molecule most main-chain residues carry a side chain on C-6. About half of these side chains are β-(1→6)-linked Gal*p* dimers, and about a quarter are single Gal*p* residues. The rest contain three or more residues and include most of the Ara (arabinose) found in the polysaccharide. About one-fifth of the original Ara is consumed by the peeling reaction, and Ara groups at the non-reducing end of the main chain are proposed, predominantly as arabinobiosyl groups [β-L-Ara*p*-(1→3)-L-Ara*f*-(1→]. In general for the larger side chains abundance decreases with size, while branching and Ara content increase with size. Most terminal Ara*f* residues occur in three- and four-residue side chains, while most arabinobiosyl groups are found in side chains of more than three residues. The Ara probably occurs only as these monomeric or dimeric groups, and since no Ara is found in side chains smaller than three residues, this implies that there are no Ara branches attached directly to the main chain.

INTRODUCTION

Arabinogalactan (AG) from Western larch (*Larix occidentalis*) is a water-soluble polysaccharide of emerging importance as a commercial polymer. Its relatively narrow weight distribution has a maximum at about 37 000 (Swenson *et al.*, 1969; Prescott *et al.*, 1995) and in the previous paper of this series we propose that this is a multiplex weight (Ponder & Richards, 1997b), the multiplexes being ordered assemblies of molecules hydrogen bonded to each other. This is the naturally occurring state of the major, high weight component of AG known as AG-A. The focus of the present study was a purified sample of this polysaccharide which has been described previously (Ponder & Richards, 1997a).

Major features of AG's structure were determined by White (1942) and by Bouveng & Lindberg (1961). Their results indicated a β-D-(1→3)-galactopyranan main chain, comprising about one-third of the molecule, with nearly every main chain residue (1→6)-linked to a side chain. Results also suggested that many side chains consist of single β-D-Gal*p* or α-L-Ara*f* residues or the dimer structures β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→ or β-L-Ara*p*-(1→3)-L-Ara*f*-(1→. Studies of AG from other larch species have indicated that some side chains are longer than two residues and contain branches (Jones & Reid, 1963; Fu & Timell, 1972). This is illustrated in Fig. 1, which summarizes current views by showing some of the general structural features involved (Stephen, 1983).

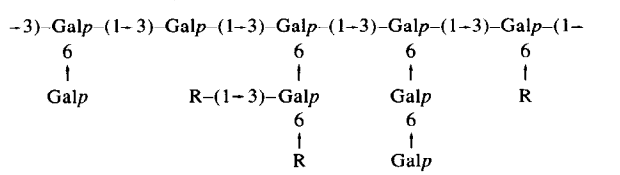


Fig. 1. Current views on AG structure.

Degradation with alkali has been generally avoided as a tool for structural analysis of polysaccharides because the reactions can be various and complex. However, a polysaccharide with a (1→3)-linked main chain is uniquely predisposed toward a single, rapid type of alkaline degradation. In mild alkali, such a polysaccharide almost exclusively undergoes sequential β-alkoxycarbonyl elimination from the reducing end of the main chain (Corbett & Kenner, 1955). Subsequent rearrangements of the former end groups convert them to 3-deoxyaldonic ('metasaccharinic') acids (Kenner & Richards, 1957). These rearrangements involve sequential loss and reformation of chirality at C-2, thus forming an epimeric pair of the acids. The value of this degradation as a structural tool lies in its application to branched polysaccharides since the side chains end up as substituents on the acids (Whistler & BeMiller, 1958). This is illustrated in Fig. 2, which depicts these reactions for a generalized reducing end group (**1**) of an AG molecule. R denotes the rest of the molecule 3-linked to the endgroup, which also bears a 6-linked side chain, R′. The main chain residue that is eliminated (RO^-) becomes the new end group and subsequently undergoes the same sequence of reactions. Main-chain residues are thus 'peeled' away as 3-deoxy-D-*xylo*- and 3-deoxy-D-*lyxo*-hexonic ('galacto-metasaccharinic', GalMS) acids bearing side chains from the original polysaccharide. These products can then be separated and analyzed.

Even though this approach has been recognized as valid in theory for many years, there are few reports of alkaline degradation analysis of AG in the literature. The first such study to yield significant results was conducted by Young & Sarkanen (1977). These workers separated the products into two fractions, which they characterized by compositional analyses. That study led to some novel conclusions (see below) and succeeded in demonstrating the feasibility of alkaline degradation as a structural tool. Since then, however, there has been only one further report of such a study, conducted by Odonmažig *et al.* (1994) as part of a wider study of AG from Mongolian larchwood (*Larix dahurica*). These workers attempted to separate the products by gel permeation chromatography (GPC) using pure water as eluent. The separation was poor and subsequent analyses yielded little meaningful information. We have found that a much improved separation of the products can be achieved with a similar system using an ionic eluent and we now report the novel structural conclusions derived from analyses of the fractions.

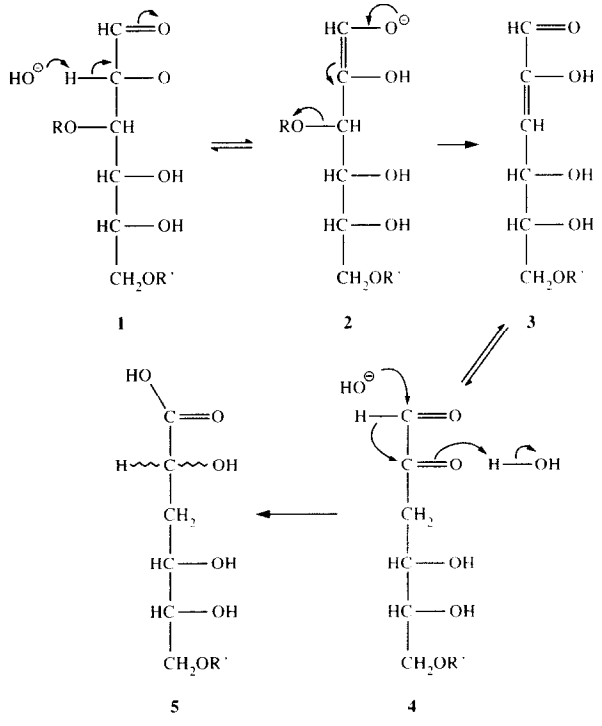


Fig. 2. Alkali-peeling mechanism for a (1→6)-branched β-D-(1→3)-galactopyranan.

With regard to terminology, since the multiplex is the form which AG maintains in the natural state and in neutral aqueous solution, it is the form we have previously referred to by the acronym 'AG' (or more specifically, 'AG-A'). However, since the multiplex dissociates into individual molecules before commencement of the peeling reaction in alkali, we frequently use 'AG' in this paper to denote these independent, unassociated molecules. In the previous paper of this series, this form of AG was referred to as 'DAG' (disordered AG). In this paper, the exact meaning of the term 'AG' must be judged from the context.

RESULTS AND DISCUSSION

Alkaline degradation

AG was degraded by boiling under reflux in deoxygenated, aqueous $Ca(OH)_2$ (lime water). This base was chosen because calcium ions catalyze the benzilic acid rearrangement (last step in Fig. 2), thus effecting a much 'cleaner' degradation with less competition from non-peeling reactions (O'Meara & Richards, 1960). Reactants in separate solutions were heated to boiling before being mixed. The combined solution immediately developed an intense, opaque yellow color, which gradually darkened to brown during the first two or three minutes of the reaction.

In one experiment, samples were removed from the ongoing reaction at intervals for later analysis to measure the rate of reaction. After rapid cooling, neutralization and filtering each sample was analyzed with an HPLC system described previously (Ponder & Richards, 1997a). In this system, with 50 mM $NaNO_3$

eluent, unmodified AG elutes at a retention time of 8.30 min. The samples of partially degraded material eluted as two peaks, the first of these being the disordered and partly degraded polysaccharide, which eluted at a retention time that increased from 8.74 to 8.80 min with increasing extent of degradation. The second of the two peaks eluted at 9.8 min and contained all of the lower molecular weight products of the degradation. The size of this peak relative to that of partly degraded polysaccharide gives a measure of the degree of degradation since the combined peak areas remained approximately constant. Thus, the extent of reaction was taken as the percentage of total peak area which occurred in the product peak at 9.8 min. The extent of reaction (%) thus determined for different sampling times (t, min) were as follows:

t: 0.25 0.50 0.75 1.00 2.00 3.00 5.00 8.00 12.0 16.0 30.0 60.0
%:27.8 41.0 54.1 62.7 77.7 84.0 89.6 91.7 92.6 93.3 93.9 94.5

These results show that 90% of the AG had degraded within the first 5 min, following second-order kinetics, i.e. a plot of 1/[AG] vs. t is linear ($R^2 > 0.99$), where [AG] = 100 − %. This is consistent with the rate data for the alkaline degradation of 3-O-methyl-D-glucose (3-MeGlc) obtained by Kenner & Richards (1954). In that study 94% of the substrate was converted in lime water almost entirely into glucometasaccharinic acid by second-order kinetics. This agreement between the results for AG and for 3-MeGlc suggests that both the polysaccharide and the model compound degrade by the same, single mechanism. The above HPLC data also shows that between 5 min and 1 h, an additional 4% of the original AG was degraded, but the data points for this interval do not align with the earlier ones, suggesting that a slower and probably less specific type of degradation (e.g. autoxidation and subsequent alkaline degradation) continues to occur after the initial 5 min. In addition, other results (below) suggest that much of the polysaccharide remaining after 5 min is stable to peeling. Such alkali-stable AG was probably included in the high-MW fraction reported by Young & Sarkanen (1977), which led them to propose side chains of up to ten glycose residues.

A ^{1}H NMR spectrum of the sodium salts of the products from the 1 h reaction showed the signals for the C-3 methylene groups of the metasaccharinate residues as an isolated complex of peaks from 1.60 to 2.05 ppm. The proportion of area in these peaks indicates that 31.5% of the total area in the spectrum derives from the metasaccharinate residues and this may be taken as a measure of the proportion of glycosyl residues in the main chain of AG. (A correction for undegraded material would raise this value.) In addition to signals for the expected products, this spectrum also showed signals for small amounts of the salts of small acids, which are probably products of the non-specific degradation reactions, e.g. formic, acetic and lactic acids. In another experiment, the products in the free acid form were titrated with 0.025 M NaOH and results suggested that 35.0% of glycose residues are in the main chain, under the assumption that all acidity comes from acids derived from the main chain. (A correction for acids from competing reactions would lower this value.) Our results therefore concur with previous estimates that one-third of all glycose residues in an average AG molecule are located in the main chain.

The products of the 1 h reaction in free acid form were lactonized, trimethylsilylated and analyzed by GC, and results are listed in Table 1. Products derived from side chains would be expected to elute after the glucitol internal standard and there were four such major peaks in the chromatogram. Based on their retention times, these can be presumed to be a dimer, a trimer and two kinds of tetramer, corresponding to monomeric, dimeric and trimeric side chains, respectively. Larger products would not have been seen in this analysis since TMS-pentasaccharides are too large to elute from the capillary column that was used.

A mass spectrum of the dimeric compound was consistent with galactosyl galactometasaccharinolactone(s) (Gal–GalMSL) and shows a $(M-CH_3\cdot)^+$ peak. The GC peaks for the dimer and trimer were each partially resolved into two peaks when loading on the column was light, consistent with the presence of pairs of epimers. The tetramers eluted as two peaks and were later deduced to represent branched and linear tetramers (see below). Among the monomeric products were the lactones of

Table 1. GC analysis of trimethylsilylated lactones of alkaline degradation products

Retention time[a]	Corresponding GPC fraction	GC peak area (%)	Identification (TMS derivatives)
0.66 and 0.67	7	0.94 and 1.24	Epimers of AraMSL[b]
0.87	7	1.35	GalMSL[b] (unresolved epimers)
1.55	6	18.3	β-D-Gal*p*-(1→6)-GalMSL[c]
2.6	5	65.4	β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→6)-GalMSL[c]
5.1 and 7.5	4	8.9 and 3.8	Branched and linear tetramers[c]

[a]Relative to TMS-glucitol. MSL = metasaccharinolactone
[b]Based on retention times and mass spectra of authentic isomeric compounds.
[c]Based on retention times of malto-oligosaccharides and on subsequent analyses.

galactometasaccharinic acid (GalMSL, two epimers unresolved) and the lactones of 3-deoxy-*erythro*- and 3-deoxy-*threo*-pentonic acid ('arabinometasaccharinic' acid lactones, AraMSL). The designation 'AraMS' for the 3-deoxypentonic acids implies that they are derivatives of Ara residues in the original polysaccharide. We regard this as a safe assumption despite the fact that 5-carbon metasaccharinic acids have been reported as products of the alkaline degradation of cellulose (Richards & Sephton, 1957) since conditions for the latter degradation were considerably more drastic than those used in the present study. The epimers of AraMSL have previously been detected in the alkaline degradation products of AG by Young & Sarkanen (1977), who interpreted them as evidence of Ara residues in the main chain.

Separation of products

The products of the 1 h reaction were separated as their ammonium salts in 0.1 M NH_4HCO_3 on Bio-Gel P-2 and collected as fractions. Figure 3 is the chromatogram from a single run. Fraction 1 eluted at the exclusion limit of the column and corresponds to the alkali-resistant AG mentioned above. This peak constitutes 6.9% of the total area in Fig. 3 and when the degradation time is extended to one day or to one week, this proportion becomes 6.4 or 4.3%, respectively. A separate SEC analysis of this fraction from the 1 h reaction, by means of a Shodex KB-804 column and 50 mM $NaNO_3$ eluent, showed an asymmetric peak at a slightly longer retention time than that of disordered AG. A similar analysis using 0.2 mM $NaNO_3$ eluent showed two peaks, one of ion-excluded material (Ponder & Richards, 1997a) and one of neutral material, with an area ratio of 2.3:1. Fraction 1 samples from the extended degradation times mentioned above were similarly analyzed and results showed that this ratio changed little with time. The nature of these Fraction 1 components and the role of the peeling reaction in their continued losses are unclear, but it is possible that the charged material consists of AG that has been stabilized against further peeling by a 'stopping reaction' (Machell & Richards, 1957), which converts a main-chain residue to an acidic residue without elimination of the rest of the main chain, e.g. due to occurrence of a (1→4)- or (1→6)-link in the main chain. The other major peaks seen in Fig. 3, Fractions 2 to 7, were subsequently found to be the major products of the alkaline degradation. Two additional peaks, not shown in Fig. 3, were observed at later elution times (8.1 and 9.0 h) and these were due mostly to $Ca(OH)_2$ and NH_4HCO_3, respectively. The $Ca(OH)_2$ peak was also found by ^1H NMR to include small amounts of formic and acetic acids in an approximate molar ratio of 8:1. The combined amount of these acids was less than 1% (w/w) of the total degradation products.

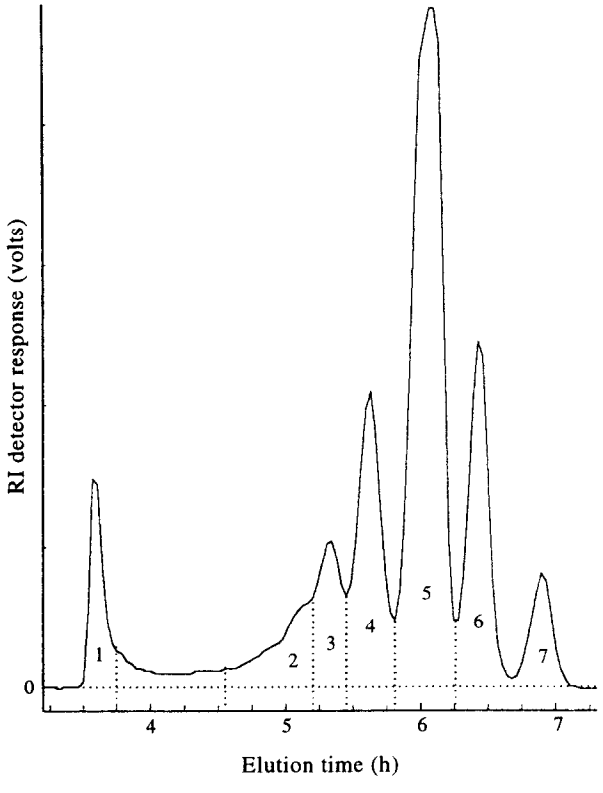


Fig. 3. Alkaline degradation products on Bio-Gel P-2.

Fractions 3 to 7 in free acid form were lactonized, trimethylsilylated and analyzed by GC. That the Fraction 3 material did not elute from the capillary column indicated that it was larger than a tetramer. The other fractions gave GC peaks which corresponded to peaks observed before separation (Table 1). Fractions 1 to 6 were hydrolyzed and lactonized, reduced, acetylated and analyzed by GC. GalMS residues were thus converted to 3-deoxyhexitol peracetates (AraMS occurred only in Fraction 7, i.e. only as monomer). Results are given in Table 2.

The implied formulae given in the table for the Ara-containing fractions should be regarded as average formulae for mixtures of products. Formulae for Fractions 6, 5 and 4 are consistent with the assessment (Table 1) of these products as being dimeric, trimeric and tetrameric, respectively. The formula for Fraction 3 suggests that this fraction contains pentamers derived from tetramer side chains and the formula for Fraction 2 suggests that it contains hexamer and probably larger products derived from side chains of five or more residues. The GPC chromatogram in Fig. 3 shows a somewhat arbitrary line defining the left side of Fraction 2 and it is apparent that a small amount of additional side-chain material elutes earlier and probably represents still larger side chains. This material merges with the tail of Fraction 1, which may consist of short polymeric segments stabilized against further

Table 2. Compositional analyses[a] of AG and its alkaline degradation products

	Gal	Ara	GalMS	Implied formula[b]
AG	85.2±0.3	14.8±0.3	—	$(Gal_{5.8}Ara)_n$
Fraction 1	84.4±0.5	14.0±0.4	1.5±0.6	$Gal_{6.0}Ara_{1.0}GalMS_{0.1}$
Fractions 2 to 6	56.2±1.5	12.4±0.3	31.4±1.6	$Gal_{4.5}Ara_{1.0}GalMS_{2.5}$
Fraction 2	40.5±1.0	44.2±0.8	15.6±1.6	$Gal_{2.6}Ara_{2.8}GalMS$
Fraction 3	37.5±1.0	42.8±0.7	19.8±1.8	$Gal_{1.9}Ara_{2.2}GalMS$
Fraction 4	49.8±0.6	23.5±0.5	26.7±0.8	$Gal_{1.9}Ara_{0.9}GalMS$
Fraction 5	65.1±1.3	—	34.9±1.2	$Gal_{1.9}GalMS$
Fraction 6[c]	50.0	—	50.0	$Gal_{1.0}GalMS$

[a] $n = 3$ (minimum), values (mole%)±1 standard deviation.
[b] Subscripts have been rounded to two significant digits.
[c] Used to determine response factor for GalMS; see Experimental section.

peeling, e.g. when this tail is included in the analysis of Fraction 1, the relative amount of GalMS detected is several times larger than that reported for Fraction 1 in Table 2.

Fractions 2 to 6 are the peeling products which contain aldosyl residues. Analyses of these combined products from the 5 min reaction and from the 1 h reaction (Fractions 2 to 6, Table 2) resulted in Gal:Ara ratios of 4.5±0.2 in both cases. This value is greater than the expected theoretical value of 3.5, assuming complete consumption of a homogenous galactan main chain composed of one-third of all glycose residues. Therefore a significant proportion of Ara is consumed and these losses coincide with the occurrence of peeling. A calculation using the measured Gal:Ara ratio of 4.5, and based on the assumption that peeling consumes 33% of all glycosyl residues in AG, leads to an estimated 19% of total Ara lost during peeling (i.e. 2.8% out of the original 14.8% Ara).

The peak shapes and relative areas for the Ara-containing fractions (2, 3 and 4) in the gel chromatogram (Fig. 3) are virtually the same in the corresponding chromatograms obtained after boiling in lime water for as short a time as 5 min or for as long as a week. Indeed, all of the peak proportions in the gel chromatogram of the products (excluding Fraction 1) remained almost unchanged during this entire span of time, the only significant difference being a 67% increase in the relative size of the Fraction 7 peak, which reflects a gradually increasing lactic acid yield. This indicates that all glycosidic linkages in these products possess long-term stability under these conditions. Thus we cannot invoke the concept of alkali-labile arabinosidic linkages in the side chains as a possible explanation for Ara consumption during peeling. Rather, these Ara losses must be due to peeling itself, from which it follows that some Ara is in the main chain, as proposed by Young & Sarkanen (1977). As for Ara in the side chains, a noteworthy feature of the data in Table 2 is a conspicuous absence of any detectable products corresponding to side chains composed only of monomeric or dimeric Ara. This observation refutes the assumption, frequently made in the past, that the predominate disposition of these groups involves direct attachment to the main chain.

Table 3 lists results of methylation analyses of undegraded AG (Ponder & Richards, 1997b), of Fractions 2 to 6 (combined) and of Fractions 2, 3 and 4 from the 1 h degradation. For Fraction 5, this analysis only indicated the presence of equimolar amounts of 6-linked and terminal Gal*p*, and for Fraction 6, only terminal Gal*p* was indicated. No

Table 3. Methylation analyses[a] of AG and its alkaline degradation fractions

Linkage	AG	Fractions 2 to 6[b]	Fraction 2	Fraction 3	Fraction 4
Ara*f*-(1→	6.9±0.3	6.2±0.4	17.5±0.8	19.8±0.7	25.1±1.0
Ara*p*-(1→	3.9±0.1	2.8±0.2	17.1±1.3	15.8±1.5	2.4±0.2
→3)-Ara*f*-(1→	4.1±0.3	2.9±0.3	17.5±1.5	16.9±1.6	2.5±0.4
Gal*p*-(1→	26.0±0.6	28.1±1.2	8.9±1.1	11.2±0.8	27.6±1.7
→3)-Gal*p*-(1→	2.6±0.8	0.5±0.3	1.7±0.2	1.8±0.1	2.2±0.5
→6)-Gal*p*-(1→	21.4±1.0	20.0±1.3	15.9±1.2	13.7±0.7	18.9±0.5
→3,4)-Gal*p*-(1→	2.9±0.4	1.7±0.2	10.4±0.6	9.8±0.6	2.9±0.4
→3,6)-Gal*p*-(1→	30.5±0.8	3.6±0.3	8.1±0.5	9.4±1.7	18.4±1.4
→3,4,6)-Gal*p*-(1→	1.7±0.3	0.3±0.1	2.9±0.5	1.6±0.2	
		Total Ara			
linkage analysis	14.9±0.4	11.9±0.5	52.1±2.1	52.5±2.3	30.0±1.1
sugar analysis[c]	14.8±0.3	12.2±0.1	52.2±1.0	53.5±0.9	32.1±0.3

[a] $n = 4$ or 5; values (mole%)±1 standard deviation; GalMS component excluded.
[b] Normalized to 66% total for comparison with AG results.
[c] From data in Table 2, excluding GalMS component.

derivatives corresponding to the GalMS residues were detected in these GC analyses and it is assumed that they, like uronic acids, are not amenable to the usual methylation analysis.

As with all earlier linkage analyses of AG, the Ara is represented by three types of glycosidic linkages. Earlier work has indicated that the Ara*f*-(1→ residues are attached directly to Gal*p* residues and that Ara*p*-(1→ is the 3-linked substituent on →3)-Ara*f*-(1→ in an arabinobiosyl group (Ara_2). Bouveng & Lindberg (1961) isolated this group from AG as a disaccharide in 2.0% yield using partial acid hydrolysis and by similar means we have isolated it in approximately 2.7% yield (w/w). Comparison of this yield with the methylation analysis results for AG (Table 3) shows that most of the Ara*p*-(1→ and most of the →3)-Ara*f*-(1→ are accounted for by Ara_2 and it is unlikely that a yield of this magnitude could be obtained by partial acid hydrolysis if the relevant linkages occurred in any other structures to a significant extent. This question is relevant to the proposal of Ara in the main chain because such Ara could occur in only two possible ways, which can be described as follows.

Possible types of Ara in the main chain:

Type 1: →3)-Ara*f*-(1→ residues interrupting an otherwise homogenous galactan main chain

Type 2: Ara groups, either monomeric or dimeric, at the main chain's non-reducing end

The amount of Type 1 could not exceed about one mole% and be consistent with the linkage analysis and partial hydrolysis results for AG. The amount of Type 2 is limited by the size of the AG molecule since only one Ara group per molecule could be present at the non-reducing terminus. If we use a molecular weight of 12000 as an estimate for this size, i.e. dp *c*. 75 (Ponder & Richards, 1997b), then the Ara residues in a single Ara_2 group would comprise 2.7 mole% of glycose residues or 18 mole% of Ara in AG. Thus in total we may suppose that as much as 4 mole% of residues in AG could be main-chain Ara residues, i.e. about one quarter of all Ara in AG, and our measured Ara losses are well within this estimate.

Fractions 2 to 6 are the peeling products that contain glycosyl residues. The mole% values for the combined Fractions 2 to 6 in Table 3 are normalized to 66% so that they may be compared directly to the values for undegraded AG (i.e. we assume that 33% of all glycose residues are consumed by peeling). These results suggest that the Ara losses are distributed among all types of Ara, with highest losses for the two types of Ara contained in Ara_2 groups. Losses for Ara*p*-(1→ and for →3)-Ara*f*-(1→ compare favorably with the losses expected if each AG molecule had an Ara_2 group at the non-reducing end of the main chain. Moreover, Table 3 shows that the approximate equimolar relationship between 3-linked Ara*f* and terminal Ara*p*, which is seen in the original polysaccharide, is preserved in the unfractionated alkaline degradation products (Fractions 2 to 6) as well as in all three of the Ara-containing fractions (Fractions 2, 3 and 4). This supports the idea that these Ara linkages are nearly always associated with each other in Ara_2 groups and thus also militates against the idea that a significant amount of the 3-linked Ara*f* occurs without terminal Ara*p* in the main chain. Thus, main-chain Ara of Type 1 appears unlikely. As for the observed losses for terminal Ara*f* (0.7±0.5 mole%, Table 3), this corresponds to the loss of 0.5±0.4 of a single Ara*f*-(1→ residue from each AG molecule, assuming average dp 75. The statistical significance of this value is marginal at best and the simplest explanation for it is that the non-reducing end of the main chain is occasionally terminated by an Ara*f*-(1→ group rather than by the usual Ara_2 group.

Methylation analysis results for Fractions 5 and 6 were unambiguous in terms of the structures of compounds found in these fractions. However, results for Fractions 2, 3 and 4 (Table 3) are open to several interpretations and it is clear that these fractions contain mixtures of oligomers representative of different types of side chains. By coincidence, each of the Ara-containing fractions contains approximately the same proportion (21%) of branched residues, but this implies a different proportion of branch-containing side chains in each case. Thus, the 21% of branched residues in Fraction 4 implies that 63% of trimer side chains are branched, the 21% of branched residues in Fraction 3 implies that 84% of tetramer side chains are branched, and the 21% of branched residues in Fraction 2 implies that virtually all of the pentamer and larger side chains are branched and/or that many of them contain more than one branched residue. The overall conclusion to be drawn from these observations is that linear side chains are the exception rather than the rule for the larger side chains.

Each of the fractions, as their sodium salts, were analyzed in D_2O by ^1H NMR (see signal assignments in Ponder & Richards, 1997b). The spectrum of Fraction 1 was similar to that of disordered AG, with signals for C-3 methylene protons discernable but too weak to integrate with precision. The spectra of Fractions 2 and 3 were similar to each other, with all of the Ara anomeric signals (4.9–5.7 ppm), which are relatively weak in original AG, seen as conspicuous, major signals that measured about half of the total area for anomeric protons. For Fraction 4, the Ara anomer signals measure about one-third of the total anomer area, the majority of this proportion being found in a well-defined doublet (1.2 Hz) at 5.2 ppm. This is due to the terminal Ara*f* indicated as the dominant Ara residue in Fraction 4 by the methylation data (Table 3), and the small coupling constant indicates an α linkage. No Ara signals are seen in the

spectra of Fractions 5 or 6. These observations are consistent with the compositional results and proton integrations agree with the implied formulae, as shown in Table 4.

In the spectra of Fractions 2, 3 and 4, the major Gal anomer signal at 4.4 ppm is broad and complex, whereas in Fractions 5 and 6 it is relatively sharp and simple, being a doublet in the latter case and a pair of overlapping doublets in the former. The spectra for Fractions 5 and 6 have been assigned using a variety of 1D and 2D NMR techniques (Manley-Harris, 1997). These studies confirm that the dimer of Fraction 6 is *β*-D-Gal*p*-(1→6)-GalMS and that the trimer of Fraction 5 is *β*-D-Gal*p*-(1→6)-*β*-D-Gal*p*-(1→6)-GalMS, the product in each case occurring as a pair of C-2 epimers of the GalMS residue.

NMR shows that there is one predominant type of Ara residue in Fraction 4, namely terminal α-L-Ara*f*. Its abundance in this fraction (25 mole%), coupled with the estimate that 63% of trimeric side chains are branched, leads to the conclusion that this Ara occurs in branched side chains. Other constraints from the methylation data (Table 3) require that the major branch point in question be →3,6)-Gal*p*-(1→ and that the third residue be terminal Gal*p*. Moreover, an HMBC spectrum of Fraction 4 shows long-range coupling between C-1 of the Ara*f* residue and H-3 of the →3,6)-Gal*p*-(1→ residue (Manley-Harris, 1997). It follows that the major trimer side chain consists of an elaboration on the Gal dimer side chain, in which an Ara*f* residue is α-(1→3)-linked to the inner Gal*p* residue. These NMR studies further demonstrate that another major component of Fraction 4 is derived from yet another elaboration on the Gal dimer side chain, namely a linear (1→6)-linked Gal trimer side chain. These two products account for 83% of the linkages indicated for Fraction 4 in the methylation analysis. They are illustrated in Table 5 along with two probable minor products that account for the remaining linkages in Fraction 4.

The ^{1}H NMR spectrum of Fraction 7 shows that lactic acid is another major component in this fraction in addition to AraMS and GalMS. Lactic acid is a general alkaline degradation product of glycoses and its presence in the products from AG is an indication of the occurrence of some non-peeling pathways. Integration of the doublet at 1.4 ppm indicates that lactic acid comprises about 38 mole% (or 27 wt%) of Fraction 7 and this corresponds to 1.4 wt% yield of lactic acid from original AG, based on GPC peak area. The other 73 wt% of Fraction 7 consists of AraMS and GalMS in an approximate wt/wt ratio of 1.6:1 (Table 1). This ratio (±10%) was confirmed by GC analyses both of the TMS-lactones (1.5:1) and of the 3-deoxyalditol peracetates (1.8:1) of Fraction 7. It leads to an estimated yield from AG of 2.3 wt% for AraMS and of 1.5 wt% for GalMS.

The most likely origin of the GalMS is unbranched main chain residues and the AraMS probably derives from Ara residues consumed by the peeling reaction. Most of the latter probably occur as Ara_2 groups at the non-reducing ends of the main chains. Conversion of →3)-Ara*f*-(1→ residues in these Ara_2 groups to AraMS is a straightforward consequence of peeling, whereas conversion of Ara*p*-(1→, initially released as free Ara, to AraMS requires the slower *β*-hydroxycarbonyl elimination (cf. step **2**→**3** of Fig. 2). Other workers have shown that pentoses in lime water at 25°C yield the C-2 epimers of the corresponding 3-deoxyaldonic acid as the major product (Ishizu *et al.*, 1967). To test the efficiency of this process under the present reaction conditions, L-arabinose was boiled in lime water for 5 min, after which the solution was neutralized and analyzed on the GPC system. Only one major peak was observed in the chromatogram and it eluted with the same retention time as that of Fraction 7 in Fig. 3. Analysis of the material collected at this major peak by GC (as TMS-lactones) and of the unfractionated material by NMR (as sodium salts) showed that about half of the product mixture was AraMS and about one quarter was lactic acid (w/w). Additional support for the idea that AraMS derives from residues at the non-reducing end consists of the observation that at early stages of the peeling reaction AraMS is only a minor constituent of Fraction 7, e.g. when the reaction is stopped at 27% completion (as determined by GPC areas), the AraMS/GalMS ratio (as determined by GC of TMS lactones) is only 0.1. This ratio increases to 1.6±0.2 during the course of peeling and afterwards it does not change. This behavior is consistent with the

Table 4. ^{1}H NMR spectra of alkaline degradation products

Fraction	Formulae from Tables 1 and 2	Integrated area (% of total)			
		C-3 methylene protons		Anomeric protons	
		Theoretical	Observed	Theoretical	Observed
2	$Gal_{2.6}Ara_{2.8}$ GalMS	4.8	4.6	12.8	14.1
3	$Gal_{1.9}Ara_{2.2}$ GalMS	6.0	6.0	12.2	12.4
4	$Gal_{1.9}Ara_{0.9}$ GalMS	7.8	7.8	10.9	11.0
5	Gal_2GalMS	9.5	9.6	9.5	9.4
6	Gal–GalMS	14.3	14.9	7.1	7.2

Table 5. Probable composition of Fraction 4

Product	Mole% of Fraction 4
β-D-Gal*p*-(1→6) β-D-Gal*p*-(1→6)–GalMS α-L-Ara*f*-(1→3)	55
β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→6)–GalMS	28
α-L-Ara*f*-(1→4) β-D-Gal*p*-(1→6)–GalMS α-L-Ara*f*-(1→3)	9
β-L-Ara*p*-(1→3)-L-Ara*f*-(1→3)-β-D-Gal*p*-(1→6)–GalMS	7

idea that GalMS derives from residues interspersed along the length of the main chain whereas AraMS derives from terminal residues, which become accessible to peeling only in the later stages of the reaction.

Summary and conclusions

Key results from this study are given in Table 6, which lists the alkaline degradation products ascribable to peeling. Listed are fraction numbers as given in Fig. 3 and the percentage of total area in each section of the chromatogram for Fractions 2 to 7. The area given for Fraction 7 has been adjusted to allow for the fact that only a portion (73%) of the observed peak area corresponds to GalMS and AraMS. Also listed are estimated percentages of types of main-chain residues represented in each of the fractions, each Gal*p* type being characterized by the type of attached side chain, if any. For each fraction, this number was arrived at by dividing the area percent by the (average) number of monomers in the product(s) of that fraction, and then normalizing to 100%. (Prior to this calculation, areas for the Ara-containing fractions were adjusted to allow for the smaller response of an Ara residue, on the assumption that the RI detector response of a glycosyl residue is a function of its size or weight.) The two monomer products of Fraction 7 are treated separately, with the adjusted area of the fraction divided according to the estimated proportions of the products.

These results show that about half of all side chains are Gal dimers and that about one quarter of them are single Gal residues. The remaining quarter of side chains are those that contain Ara and are composed of more than two glycose residues. (Note that these estimates describe an *average* AG molecule. Significant variation of these quantities occurs among individual molecules and this aspect of AG will be considered in the next paper of this series.) The Ara-containing side chains exhibit a variety of structures and generally appear to incorporate greater amounts of branching and of Ara content with increasing size. The largest side chains are the least abundant and probably become insignificant beyond a dp of about 7.

Practically all of the Ara occurs as either terminal Ara*f* or as the Ara_2 group [β-L-Ara*p*-(1→3)-L-Ara*f*-(1→] and all Ara detected in the alkaline degradation products occurs in side chains larger than two residues. This rules out the possibility that the Ara in these products was attached directly to the main chain in the original polysaccharide and since this Ara constitutes four-fifths of the original Ara, we conclude that Ara occurs mostly on the periphery of the AG molecule at the ends of relatively long side chains. A precedent for this concept is the peripherally attached terminal Ara*f*, which has been proposed for many arabinogalactan proteins (Stephen, 1983). The most plausible explanation for the Ara that is missing from the alkaline degradation products is that it corresponds to Ara or Ara_2 groups attached 1→3 at the non-reducing end of the main chain.

Table 6. Relative amounts of peeling products from an average AG molecule

GPC fraction	Area(%)[a]	MC(%)[b]	Products[c]
2	7.2	3.2	$Gal_{2.6}Ara_{2.8}$GalMS, extensively branched; Ara mostly as Ara_2
3	8.3	4.8	$Gal_{1.9}Ara_{2.2}$GalMS, 84% branched, mostly at →3,6)-Gal*p*-(1→ and →3,4)-Gal*p*-(1→; Ara mostly as Ara_2
4	15.7	11.3	$Gal_{1.9}Ara_{0.9}$GalMS, 63% branched, mostly at →3,6)-Gal*p*-(1→; Ara mostly as terminal Ara*f*
5	49.0	46.9	β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→6)–GalMS
6	15.8	22.7	β-D-Gal*p*-(1→6)–GalMS
7	3.8	4.2	GalMS
		6.8	AraMS

[a]Mean values of peak areas from five injections; relative standard deviations < 4%.
[b]Percentage of source main-chain (MC) residues as a proportion of total residues in the main chain.
[c]Average formulae given for Fractions 2, 3 and 4.

Figure 4 shows the structural implications of this study. The figure shows ten main-chain residues, seven of which carry side chains at C-6. Atypically linked main-chain residues (e.g. branched at C-4), though probable, are not depicted and neither is the known small amount (*c.* 0.2%) of uronic acid residues (Ponder & Richards, 1997a). In addition, the Ara-containing side chains show only the major types of linkages which occur in them. Finally, the Ara group shown at the non-reducing end is an Ara_2 group, though single Ara*f*-(1→ residues are also possible. Thus, the figure is intended to illustrate only major features of an average or typical AG molecule. Percentages above the figure are estimates of the percentage of main-chain residues carrying the type of attached side chain (if any) shown beneath. Four types of novel side chains are depicted and labeled I through IV. I and II are found in Fraction 4 of the alkaline degradation products, and III and IV are found in Fraction 3. Percentages for side-chain types I and II account for most of the trimeric side chains in AG. Other types of trimeric side chains (not depicted) contribute an additional 2% (Table 5). Percentages for side-chain types III and IV are combined since methylation analysis could not distinguish between them and only singly branched side chains are depicted; other types of tetrameric side chains (not depicted) contribute an additional 1%. Side chains that contain five or more glycosyl residues (Fraction 2) are denoted R′. Somewhat more speculative than the proposed side chains is the Ara_2 group shown at the non-reducing end and the percentage value given for this is based on an estimate of 25 glycosyl residues in an average main chain.

The largest side chains, types III, IV and R′, are depicted with considerable ambiguity as the evidence does not justify the proposal of specific structures. Complexities in the NMR spectra suggest that the structures of these side chains are highly variable, with numerous possible linkage environments for the Ara residues. This makes it difficult to envisage a regular repeating unit in AG. Moreover, the relatively small weight of an average AG molecule (*c.* 12000) corresponds to a dp of about 75, of which some 11 residues are Ara, with perhaps two of these typically at the non-reducing end of the main chain. It is therefore unlikely that all types of even the major large side chains are represented in any single AG molecule.

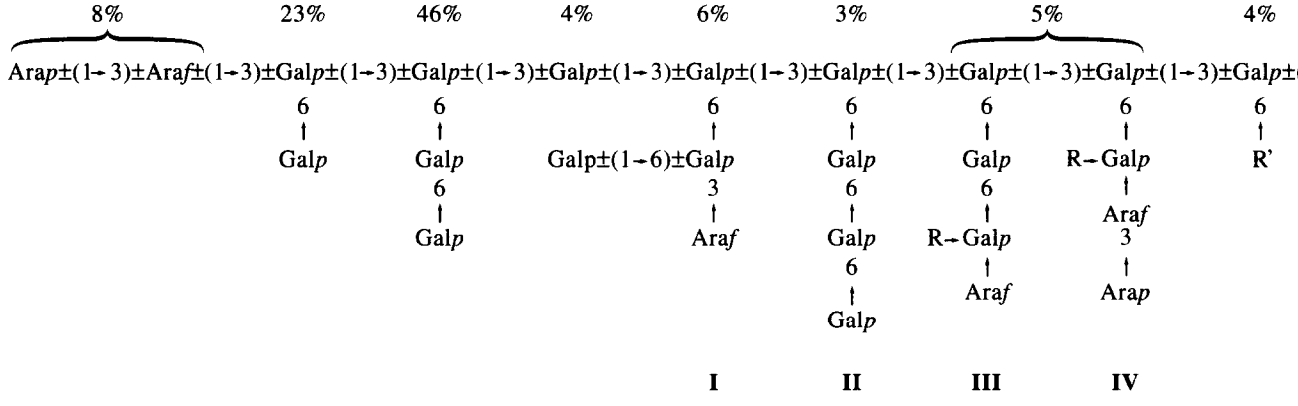
EXPERIMENTAL

Alkaline degradation

AG starting material, described previously (Ponder & Richards, 1997a), was used as received, i.e. in the ordered form. In a typical experiment, AG (5.0 g) in water (50 ml, N_2 sparged) was heated to boiling, then added to refluxing, saturated calcium hydroxide (lime water, 50 ml, N_2 sparged). The solution was boiled under reflux for 1 h with an excess of solid $Ca(OH)_2$ in the flask. It was then filtered hot and, after the clear, brown filtrate had cooled, was worked up for analysis in one of the ways described below. In the reaction rate experiment, samples (5 ml) were removed from the flask and immediately added to ice (4 ml) containing Amberlite IR-120(H^+) resin (2 ml). These solutions were filtered (0.45 μm) prior to HPLC analysis.

Chromatography

Preparative separation of the alkaline degradation products was achieved on a Bio-Gel P-2 (bead size < 45 μm) column (90×2.6 cm i.d.) that was pumped continuously with 0.10 M NH_4HCO_3 at 0.6 ml/min with



Gal*p* = β±D±Gal*p*, Ara*p* = β±L±Ara*p*, Ara*f* = α±L±Ara*f*.
R = Gal*p* or Ara*f*, R'= side-chains containing more than 4 residues.
Branched Galp residues in III and IV are linked →3,6)± or →3,4)±
Ara mole fractionin III, IV and R' ≈ 0.5, with Ara on periphery.

Fig. 4. Major structural features of a typical larch arabinogalactan molecule.

a Waters 501 HPLC pump. The column was calibrated with malto-oligosaccharides but neutral compounds eluted later than charged compounds of comparable molecular size, e.g. galactose eluted at 9.5 h retention time, while ammonium galactometasaccharinate (GalMS) eluted at 6.9 h retention time. For preparative purposes, 55 mg samples were loaded using a 1.1 ml loop on a Rheodyne 7125 injection valve. Prior to injection on the column, the products were converted from calcium to ammonium salts by addition of NH_4HCO_3 to the cooled solution, followed by centrifugation and filtration to remove $CaCO_3$ precipitate. Enough NH_4HCO_3 was added to effect this exchange and to bring the solution to *c.* 0.1 M NH_4HCO_3, as necessary to prevent a negative 'water peak' in the chromatogram; slight overestimation of this amount often resulted in a peak at 9.0 h retention time. In addition, the persistence in solution of a small amount of $Ca(OH)_2$ resulted in a GPC peak at 8.1 h retention time. A Waters R401 differential refractometer was used for detection and the signal was monitored and accumulated using a PC fitted with an A/D convertor (sampling interval: 2 min). Effluent was collected with a Bio-Rad Model 2110 Fraction Collector set to index every 6 min (i.e. 3.6 ml). For each SEC fraction, peak area was correlated with the weight of material (as the sodium salt) which eluted in that fraction after evaporation to dryness, i.e. response factors were determined and these were found to be approximately the same (±5%) for Fractions 1 to 7. The material corresponding to each fraction was collected in three to five test-tubes, allowing analyses to be performed on material from the center of each fraction.

After separation of the products, excess Amberlite IR-120(H^+) resin was used (batch treatment at 25°C for 30 min) to decompose the NH_4HCO_3 and to convert the MS residues to the acid form. After filtration, excess IR-120(Na^+) resin was used (column treatment at 25°C) to convert the products to sodium salts for NMR analyses. Alternatively, the products were lactonized for trimethylsilylation as described below. Compositional and methylation analyses gave the same results regardless of whether the products were used in the acid or salt forms.

The GC system has been described previously (Ponder & Richards, 1997b). For TMS derivatives, the temperature program began at 55°C isothermal for 1 min followed by a rate of 30°C/min to 180°C, then 10°C/min to 280°C, then 30°C/min to 320°C. Monomeric products and glucitol internal standard eluted during the 10°C/min ramp, whereas dimeric and larger products eluted during the 320°C isothermal part of the program. Under these conditions, TMS-glucitol eluted from the Ultra-2 column at *c.* 13.5 min and relative retention times of the products are given in Table 1. The TMS derivatives of maltose, maltotriose and maltotetraose eluted at 1.43, 2.47 and 7.1, respectively, relative to TMS-glucitol. Trimethylsilylated, C-2 epimeric lactones of the 5-carbon metasaccharinic (i.e. 3-deoxypentonic) acids (TMS-AraMSL) were identified by matching their retention times and mass spectra to those of the analogous compounds derived from alkaline degradation of 3-O-methyl-D-xylose (Ponder & Richards, 1991). No significant differences were discerned between the mass spectra of the L-arabinose-derived compounds and those of the D-xylose-derived compounds. Likewise, the retention time and mass spectrum of the unresolved trimethylsilylated lactones of the metasaccharinic acids derived from galactose (TMS-GalMSL) were similar to those of the analogous derivatives from glucose (Ponder *et al.*, 1992). Among the larger products, a complete mass spectrum was obtained only for the trimethylsilylated lactones of the dimer because the *m/z* limit for the instrument is 800. Major *m/z* signals (and relative intensities) for the combined epimers of this compound [TMS-β-D-Gal*p*-(1→6)-GalMSL] are as follows: 129 (23), 147 (32), 204 (100), 217 (21), 243 (5), 305 (3), 361 (9), 407 (26), 495 (3), 612 (6), 741 (2) $[(M-CH_3\cdot)^+$ (DeJongh *et al.*, 1969)].

Compositional analysis

GC analysis of peracetylated galactitol and arabinitol has been described previously (Ponder & Richards, 1997b). The C-2 epimeric 3-deoxyhexitol peracetates derived from reduction of lactonized GalMS followed by acetylation were identified from their mass spectra, each of which had the following major *m/z* signals (and relative intensities): 69 (100), 81 (96), 94 (22), 103 (28), 112 (15), 129 (43), 154 (12), 201 (25), 231 (16), 303 (10), 317 (2) $[(M-OAc\cdot)^+$ (Lönngren & Svensson, 1974)]. Retention times relative to the erythritol peracetate internal standard were 1.24 and 1.25. Their combined FID response factor (0.93±0.06) was arrived at by assuming that their combined molar yield was equal to that of galactitol peracetate in the analysis of the dimer product of GPC Fraction 6; thus the perfect 1:1 relationship between Gal and GalMS reported for this fraction in Table 2. In performing these analyses, care had to be taken to add M NH_4OH (50 μl) and 2% $NaBH_4$ in DMSO (500 μl) *simultaneously* to effect the reduction; even brief exposure to the base alone resulted in significant hydrolysis of the lactone and consequent failure to effect complete reduction. The C-2 epimeric 3-deoxypentitol peracetates derived from AraMS were produced by reduction and acetylation of lactonized Fraction 7. Retention times relative to erythritol peracetate internal standard were 1.07 and 1.08, and their mass spectra each had the following major *m/z* signals (and relative intensities): 69 (100), 82 (14), 83 (5), 103 (7), 129 (35), 142 (10), 231 (9), 245 (1) $[(M-OAc\cdot)^+]$.

Trimethylsilylation

Lactonization of alkaline degradation products in free acid form was achieved by evaporation to dryness under reduced pressure at 40°C followed by rigorous drying in a vacuum desiccator at 40°C for 24 h. The sample (2–5 mg) was combined with glucitol (*c.* 1 mg) internal standard. Silylation-grade pyridine (0.8 ml) was then added and the sample heated and ultrasonicated to achieve dissolution. Tri-Sil reagent (Pierce, 0.2 ml) was added and the sample heated at 90°C for 1 h. The sample was then blown dry with filtered dry air and hexane (1 ml) was added. Hexane-insolubles were removed by centrifugation and TMS derivatives in hexane were analyzed by GC as described above.

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