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Arabinogalactan from Western larch. Part IV. Polymeric products of partial acid hydrolysis

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Abstract

Under identical conditions of acid-catalyzed hydrolysis, disordered arabinogalactan (DAG) degrades about twice as fast as ordered arabinogalactan (AG). Size-exclusion chromatography (SEC) shows that the polymeric product from DAG becomes increasingly polydisperse as hydrolysis proceeds, whereas the polymeric product from AG persists as a narrow SEC peak. Polymeric products isolated from hydrolysates of AG and DAG at different stages of degradation, like original AG, are disordered with alkali and subsequently reordered by drying, which indicates that order is maintained in the degrading polysaccharide. Conformational restraints imposed by a network of hydrogen bonds in the ordered portion of the AG multiplex are a likely cause for its slower degradation, and this interpretation is supported by results from ^{1}H NMR. After disordering, the partially hydrolyzed AG products return to the ordered state more readily than DAG returns to AG, and this preference for order increases with extent of degradation, suggesting that prior hydrolytic removal of non-ordered portions of the AG multiplex facilitates re-establishment of order from disorder. By contrast, the partially hydrolyzed DAG products show less tendency to reorder than DAG, the more so with increasing extent of degradation. Compositional analyses of the products from AG and DAG indicate little difference in the rate of initial rapid loss of Ara residues, consistent with the view that these residues are on the periphery of the molecule and not involved with order in the multiplex. Results of linkage analyses and of alkaline degradation analyses of these products indicate that maintenance of order during hydrolysis of AG coincides with preservation of 3,6-linked main-chain Gal*p* residues with single, terminal Gal*p* residues at C-6, from which it follows that these moieties are involved in the ordered network. Part of an earlier study, which led to the conclusion that hydrolysis of AG yields two distinct polymeric fragments, was repeated, and this conclusion is critically reassessed.

Keywords: Arabinogalactan; Acid-catalyzed hydrolysis; Size-exclusion chromatography

1. Introduction

This paper is the fourth in a series devoted to renewed chemical investigations of the arabinogalactan polysaccharide that can be extracted with water from the wood of Western larch (*Larix occidentalis*). The first paper of the series discusses the unique behavior of arabinogalactan (AG) on size-exclusion chromatography (SEC) columns using low ion strength eluent (Ponder & Richards, 1997a). This behavior is an example of the ion-exclusion effect, and is caused by trace amounts of uronic acid residues in AG. The second paper shows that hydrated AG species exist naturally as ordered assemblies of molecules (multiplexes) that can be disrupted by alkali to form individual, unassociated molecules, i.e. disordered AG (DAG) (Ponder & Richards, 1997b). This order–disorder transition can be reversed by drying or freezing. The third paper introduces alkaline degradation analysis, a technique that is particularly well suited for structural analysis of AG, which possesses an alkali-labile (1 → 3)-linked main-chain (Ponder & Richards, 1997c). The present paper describes application of the above concepts and techniques to samples of AG and DAG that have been degraded by partial acid hydrolysis. These two forms of the biopolymer degrade by substantially different pathways, a comparative study of which yields clues regarding the nature of the ordered multiplex and of the forward and reverse transitions.

The literature contains numerous reports of studies on the monomeric and oligomeric products of partial acid hydrolysis of various larch arabinogalactans (Aspinall et al., 1968; Bouveng & Lindberg, 1956; Haq & Adams, 1961; Jones & Reid, 1963; Karácsonyi et al., 1984), but the partially degraded polymeric products have been largely neglected. The only significant study of such products was conducted by Bouveng (1959) on one of two components of AG that Bouveng & Lindberg (1958) had separated by a fractional

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precipitation procedure. They reported that these components possessed different molecular weights, and they referred to the major, high-weight component as 'A', and to the minor, low-weight component as 'B'. The hydrolysis study in question was conducted on A, and the observations reported can be correlated with results of the present study by reinterpreting the nature of A and B. The means by which Bouveng and Lindberg separated and distinguished these components can be influenced by the presence of low levels of uronic acid groups (Scott, 1955; Wieland, 1959), now known to be trace constituents of AG. A reinvestigation of their fractionation procedure, detailed below, leads to the conclusion that Bouveng and Lindberg inadvertently separated AG multiplexes bearing very low levels of uronic acid groups (their A fraction) from multiplexes bearing no such groups (their B fraction). Their work has had extensive influence on the subsequent literature, and it is possible that misinterpretation of these A and B fractions has been reinforced by later, misinterpreted chromatographic results (Ponder & Richards, 1997a). These considerations underscore the need for clear definitions of 'AG-A' and 'AG-B'. In the present series of papers, these terms are used as follows.

AG-A is the form of AG whose chemical species exists naturally as an ordered multiplex, probably a triple helix (Ponder & Richards, 1997b). It constitutes some 95% or more of a typical sample of AG extracted from Western larch. Its average Ara content is about 15 mol%, and its MW (of the multiplex) is about 37 000 (Prescott et al., 1995; Swenson et al., 1969). It can be disordered by alkali to form the unassociated molecules (DAG), and these can be reordered by drying or freezing. Some uronic acid residues are distributed evenly through the molecular weight distribution, and their trace amount (ca. 0.2%) is such that some molecules include these residues while others do not (Ponder & Richards, 1997a).

AG-B is the form of AG that exists naturally as discrete molecules. It constitutes some 5% or less of a typical AG sample, and its average Ara content is about 38 mol%. It is distinguished from DAG by GPC, having a longer retention time, and its MW is about 7000 to 10 000 (Swenson et al., 1969; Simson et al., 1968). Neither drying nor freezing causes it to assume a multimolecular structure, and it contains no uronic acid residues. AG-B will be described in more detail in a later paper in this series.

To a first approximation, AG in general can be equated with AG-A, since the latter is the overwhelmingly dominant component in most AG samples. For this reason the term 'AG' is used implicitly as a synonym for AG-A throughout most of this paper; however, in the section describing the work of Bouveng, 'AG' is used in the broader sense. With regard to other terminology used in this paper, SEC denotes high-pressure size-exclusion chromatography and GPC denotes low-pressure size-exclusion chromatography. The simple designations 'A' and 'B' will be used to denote the components of AG isolated by Bouveng and Lindberg.

2. Experimental

2.1. General methods

Compositional, linkage and ^{1}H NMR analyses were performed using methods and instruments described previously (Ponder & Richards, 1997b). Unless otherwise stated, AG is the purified material used in the earlier work of this series, and DAG is derived from it by a previously described mild alkaline treatment (Ponder & Richards, 1997b). The heartwood AG sample used for CTA-OH fractionation was pressed from Western larch center poles, and has been described previously (Ponder & Richards, 1997a).

2.2. Acid hydrolyses

Comparative acid hydrolyses of AG and DAG, which provided the data for Fig. 1, were conducted by combining separately heated (90°C) aqueous solutions (50 ml ea.) of 10% AG or DAG and 1.0 N H_2SO_4 in a conical flask (300 ml). The resulting solution was maintained at 90°C in a thermostatically controlled water bath, and samples (5 ml) were removed at intervals. These samples were added to excess $BaCO_3$, centrifuged, diluted to 1%, and filtered (0.45 Gμm) prior to HPLC analysis. The above conditions were also used for production (5 g scale) of polymeric products. For each product, the reaction flask was removed from the water bath at a specific time, cooled under tap water, neutralized with an equivalent amount of NaOH, then dialyzed against running water for 48 h. For the agh products, the dialysis was performed with MWCO 3.5 k tubing, and this was sufficient for cleanup of agh30m and agh1h.[1] The other agh products, however, needed additional cleanup (especially agh8h) by ultrafiltration with a 10 k membrane (Diaflo YM10) in a 50 ml ultrafiltration cell (Amicon, Inc.). The dagh products were dialyzed with tubings of different pore sizes as follows:

product:	dagh15m	dagh30m	dagh1h	dagh2h	dagh4h
tubing MWCO:	15 k	12–14 k	6–8 k	3.5 k	1 k

Spectra/Por tubing (Spectrum Medical Industries, Inc.) was used in all cases. Adequate cleanup conditions were judged by assessing the symmetry of the SEC peak obtained for the resulting polysaccharide. The dialyzed solutions were concentrated, and the products were obtained as white, freeze-dried powders. For the dagh products, small aliquots of the solution were removed prior to freeze-drying, and these were used for SEC analyses (Figs. 2, and 5).

Partial acid hydrolysis of the charged AG-A (26 mg) isolated from heartwood AG was achieved by heating in 1 M HCl (2 ml) at 100°C for 15 min, followed by dilution with water and ultrafiltration with a 10 k membrane (50 to 5 ml × 3).

[1] The products are named according to the substrate and reaction time; e.g. 'agh30m' denotes AG that has been acid-degraded for 30 min.

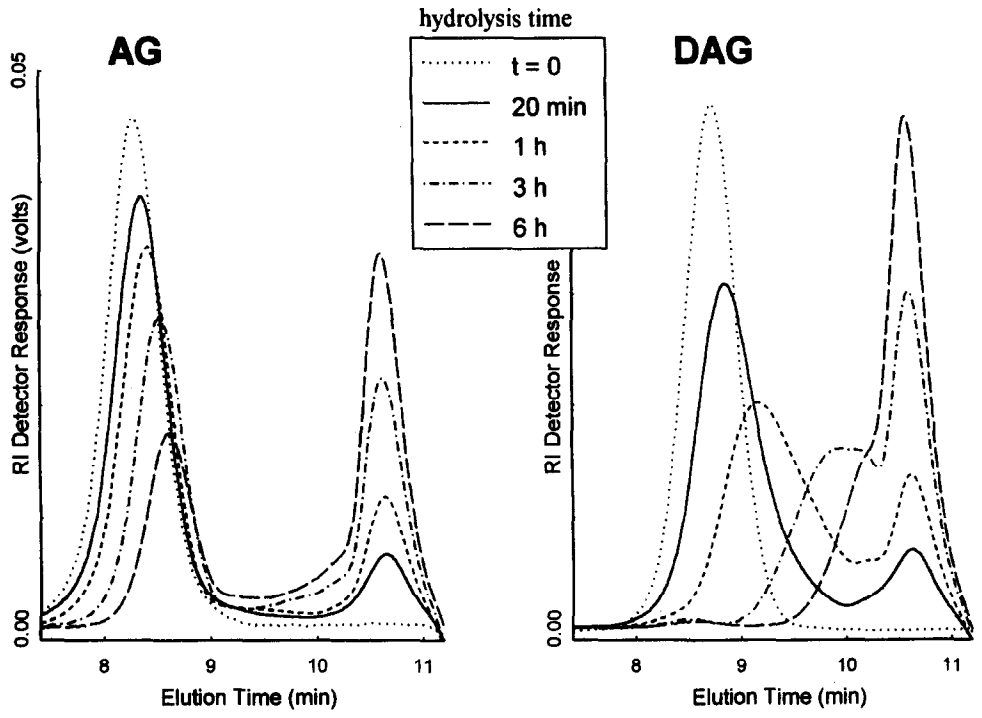

Fig. 1. SEC chromatograms of acid-catalyzed hydrolysates of AG and DAG. Hydrolysis conditions: 0.05 N H_2SO_4, 90°C. SEC conditions: Shodex KB-804 column, 50 mM $NaNO_3$ eluent, 1.0 ml min^{-1} flow rate.

2.3. Chromatography

SEC analyses were conducted with a previously described HPLC system that incorporates a Shodex KB-804 column (Ponder & Richards, 1997a). All chromatograms in Figs. 1, 2, 4 and 5 were obtained using 50 mM $NaNO_3$ eluent in the system, and chromatograms in Figs. 10 and 11 were obtained using 0.2 mM $NaNO_3$ eluent. GPC chromatograms shown in Figs. 7 and 8 were obtained with a previously described system using Bio-Gel P-2 (Ponder & Richards, 1997c). Isolation of charged AG-A from the heartwood sample was accomplished with the above GPC system using Sephacryl S-300 and 0.2 mM $NaNO_3$ eluent at a flow rate of 1 ml min^{-1}. The sample load was 50 mg;

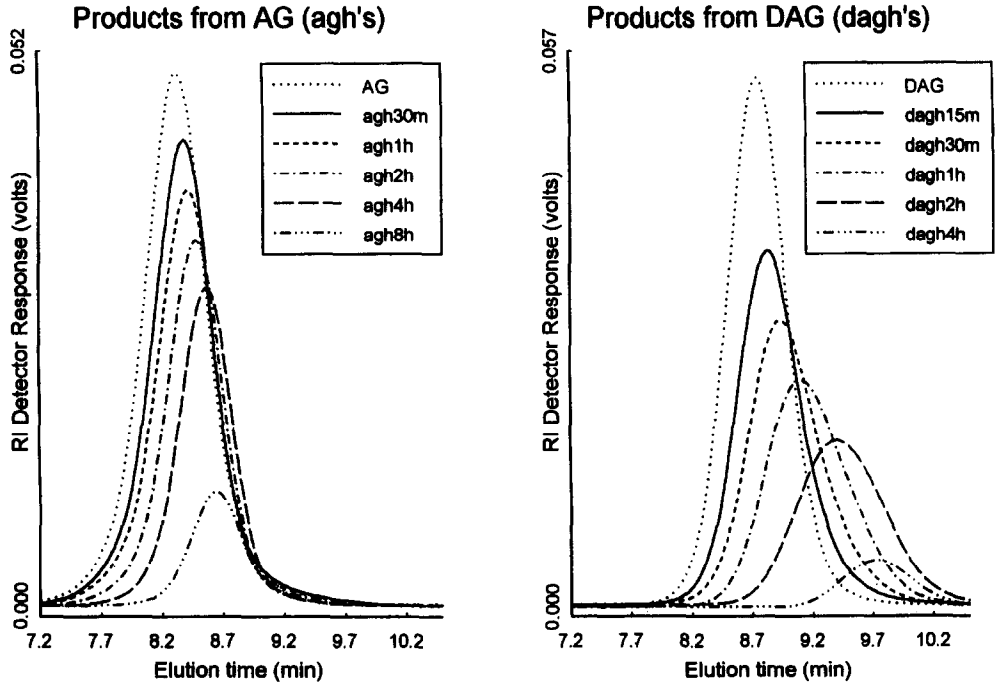

Fig. 2. SEC chromatograms of polymeric products of partial acid hydrolyses of AG and DAG.

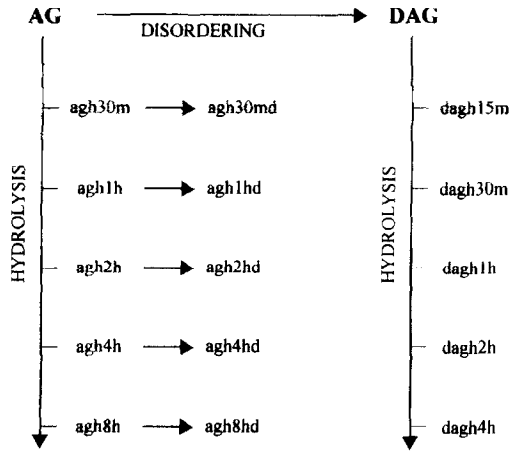

Fig. 3. Reaction pathways for products in this study.

charged AG-A eluted from 3.5 to 4.3 h, uncharged AG-A eluted at 5.5 h, and AG-B eluted at 6.1 h.

2.4. Alkaline degradation analyses

Compared to the previously reported alkaline degradation analyses of AG, those in this study were conducted on a smaller scale. In each case, the freeze-dried, degraded polysaccharide (250 mg) was placed in a glass vial (7.5 ml, sealed with a Teflon-lined cap) with saturated $Ca(OH)_2$ (5 ml) and heated (100°C) in a thermostatically controlled heating block for 1 h. After cooling under tap water, the solution was worked up with NH_4HCO_3, as described previously, in preparation for GPC analysis (Ponder & Richards, 1997c).

2.5. Fractional precipitation with CTA-OH

The crude, freeze-dried sample of heartwood AG was purified as follows. EtOH (200 ml) was added dropwise with stirring to the dissolved solids (20 g) in water (200 ml). After centrifugation, the dark pellet (0.7 g dry) was discarded. More EtOH (200 ml) was added, followed by acetone (400 ml), and the precipitate was isolated by centrifugation and washed twice with 95% EtOH (150 ml). This was then redissolved in water and re-precipitated with two volumes each of ethanol and acetone. The final precipitate was freeze-dried from aqueous solution to give an off-white solid (18.3 g). A portion (10.0 g) of this material was fractionated as precipitates from 0.176 M H_3BO_3 (354 ml) by sequential additions of cetyltrimethylammonium hydroxide (CTA-OH, 0.095 M) and NaOH (0.5 M), as described by Bouveng & Lindberg (1958). CTA-OH was made by passing the bromide (Aldrich) through a column of excess Amberlite IRA-400 (OH^-) ion exchange resin. Precipitates were isolated by centrifugation, washed with water and dissolved in 6% HOAc (25 ml). The regenerated polysaccharide was then precipitated with EtOH (200 ml). In the case of Fr.5, the final supernatant was

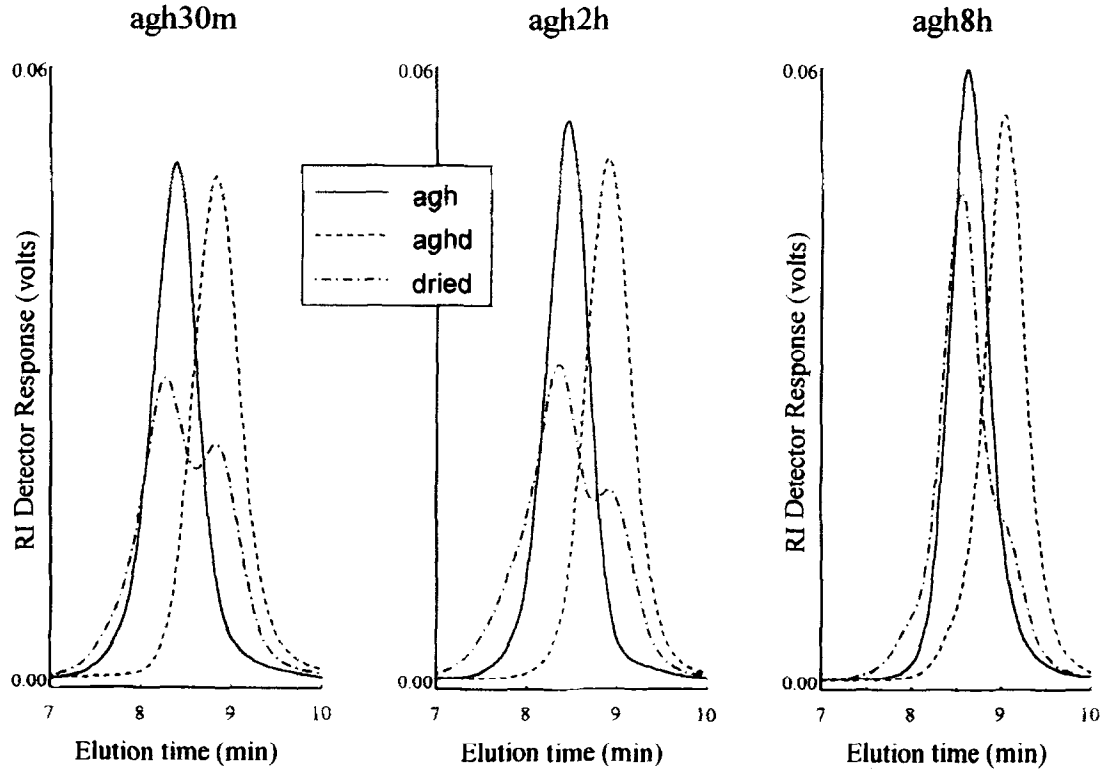

Fig. 4. SEC chromatograms of agh products, of corresponding aghd products and of partially reordered products obtained from aghd products by a single evaporation to dryness.

dialyzed (3.5 k tubing, 24 h), concentrated, evaporated with MeOH (× 3), and poured into EtOH to recover the residue (68 mg, AG-B). All fractions were dried in a vacuum desiccator at 40°C and obtained as white powders. Total recovery was 87%, and percentages of each fraction are given in Table 3.

3. Results and discussion

3.1. Comparison of acid-catalyzed hydrolyses of AG and DAG

Fig. 1 shows two sets of superimposed SEC chromatograms that illustrate the acid-catalyzed hydrolyses of AG and DAG. Each hydrolysis was conducted with 5% of polysaccharide in 0.05 N H_2SO_4 at 90°C, and samples were removed and neutralized at intervals for analysis. Each set of chromatograms shows a growing monomer peak (elution time, 10.6 min) and a diminishing polymer peak. In the case of AG, the latter peak (elution time, 8.3 to 8.6 min) is well defined throughout the hydrolysis. The lack of significant area between it and the monomer peak, even at late stages of the degradation, combined with the persistence of a narrow peak-width for the polysaccharide, suggests that the degradation entails removal of fragments from the periphery of the multiplex, with the ordered core held intact. In contrast, the set of chromatograms for DAG shows the monomer peak growing more rapidly, as the polymer peak loses its shape and becomes a wide oligomer peak that is finally a mere shoulder on the monomer peak. This result suggests that the breakdown of order prior to commencing hydrolysis imparts conformational freedom to the main-chain residues, which makes their glycosidic linkages more susceptible to hydrolysis.

3.2. Production and preliminary analysis of polymeric products

Polymeric products like those discussed above were produced and isolated on a larger scale; different products were made by varying the reaction time. Five products each were made from AG and DAG, the reaction times for the latter being generally shorter, since DAG degrades more rapidly. After neutralization, low MW products were removed from the hydrolysates by dialysis or ultrafiltration. For the more degraded products, especially those from DAG, these procedures were aimed at producing products that gave symmetrical SEC peaks. Fig. 2 shows the two sets of chromatograms resulting from SEC analyses of the final products. Chromatograms of the starting materials (AG and DAG) are included for comparison, and the other chromatograms are scaled according to the yields of the corresponding products so obtained.

Compositional analyses (Table 1) of the products indicated rapid loss of Ara residues from both AG and DAG. It has long been recognized that these residues, which are attached to the galactan core via furanoside linkages, are less easily hydrolyzed than furanosides in general (Bouveng, 1961). This relative stability of the Ara*f* linkages, combined with the fact that Gal*p* residues are more susceptible to acid hydrolysis than pyranosides in general (Capon, 1969), means that there is much less difference between the hydrolysis rates of these two types of residues than might otherwise be expected. As a result, the goal of removing practically all Ara residues by acid hydrolysis, while leaving the galactan core relatively undisturbed, is unrealistic. The agh1h product, in which about 85% of the Ara has been removed at the expense of losing about 15% of the Gal, represents a typical compromise in this respect. The fact that the rate of Ara loss is about the same for both AG and DAG indicates that these residues are not included in that part of the multiplex that is resistant to acid hydrolysis.

Portions of each of the agh products were subjected to the same disordering conditions that were used to generate DAG from AG (Ponder & Richards, 1997b), and SEC analyses indicated that all of these products underwent an order–disorder transition. In this respect, the agh products are analogous to partially hydrolyzed variants of xanthan, which display order–disorder properties similar to native

Table 1
Polysaccharides derived from AG and DAG by partial acid hydrolysis

Product	%Yield	Mol% Ara	SEC r.t. (min)	[aghd][a]	App. MW × 10^{-3}	[aghd][a]
from AG		14.8	8.30		17.0	
agh30m	86	4.7	8.37	[8.82]	15.5	[8.3]
agh1h	75	2.1	8.42	[8.86]	14.8	[7.9]
agh2h	62	0.7	8.49	[8.89]	13.1	[7.5]
agh4h	50	0.2	8.59	[8.98]	11.4	[6.7]
agh8h	18	0.2	8.63	[9.05]	10.8	[6.0]
from DAG		14.8	8.74		9.2	
dagh15m	74	6.8	8.83		8.2	
dagh30m	64	2.5	8.93		7.1	
dagh1h	58	0.8	9.10		5.6	
dagh2h	46	0.4	9.42		3.6	
dagh4h	12	0.2	9.72		2.4	

[a]Value for disordered form of corresponding product.

xanthan (Christensen et al., 1993). Thus, by means of disordering, another set of five products was made, and these will be referred to as 'aghd' products. ^{1}H NMR analyses of the agh and aghd products revealed the same spectrometric effects observed for AG and DAG (Ponder & Richards, 1997b). The agh spectra were poorly resolved in comparison to the corresponding aghd spectra, and the agh spectra included the two peaks at 4.9 and 6.2 ppm, previously and tentatively identified as non-exchangeable hydroxyl proton signals, whereas the aghd spectra lacked these peaks. Moreover, the relative size of these peaks in the agh spectra increased with extent of acid degradation, reaching their maximum size in the spectrum of the agh8h product, where they constituted 5.1 and 2.9%, respectively, of total area (compared with 1.8 and 1.3%, respectively, for AG).

Fig. 3 shows the reaction pathways used to make all of the products in this study, and Table 1 lists the yield, composition and SEC data. Apparent molecular (or multiplex) weights, calculated from SEC retention times (r.t.) and based on pullulan calibration, are also included in the table, although it should be emphasized that these numbers have only relative value; the actual weights are probably much greater in all cases. SEC data for aghd products are given in square brackets in the table. Fig. 3, in which horizontal arrows denote order–disorder transitions, refers to these products by a similar convention; e.g. 'agh30md' denotes AG that has been acid-degraded for 30 min and then disordered. The SEC data (Table 1) indicate that the products obtained by acid degradation followed by disordering (aghd products) are not the same as the products obtained by disordering followed by acid degradation (dagh products).

3.3. Reordering the disordered products by drying

Further confirmation of the difference between the aghd products and the dagh products is obtained by observing the effects of drying. Fig. 4 shows separate SEC chromatograms for three of the agh products, and superimposed in each case is the chromatogram for the respective disordered product (aghd). Also in each case, a third chromatogram shows the effect of evaporating the aghd product to dryness under reduced pressure. Chromatograms for disordered products were obtained with samples that had been neither evaporated dry nor freeze-dried since being disordered, and the 'dried' chromatograms shown in the figure were obtained from these aghd samples by drying once on a rotary evaporator, then redissolving for SEC analysis. An aliquot of the same size and concentration was dried under the same conditions in each case. Fig. 4 indicates that the tendency for aghd products to reorder is proportional to the extent that the original agh product was degraded. (The small shoulder on the left side of the 'reordered' peaks is assumed to be due to some non-ordered aggregates, perhaps analogous to aggregates that form from some disordered β-(1 → 3)-glucans, Yanaki et al., 1985.) Moreover, all of the aghd products reorder more easily than DAG, and this trend extends to the dagh products. Fig. 5 shows SEC chromatograms for three of the dagh products, and superimposed in each case is the chromatogram that shows the effect of evaporating to

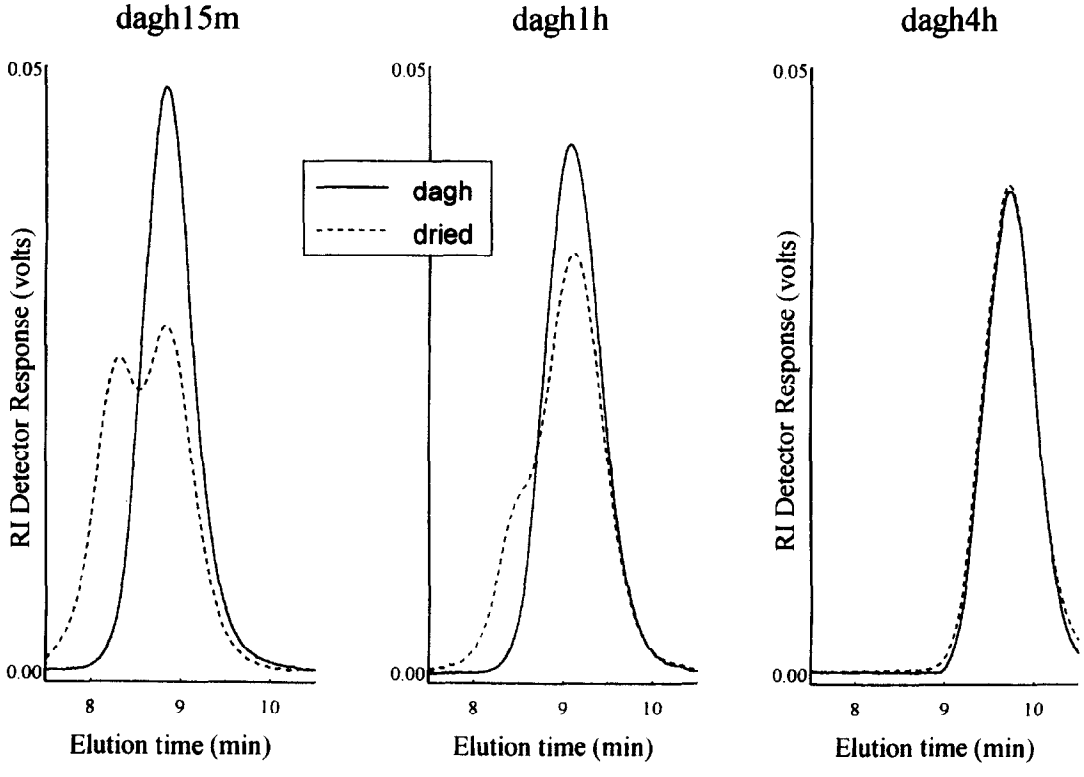

Fig. 5. SEC chromatograms of dagh products and of partially reordered products obtained from dagh products by a single evaporation to dryness.

Table 2
Methylation analyses of agh products and dagh products[a]

Deduced linkage	agh30m	agh1h	agh2h	agh4h	agh8h
Gal*p*-(1 →	33.6	35.2	37.1	37.9	36.2
→ 3)-Gal*p*-(1 →	2.3	3.8	5.0	10.6	19.5
→ 6)-Gal*p*-(1 →	24.2	23.5	20.3	13.4	8.3
→ 3,6)-Gal*p*-(1 →	32.4	33.6	35.2	36.3	34.2
	dagh15m	**dagh30m**	**dagh1h**	**dagh2h**	**dagh4h**
Gal*p*-(1 →	30.5	32.3	32.9	32.3	30.4
→ 3)-Gal*p*-(1 →	4.0	5.6	7.7	10.0	16.2
→ 6)-Gal*p*-(1 →	24.6	25.9	26.1	25.8	23.6
→ 3,6)-Gal*p*-(1 →	31.9	31.4	30.6	29.6	28.1

[a]Values (mol%) are averages from three or four analyses (relative standard deviation < 6%). Values for Ara linkages are not included.

dryness under the same conditions used for Fig. 4. All of the dagh products show less tendency to reorder than DAG, the more so with increasing extent of degradation. A rough measure of the tendency to reorder is the relative area in the reordered peaks of the 'dried' chromatograms in Figs. 4 and 5. In those cases where two peaks do not resolve (dagh1h-dried and agh8hd-dried), areas can be measured from a line dropped from the point of inflection. When this is done, the trend can be quantified as follows:

	dagh4h	dagh1h	dagh15m	DAG	agh30md	agh2hd	agh8hd
% reordered when dried	0	15	44	55	60	71	85

This trend is consistent with the idea that the ordered multiplex preferentially loses non-ordered portions of its structure to acid hydrolysis (AG → agh). After being dissociated into individual molecules (agh → aghd), these recombine into multiplexes (aghd → agh) easily since substructures that do not contribute to order, e.g. large, Ara-containing side-chains, are no longer present to interfere. In contrast, acid hydrolysis of the dissociated molecules (DAG → dagh) results in relatively indiscriminate removal of all substructures, as well as a higher incidence of main-chain scission, so that when restoration of order is attempted, the requisite substructures and chain-lengths are not found.

3.4. Linkage analyses

All of the agh products and dagh products were subjected to methylation analyses, and results for the major galactosyl linkages are shown in Table 2. Numbers are mole percentage of linkages indicated and are averages of multiple analyses. Fig. 6 is a graphical representation of the data in Table 2 that pertain to the three major linkages in the original polysaccharide. These are terminal Gal*p* (t-Gal*p*), which is present primarily at the ends of monomeric and dimeric Gal*p* side-chains, 6-linked Gal*p*, which is present primarily in dimeric Gal*p* side-chains, and 3,6-linked Gal*p*, which is present primarily in the main-chain (Ponder & Richards, 1997c). The data points for $t = 0$ in the figure apply to undegraded AG or DAG (Ponder & Richards, 1997b). The figure shows that acid degradation of either form of the biopolymer begins with a sharp increase in the relative amounts of these three major types of galactosyl residues, and, in general, this can be attributed to the rapid removal of Ara residues. This effect is augmented in the cases of t-Gal*p* and 6-linked Gal*p*, since many of these linkage types are generated by removal of Ara. However, the effect is diminished in the case of 3,6-linked Gal*p*, since a significant proportion of these linkages are in side-chains and are converted to 6-linked Gal*p* upon removal of Ara. After the initial increase in these three linkages, their amounts remain relatively constant in the case of the dagh products. This reflects indiscriminate attack upon the remaining galactan, and the gradual decrease seen for these linkages in the figure reflects a growing relative amount of unbranched main-chain residues, i.e. conversion of → 3,6)-Gal*p*-(1 → to → 3)-Gal*p*-(1 → (Table 2). In the case of the agh products, however, there is a clear preference for preservation of 3,6-linked and terminal Gal*p* at the expense of 6-linked Gal*p*. The relative decrease in the latter type of residue indicates a preferential loss of the dimeric Gal*p* side-chain. Therefore, the principal conclusion to be drawn from these linkage analyses is that main-chain residues with single, terminal Gal*p* residues attached at C-6 are preserved within the acid resistant portion of the multiplex.

3.5. Alkaline degradation analyses

The above conclusion is reinforced by results from alkaline degradation analyses. Fig. 7 is a review of this analysis as it has been developed in this laboratory for study of AG (Ponder & Richards, 1997c). At the top of the figure is a hypothetical segment of the (1 → 3)-linked main-chain consisting of five main-chain Gal*p* residues (G), four of which bear side-chains on C-6. The two simplest side-chains shown are known to comprise all the monomeric and dimeric side-chains in AG, and the trimer side-chain, which contains an Ara*f* residue (Af), is known to be the

major trimer side-chain in AG (Manley-Harris, 1997). The tetramer side-chain shown is hypothetical, being but one of several likely possibilities. Alkaline degradation using aqueous $Ca(OH)_2$ has the effect of sequentially breaking (peeling) all linkages in the main-chain while converting the main-chain residues to 3-deoxyaldonic or 'metasaccharinic' acids (Gms). The resulting side-chain-derived products are then separated on Bio-Gel P-2, as shown by the chromatogram at the bottom of the figure. Ara-containing products are restricted to the peaks containing tetramers ('tetra'), pentamers and larger ('penta + ') oligomers. In addition to Gms, the monomer peak also contains significant amounts of lactic acid and the metasaccharinic acid derived from Ara, but the latter product would be of little importance in the case of the acid degraded polysaccharides, for which changes in the relative size of the 'mono' peak primarily reflect changes in the relative amount of unbranched main-chain Gal*p* residues.

Fig. 8 shows GPC results of this analysis applied to some of the acid degraded products. Two sets of three superimposed chromatograms are shown, and each includes the chromatogram corresponding to previously undegraded AG, i.e. that of Fig. 7, for comparison. This is labeled 'AG' in one set of chromatograms and 'DAG' in the other; it applies to either, since AG is converted to DAG by the alkali before the onset of peeling. (Likewise, aghs are converted to aghds before the onset of peeling.) The other chromatograms shown are the alkaline degradation results of the least and most acid degraded of the agh and dagh polysaccharides. For the least acid-degraded products (agh30m and dagh15m), these chromatograms show little difference in the distribution of side-chains. In both cases, a large decrease in the relative abundance of Ara-containing side-chains is apparent, and this is accompanied by an increase in the dimeric Gal*p* side-chain (trimer product), since many Ara-containing trimer side-chains are initially converted to this upon losing Ara. However, beyond the initial stages of acid degradation, the alkaline degradation results for the agh and dagh products are different in terms of distribution of main-chain residues bearing dimeric, monomeric and no side-chains, i.e. in trimeric, dimeric and monomeric alkaline degradation products. These differences are illustrated by the chromatograms from agh8h and dagh4h in Fig. 8, and their development is represented graphically in Fig. 9, which plots the GPC peak areas for the major alkaline degradation products from all of the agh and dagh polysaccharides.

Fig. 9 shows that after the initial increase in relative amount of dimeric side-chains ('Tri' product), these structures decrease throughout the course of hydrolysis for both forms of the biopolymer. The dimeric side-chains, which are initially the most abundant, are lost either by cleavage of the 6-linked residue or of the terminal residue. In the former case, an unbranched main-chain residue is generated, and in the latter case, a monomeric side-chain is generated. Thus, coinciding with the decrease in dimeric side chains is an increase in the relative amount of monomeric side-chains ('Di' product). The latter side-chains are also lost, but initially not as fast as they are generated by degrading dimer side-chains. In the figure, the amount of monomer

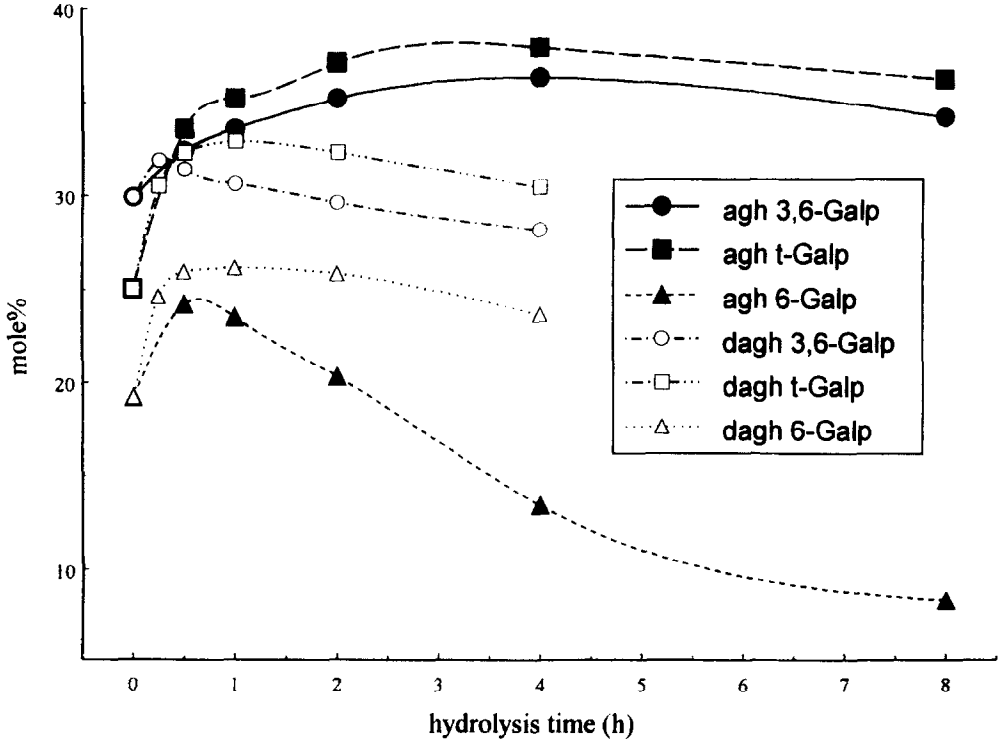

Fig. 6. Distribution of major galactosyl linkages in polymeric products as a function of hydrolysis time.

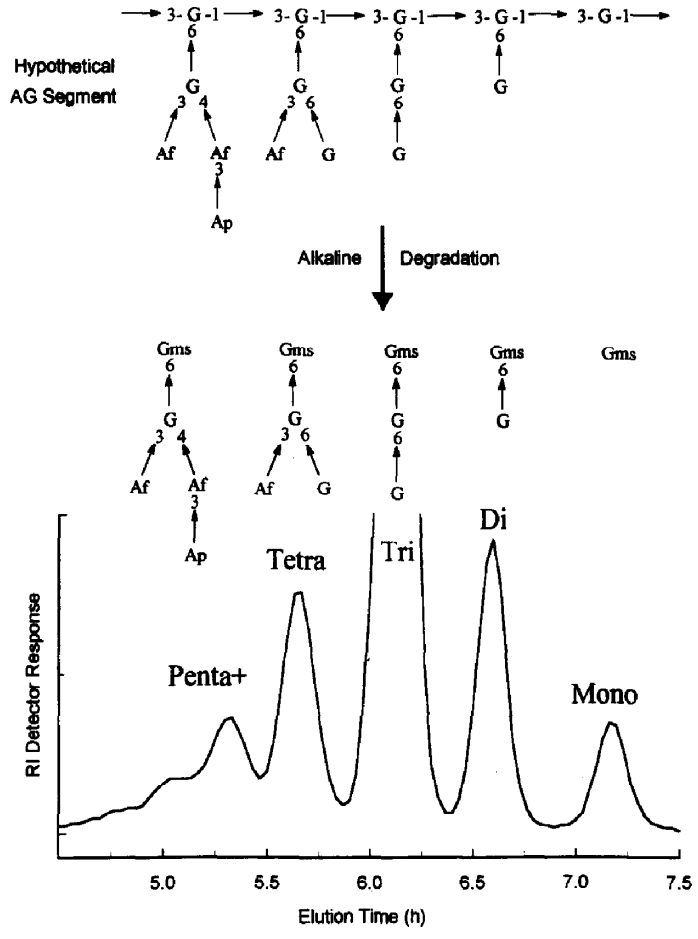

Fig. 7. Review of alkaline degradation analysis. G = β-D-galactopyranosyl; Af = α-L-arabino-furanosyl; Ap = β-L-arabinopyranosyl; Gms = galactometasaccharinate.

side-chains is seen to level off as their rate of loss catches up to their rate of formation, and unbranched main-chain residues steadily increase. All of these trends are readily apparent in the results for the dagh products. By comparison, the data for the agh products suggest an additional, significant effect, one that retards both the loss of monomeric side-chains and the formation of unbranched main-chain residues. This agrees with the results of linkage analyses, and suggests that main-chain residues bearing single Gal*p* side-chains are integral components of the ordered network and thus preserved in the agh polysaccharides.

Shorter main-chain lengths for the dagh polysaccharides were apparent from the fact that they underwent the peeling reaction with a relatively high degree of completion, leaving little or no alkali-resistant fraction, and also from the fact that they generated more lactic acid. Shorter main-chains imply a higher proportion of non-3-linked main-chain end-groups, which are prone to non-specific alkaline degradation reactions, of which lactic acid is a major product. Thus, dagh4h in saturated $Ca(OH)_2$ at 100°C for 1 h underwent peeling completely, leaving no polymeric residue, and produced a 6.2 wt% yield of lactic acid (by ^{1}H NMR). In comparison, agh4h, reacting under the same conditions, gave a 13% yield of unpeeled polymer and a 1.1 wt% yield of lactic acid.

Larch AG is subsumed within a larger category of polysaccharides known as type II arabinogalactans (Stephen, 1983). Included in this category are several types of gum exudates (e.g. gum arabic) in which a tendency toward preservation of the β-(1 → 3)-galactan main-chain during acid hydrolysis has been previously observed (Churms et al., 1977). For one such case, the proposal has been made that this acid resistance is due to electronic effects of uronic acid residues that occur as 6-linked branches along the main-chain (Churms & Stephen, 1971). In the present case, however, the uronic acid content is much too low to function significantly in this capacity. Rather, the stabilizing effect of a higher-order structure is a more likely explanation. A precedent for the involvement of single glycosyl side-chains in such a structure is found in the triple-helical polysaccharide schizophyllan (Teramoto et al., 1995). The regularly spaced, single glucosyl side-chains in this β-(1 → 3)-glucan are thought to protrude from the helix core and to form a hydrogen bonding network with intervening water molecules, 'giving rise to some longitudinal order along the helix axis' (Hirao et al., 1990). Extrapolation of this concept to the AG system suggests that the tentatively assigned 'hydroxyl proton' peaks in ^{1}H NMR spectra of AG and agh products may be due to 'bound' or 'structured' water

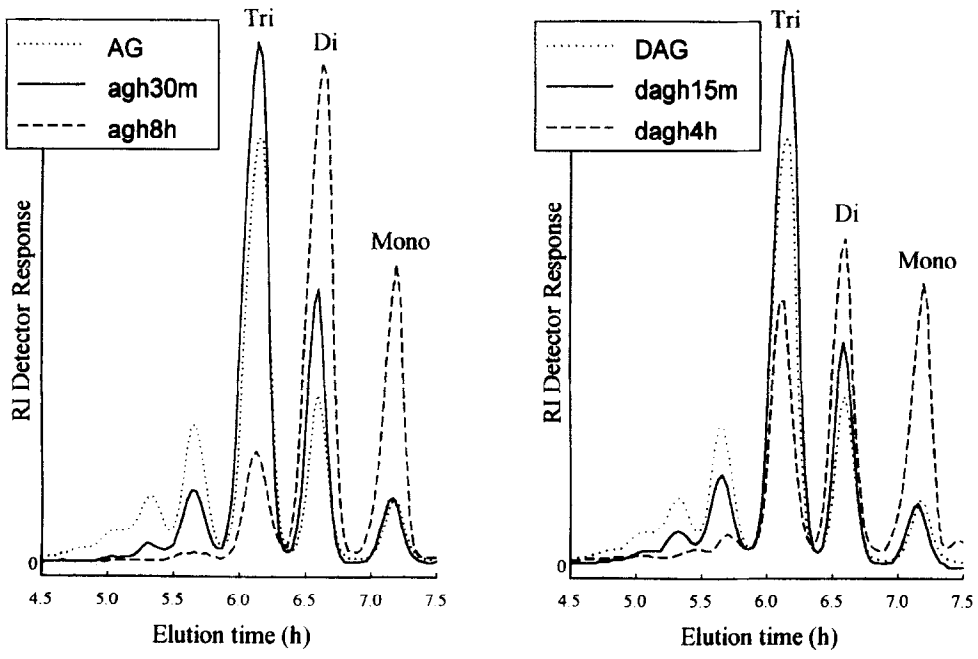

Fig. 8. GPC chromatograms of alkaline degradation products from AG (or DAG) and from some polymeric products of acid degradation. Tri, Di and Mono = trimeric, dimeric and monomeric products of alkaline degradation, respectively.

in addition to alcoholic hydroxyls protected from D_2O exchange by inclusion in ordered regions. Relevant in this context is the fact that these signals are suppressed when the HOD peak is irradiated. The schizophyllan analogy also raises the question of whether the single, Gal*p* side-chains known to be in AG are regularly spaced along the main-chain. However, the possibility that the 6-linked residues in dimeric side-chains may function in this ordering capacity in addition to, or instead of, the monomeric side-chains cannot be dismissed, since, conceivably, such residues could survive in the degraded product as single Gal*p* side-chains. The essential participation of certain side-chain structures in the ordered network of AG explains why AG that has had all side-chains removed by repeated Smith

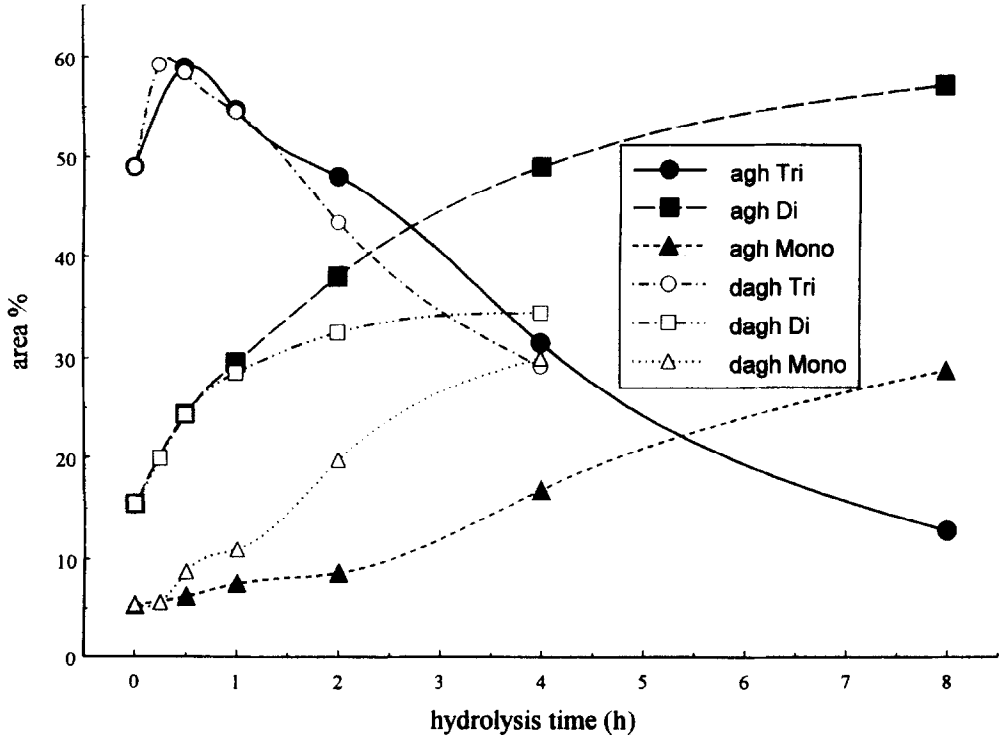

Fig. 9. Distribution of GPC peak areas for major products of alkaline degradation of polymeric products of partial acid hydrolysis, as a function of hydrolysis time. Ordinate values are percentages of total peak area. Data for arabinose-containing products are not included.

Table 3
CTA-OH fractionation of heartwood AG, and SEC analysis of fractions

	Fr.1	Fr.2	Fr.3	Fr.4 and 5
Bouveng & Lindberg				
% of recovered material	40.9	32.5	16.5	10.1
Identification	A	A + B	B	B
Current work				
% of recovered material	30.4	47.9	17.3	4.4
% area in ion excluded peaks	87	47	26	8
R.t. of 'neutral peak' (min)	8.10	8.17	8.20	8.93

degradations (while, at the same time, being disordered) shows no ability to form ordered multiplexes by either drying or freezing. Smith degradation studies of AG will be detailed in a later paper in this series.

3.6. The work of Bouveng

The first study of polymeric products of partial acid hydrolysis of AG was reported by Bouveng (1959). That study was conducted on a heartwood AG component that was isolated by precipitation with cetyltrimethylammonium hydroxide (CTA-OH) and NaOH from aqueous H_3BO_3 (Bouveng & Lindberg, 1958). Thus did Bouveng and Lindberg obtain this major component, which they called 'A', along with a minor component, which they called 'B', in a ratio of 2:1. Analytically, they distinguished A from B on the basis of their different electrophoretic mobilities in borate buffer, and they reported that A had a molecular weight several times that of B on the basis of ultracentrifugation measurements. The results that were reported for the acid hydrolysis study do not readily correlate with results of the present study and raise questions regarding the nature of A and B. It was therefore decided to repeat the CTA-OH fractionation and to analyze the fractions for uronic acid content.

The CTA-OH procedure was applied to a sample of AG from Western larch heartwood, and Table 3 compares the

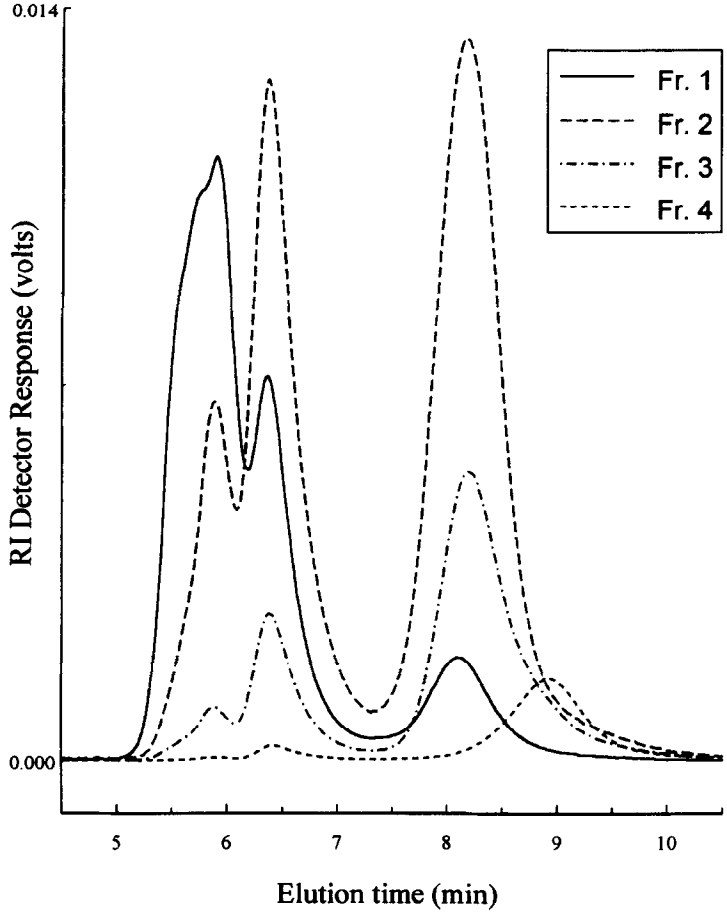

Fig. 10. SEC chromatograms, obtained with 0.2 mM $NaNO_3$ eluent, of CTA-OH-precipitated fractions of heartwood AG.

results with those of Bouveng and Lindberg. The fractions obtained by CTA-OH fractionation in the current work were subjected to SEC analysis in low ion strength eluent, and the resulting chromatograms are shown in Fig. 10. Data for Fractions 4 and 5 are combined, and the area under each of the four chromatograms has been normalized to correspond to the yield of the respective fraction. Under the SEC conditions used, neutral AG multiplexes elute at about 8.2 min retention time, while charged AG multiplexes elute at about 6.2, 5.8 or 5.5 min, depending, most likely, on whether they are singly, doubly or triply charged, respectively (Ponder & Richards, 1997a). Thus, it is apparent from Fig. 10 that the CTA-OH procedure effects a fractionation by charge, with the most highly charged species precipitating first. Moreover, the total proportion of charged material decreases through the fractions, as shown in Table 3, which lists the percentage of total area in the ion excluded peaks in each chromatogram. The trend of increasing retention times (r.t.) for the neutral peak in Fractions 1, 2 and 3 (Table 3) indicates that a fractionation by weight is occurring simultaneously with the fractionation by charge, and the retention time of Fraction 4 indicates that this fraction is primarily AG-B. Thus, a separation of the two weight fractions of AG can indeed be achieved by this procedure, but not at the point indicated by Bouveng and Lindberg. Correlation of their approximate identification scheme with the current SEC results suggests that their 'A' was charged AG-A and that their 'B' was neutral AG-A mixed with a small amount of AG-B.

This interpretation accords with other results reported for A and B, such as the greater electrophoretic mobility of A and only a small compositional difference between A and B. In addition, Bouveng reported that A underwent partial acid hydrolysis to yield not only a polymeric product with about the same electrophoretic mobility as A, but also a polymeric product with about the same electrophoretic mobility as B. These products were given the generic names 'AI' and 'AII', respectively. In one experiment that Bouveng describes, the reported observations indicate that the AII product formed gradually and was derived from the AI product. This is consistent with the idea that AI was partially hydrolyzed, charged AG-A that retained its uronic acid residue(s), and AII was partially hydrolyzed, previously charged AG-A in which uronic acid residue(s) were removed by hydrolysis. In order to test this concept, a few milligrams of the charged material in the heartwood AG used in the current work was isolated by GPC. Analysis by SEC using low ion strength eluent resulted in the 'charged AG-A' chromatogram shown in Fig. 11. This material was then partially acid hydrolyzed to give a

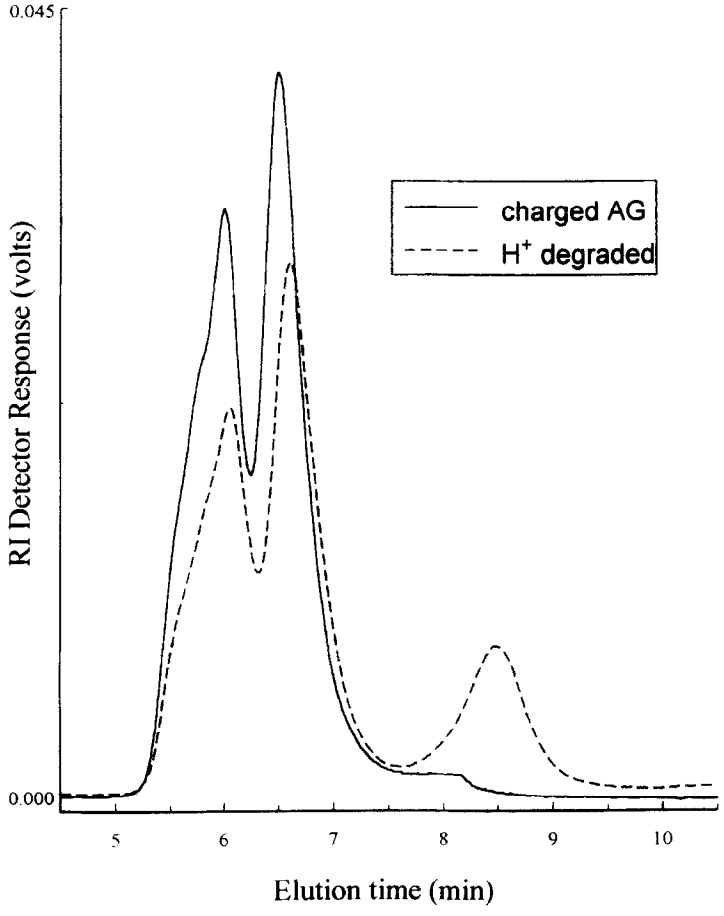

Fig. 11. SEC chromatograms, obtained with 0.2 mM $NaNO_3$ eluent, of charged fraction of heartwood AG before and after partial acid hydrolysis.

degraded polysaccharide in 43% yield after removal of low molecular weight products by ultrafiltration. Similar SEC analysis of this product resulted in the 'H^+ degraded' chromatogram of Fig. 11, which shows that neutral polymeric material had been generated from the charged material during hydrolysis. Thus, by reinterpretation of Bouveng's fraction A, the earlier results can be reconciled with the current work. However, this new interpretation implies that A and B were almost equal in MW, and this is contradicted by the ultracentrifugation measurements that Bouveng and Lindberg report. Because no resolution of this contradiction is apparent, the view of fractions A and B presented here must be regarded as tentative.

3.7. Summary and conclusion

The various types of polysaccharide products resulting from partial acid hydrolysis of larch arabinogalactans will have most or nearly all arabinosyl residues removed. This procedure is therefore valuable as a simple means of converting an abundant resource into a nearly pure galactan, a type of polysaccharide that is rare in nature (Stephen, 1983). This value is enhanced by the ability to 'tailor make' the products to suit particular applications, made possible by the fact that the structure of the products depends on whether the starting material is ordered or disordered. For example, the partial hydrolysis products from ordered AG contain relatively long main-chains that bear a high proportion of non-reducing galactopyranosyl end-groups. Such products could have potential in medical and biological applications involving interaction with galactose-specific binding sites in the human body (Gallez et al., 1994; Groman et al., 1994; Hagmar et al., 1994), or involving immunomodulating properties like those possessed by the known β-(1 → 3)-glucans of analogous structure (Bohn & BeMiller, 1995). In comparison to the products from ordered AG, the partial hydrolysis products from disordered AG have much smaller molecular weights and much reduced tendencies to self-associate in solution, properties that may be desirable in applications requiring membrane permeability. Differences in other potentially important properties, such as solubilities, synergisms with other polysaccharides and susceptibility to various derivatizing agents, are also likely.

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