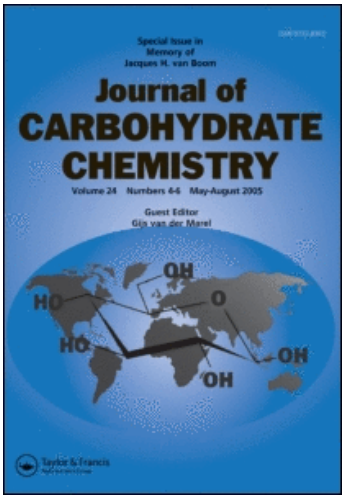

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Arabinogalactan from Western Larch, Part I; Effect of Uronic Acid Groups on Size Exclusion Chromatography

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ARABINOGALACTAN FROM WESTERN LARCH, PART I; EFFECT OF URONIC ACID GROUPS ON SIZE EXCLUSION CHROMATOGRAPHY

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ABSTRACT

Arabinogalactan (AG) from Western larch has sometimes been reported to contain a small proportion of uronic acid units. This concept is supported and extended by the present work, which shows that these units occur in some AG molecules. Molecular assemblies which contain such molecules are consequently partially excluded from the stationary phase of size exclusion chromatography (SEC) columns when low ion strength eluent is used, and the degree of exclusion is a function of the number of acid units per assembly. In this way the ion exclusion effect provides a sensitive means of measuring low levels of uronic acid content in larch AG. Recognition of this effect helps resolve inconsistencies in the earlier AG literature. The dramatic effect which a few uronic acid groups have on the chromatographic behavior of AG implies possible differences in solution properties and biological activities between the charged and uncharged species.

INTRODUCTION

Arabinogalactan (AG) is a naturally occurring, water soluble polysaccharide found in trees of the genus *Larix*, and especially in Western larch (*Larix occidentalis*) from whose wood AG can be extracted in high yield.[1] Structurally, AG is a (1→6)-branched (1→3)-β-D-galactopyranan with molar Gal/Ara ratio of about 6. AG from Western larch has been extensively studied since the early part of this century, and during that time two major attempts have been made to commercialize it.[2] Such efforts have been hampered by the

economics of extraction and purification, but technological improvements in these areas are inevitable. For example, a more efficient extraction process for AG has recently been developed,[3] and this will increase its availability and its importance as a commercial polysaccharide. This renewed commercial activity follows closely upon recent discoveries relating to AG's potential in the field of medicine.[4] These considerations, coupled with the many remaining uncertainties regarding details of AG's chemical nature, justify continued study of this polysaccharide.

AG from Western larch is composed of two fractions, the more abundant being the high weight fraction, AG-A. (Part II of this series shows that AG-A exists as ordered multiplexes or assemblies of molecules.) The low molecular weight fraction, AG-B, has been variously reported to occur in a wide range of proportions relative to AG-A, and it is unclear from the literature whether a typical A/B ratio exists. The principal material investigated in this study is the purest form of the high weight component of Western larch AG that is now commercially available. It will be referred to in this work as "AG-A".

Size Exclusion Chromatography (SEC) has been useful in our studies of AG, and our routine applications of this technique have been conducted with an HPLC system. Until recently, we have consistently used an ionic eluent with our HPLC column, as recommended by the manufacturer, in order to suppress non-SEC effects which otherwise occur with certain analytes. This has also been the approach taken by other workers who have investigated larch AG using HPLC.[5] Under such conditions AG-A elutes to give a single, narrow and symmetrical peak, suggesting a single polymeric species of narrow weight range. When a sample of crude, unfractionated AG is thus analyzed, this single peak is still the major feature of the chromatogram, but its symmetry is lost due to a low-MW shoulder corresponding to AG-B. These observations conform generally to earlier conceptions of AG-A and AG-B, but invariably and despite having analyzed many different samples, we observe the area corresponding to the B component to be less than 5% of the total; thus we find no corroboration of the many large yields of AG-B reported in the older literature. Recent experiments in our laboratory have shown that by using eluent of low ionic strength, we can obtain information relevant not only to the discrepancies mentioned above, but also to other aspects of AG, and those experiments are the subject of this paper.

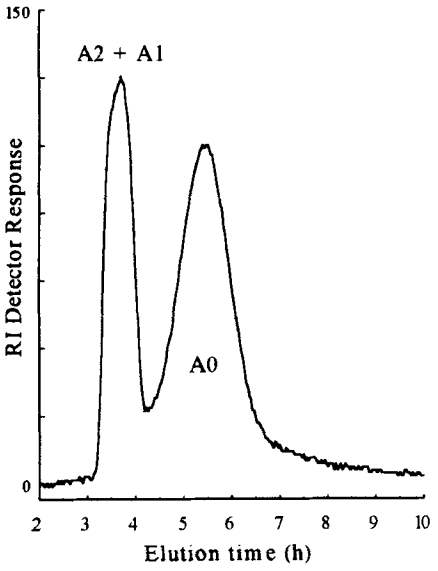

Figure 1. AG-A on Sephadex G-100. Eluent: water.

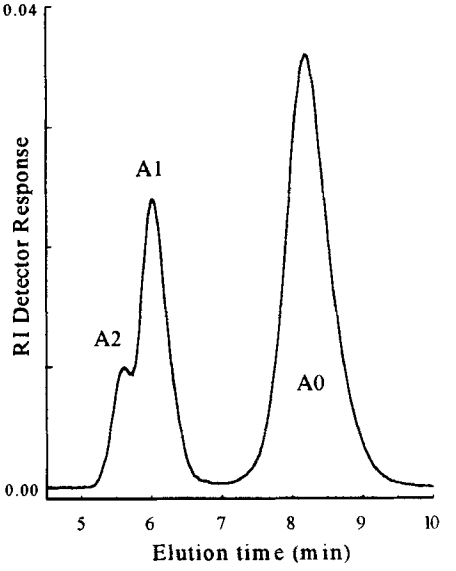

Figure 2. AG-A on Shodex KB-804. Eluent: water.

RESULTS AND DISCUSSION

The current work began with an observation of the behavior of AG-A on Sephadex G-100, using pure water as eluent. Resolution into two peaks is observed when the loading on the column is relatively light; *e.g.*, the chromatogram of Figure 1 was obtained by loading 25 mg of AG-A on the column. These peaks are immediately suggestive of the two major types of AG, AG-A and AG-B. Indeed, it may have been a chromatogram like Figure 1 which was observed by Churms, *et al.*[6] and which prompted them to report a sample of Western larch AG consisting mostly of AG-B. However, the chromatogram of Figure 1 was generated by material which, because of its prior ultrafiltration, contains no AG-B.

The above result prompted us to change the eluent of our HPLC system to pure water. Subsequent injection of any of a range of pullulan standards gave the usual single peak, but upon injecting AG-A we obtained the chromatogram of Figure 2. The first two, slightly resolved peaks begin at the exclusion limit of the column and contain about one third of the total area. The retention time of the third peak is approximately that of the single peak

that is seen when 50 mM $NaNO_3$ is used as eluent. For reasons which will become apparent, these peaks (from left to right) will be referred to as A2, A1 and A0. To establish the relation between the Sephadex results and the HPLC results, the material corresponding to each of the two peaks from Sephadex were collected and then injected separately on the HPLC system with pure water. The results showed that A2 and A1 elute unresolved near the exclusion limit of the Sephadex gel, as denoted in Figure 1. The two Sephadex fractions, "A2+A1" and A0, were also injected onto the HPLC system using the usual 50 mM $NaNO_3$ eluent, and results confirmed that these components co-elute under these conditions. Fractions from three consecutive Sephadex runs were combined, dried and weighed. The A2+A1 fraction and the A0 fraction weighed 19 mg and 37 mg, respectively.

Eluents of different ionic strengths were investigated, and it was found that nearly 100-fold dilution of the 50 mM $NaNO_3$ eluent was needed to achieve a significant separation of A2 and A1 from A0. Figure 3 shows the effect of different eluent concentrations on the elution profile for AG-A. For all of these, the A0 peak elutes at about the same retention time. Therefore the retention time for this form of AG (AG-A0) is controlled essentially by its size in all cases, while the other forms (AG-A2 and AG-A1) exhibit non-SEC behavior unless eluent of high ionic strength is used. Thus, the chromatogram obtained with 50 mM $NaNO_3$ eluent shows the "true picture" from the point of view of multiplex size, while the other chromatograms reveal the existence of other differences between AG assemblies unrelated to size. Note also that better resolution between A2 and A1 is achieved when slightly ionic eluent is used. For this reason, and because low ion strength adds a degree of reproducibility to the system,[7] 0.2 mM $NaNO_3$ became our eluent of choice for studying the non-SEC behavior of AG.[8]

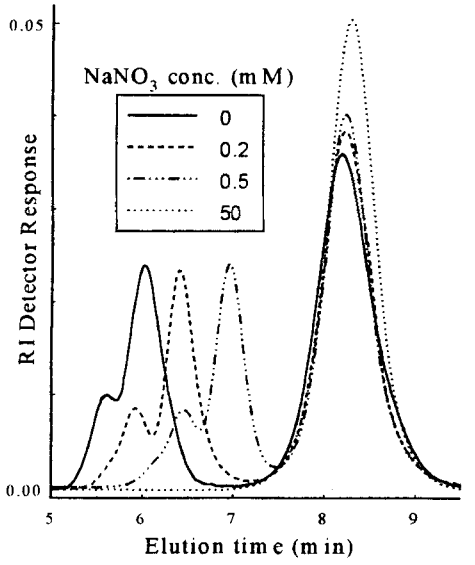

Figure 3. AG-A on Shodex KB-804. Effect of eluent concentration.

Support for the view that A2, A1 and A0 are all the same size was obtained by experiments involving further ultrafiltration of AG-A. One half of a 1% solution of AG-A

was passed through a 50 k membrane (*i.e.*, MWCO rating of 50,000 daltons) in an ultrafiltration cell. (The weight of an average AG-A assembly is reportedly about 37 kDa.)[5a,9] Both the solution which came through (permeate) and the solution which remained in the cell (retentate) were directly injected onto the HPLC system using 0.2 mM $NaNO_3$ eluent. The resulting chromatograms were virtually identical to each other and to the chromatogram of unfiltered AG-A, indicating that all three forms of AG-A passed through the membrane with equal ease. A similar experiment was conducted using a 30 k membrane, and results showed that less than 5% of AG passed through; however the small amount that did so included representative amounts of all three forms of AG-A.

Portions of the A2+A1 and A0 fractions collected from the Sephadex column were subjected to compositional, methylation and ^1H NMR analyses. Compositional analysis revealed no significant difference in Ara content between the two fractions, and methylation analysis revealed no significant differences in the distribution of linkages. The major signals between 3.0 ppm and 5.5 ppm in the ^1H NMR spectra of the two fractions in D_2O were identical both with each other and with our usual observations for AG-A (see Part II). Apparently, therefore, the non-SEC separation of these components is not due to any major compositional or structural differences.

Numerous instances of non-SEC separation of analytes in SEC systems have been documented, and there are two mechanisms for such separation that are unique to aqueous SEC. These are (1) ionic interactions between polyelectrolytes and packing and (2) intramolecular electrostatic effects of the polyelectrolyte.[10] Symptoms associated with the latter mechanism are not observed in the present case, so the first mechanism is most likely. In particular, the mechanism known as "ion-exclusion" is known to cause symptoms which are completely consistent with the SEC behavior of AG-A. This occurs when the surface of the packing has a net charge, thus causing solutes of similar charge to be excluded from the pores because of electrostatic repulsion.

The charges on Sephadex, a cross-linked dextran, derive from a small number of carboxyl groups, which have previously been exploited to separate nonionic from ionic species by means of the ion-exclusion mechanism.[11] This technique has also been used to separate ionic species from each other, since the degree of exclusion is related to the ionic charge of the analyte, with more charged species being excluded more and thus eluting earlier.[12] To test this concept with our HPLC column (Shodex KB-804), we injected dilute

solutions (0.1%) of sodium glucarate (doubly charged), sodium glucuronate (singly charged), and glucose (neutral) using 0.2 mM $NaNO_3$, and these eluted with retention times of 6.4, 6.8 and 10.5 min, respectively, consistent with a separation by charge. The source of the charges on the Shodex packing (polyhydroxymethylmethacrylate) is unclear, but their presence is tacitly acknowledged by the manufacturer who recommends use of ionic eluents precisely for the purpose of eliminating their non-SEC effects. This is the case in general for commercially available aqueous SEC columns, most of which are negatively charged.[13]

The charges on some AG-A species probably derive from uronic acid units which have been detected in varying amounts in AG's from most species of larch.[14] These amounts range from 6.8% in AG from *Larix lyallii*[15] to a "trace" in *Larix occidentalis*.[16] Previously, we have not detected these residues in AG since their quantification by chemical means,[17] which is imprecise for small quantities, requires extra effort that has not seemed justified in view of their low concentration in Western larch AG. However, the nature of the ion-exclusion mechanism is such that we would expect the SEC results to be very sensitive to even small amounts of these residues. To test the concept that the presence of carboxyl groups is the distinguishing characteristic of the AG comprising peaks A1 and A2, AG-A was eluted with water through a column of Amberlite IRA-900(OH), a macro reticular strongly basic ion-exchange resin which would be expected to preferentially retain acidic polysaccharides. The eluent was concentrated and injected on the Shodex column using 0.2 mM $NaNO_3$, and the result is shown in Figure 4. A significant amount of the polysaccharide was retained by the resin irrespective of charge, this being the case in general for carbohydrates on base resins,[18] but it is clear from the figure that the resin had a preference for AG-A1 and especially for AG-A2. (The possibility that the acidities of AG-A2 and AG-A1 derive from phosphate ester groups rather than from carboxyl groups is negated by results of an ICAPS analysis of larch AG which showed the presence of no more than 4 ppm of phosphorus, corresponding to 0.001% maximum possible phosphate ester groups.)[19]

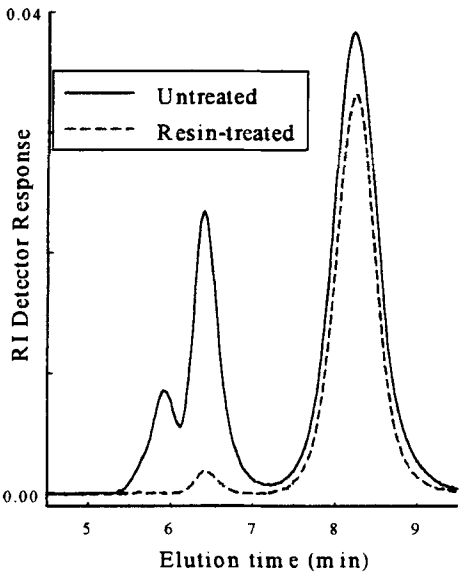

Figure 4. Removal of A2 and A1 from AG-A with base resin.

Additional support for the above concept was obtained by using methylene blue as an indicator for carboxyl groups, by analogy with its similar use for detecting these groups in oxidized cellulose.[20] This technique is based on the principle that a polymer chain bearing carboxyl groups can act as a weakly acidic cation exchanger which is capable of "taking up" the cation of methylene blue (methylthionine chloride). Application of this principle to the present work was achieved by taking advantage of the fact that the HPLC column possesses reversible binding sites for the methylene blue, so that dye previously bound to the stationary phase is picked up by carboxyl groups in analyte components passing through the column. To this end, the column was conditioned by eluting a buffered methylene blue solution through it, and this was followed by elution of weakly acidic and ionic eluents through the column to remove excess and unbound dye and to achieve a stable baseline for absorbance at 600 nm. Subsequent injection of a dilute solution of glucuronic acid (or sodium glucuronate) and glucose resulted in an intense absorbance at 600 nm coincident with the RI peak for the acid (or salt) but no such absorbance for the glucose. Thus by incorporating both a UV-VIS detector and an RI detector, this system could distinguish between acidic and non-acidic components of a mixture, and because of the high extinction coefficient of the dye, this technique was sensitive for very low concentrations of carboxyl groups.

When AG-A was injected on this system the chromatograms of Figure 5 were obtained. The chromatogram for absorbance at 600 nm shows two major peaks coincident with the RI peaks A2 and A1, thus supporting the view that these peaks correspond to acidic components. Sephadex fractions A2+A1 and A0 were also analyzed by this system, and in both cases the minor VIS peaks at 7.1 min and 8.5 min were observed, whereas the major peaks coinciding with A2 and A1 were only seen in the VIS chromatogram of the A2+A1 fraction. We therefore conclude that only the latter peaks are associated with carbohydrate components. Figure 6 is an expansion of these two major peaks, as monitored by both modes of detection, and the numbers shown are area ratios for the three vertical slices indicated by the dotted lines. The ratio of the area in the 600 nm absorbance (VIS) to that in the corresponding RI absorbance serves as a relative measure of the concentration of acid groups in each slice. These numbers indicate that the AG species in the A2 peak contain approximately twice the number of acid groups per assembly as compared to the AG species in the A1 peak. In addition, when we assess the shoulder on the left side of the A2 peak as a separate slice, its area ratio is found to be about three times that of the A1 peak, suggesting

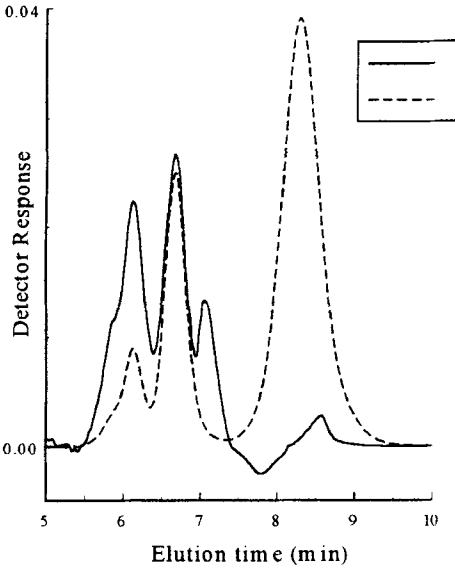

Figure 5. AG-A on methylene blue treated column.

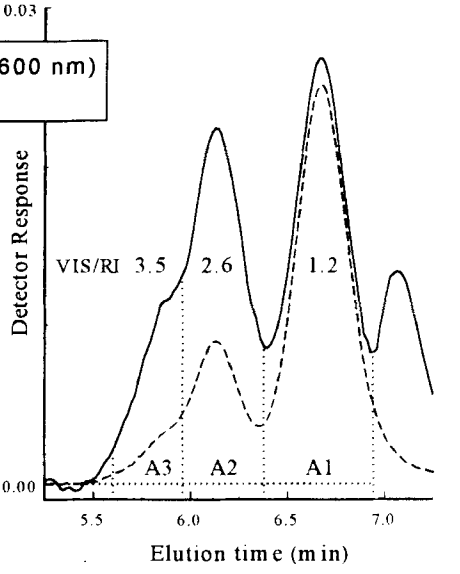

Figure 6. Expansion of Figure 5.

the presence of three times as many acid groups per assembly in this shoulder; *i.e.*, we may postulate the presence of another component, AG-A3.

In view of the above results, it appears likely that the A0 peak is due to AG-A species which bear no charged residues, the A1 peak is due to AG-A species which contain one uronic acid unit each, and the A2 peak is due to AG-A species which contain two (and in some cases three) uronic acid units each. If we assume an average weight of 37 kDa for an AG-A assembly (*i.e.*, dp ca.230), we can use the above figures to quantify the total amount of uronic acid residues in AG, and the result comes to 0.2%. This may be a more precise quantification of uronic acid in Western larch AG than can be obtained by any other means, and it illustrates the general utility of the ion-exclusion mechanism for determination of low concentrations of charged residues in polysaccharides.

With this technique we have also investigated unfractionated AG samples from three species of larch, and results are shown in Figures 7 through 10. Both of the chromatograms shown in Figure 7 are of an unfractionated sample of AG from Western larch whole wood, but they were obtained using different eluents (0.2 mM and 50 mM $NaNO_3$). The primary difference between these chromatograms and the corresponding profiles for AG-A (Figure 3) is the shoulder on the right side of the A0 (or main) peak in the chromatograms of Figure

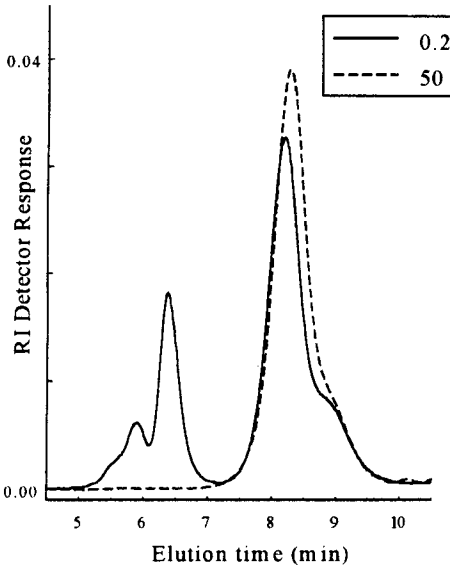

Figure 7. Western larch AG (whole wood).

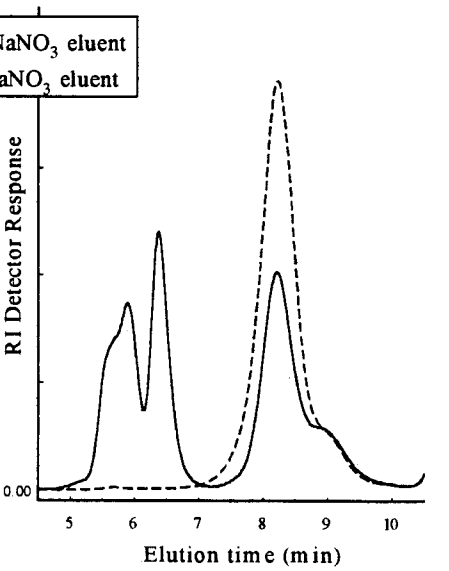

Figure 8. Western larch AG (heartwood).

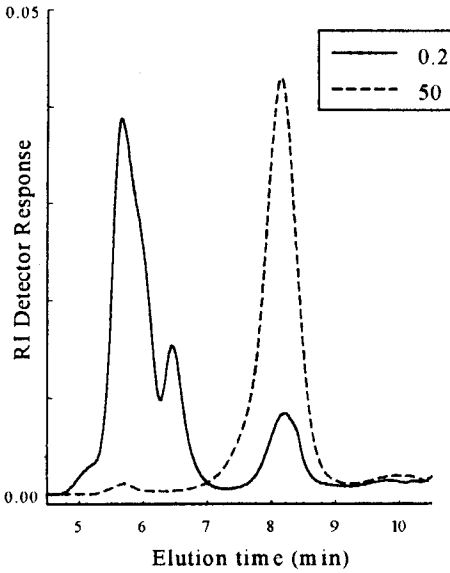

Figure 9. Eastern larch AG.

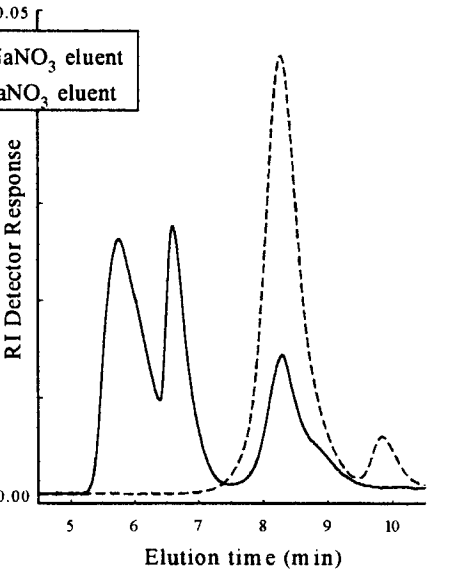

Figure 10. Japanese larch AG.

7. This is due to AG-B, which is genuinely smaller in size and significantly different in chemical composition from the three versions of AG-A. In contrast to AG-A, isolated AG-B shows no ion-exclusion effects, so it contains no ionic residues. (The other, major differences between AG-A and AG-B will be described in a subsequent paper.) It should be noted that the uronic acid content in Western larch AG might vary as a function of several parameters, including its location in the tree. This is illustrated by Figure 8, which shows chromatograms of a sample of unfractionated AG from Western larch heartwood. The chromatogram obtained using 0.2 mM $NaNO_3$ eluent shows a higher proportion of area in the fast eluting peaks as compared to the whole wood chromatogram, thus indicating a higher uronic acid content. This needs to be taken into account when assessing the older literature, where heartwood extractions were the rule.

Figure 9 shows chromatograms of an unfractionated sample of AG from Tamarack or Eastern larch (*Larix laricina*) whole wood. It is apparent that this AG contains several times more uronic acid units than does AG from Western larch, and this result is consistent with an earlier determination of 2% uronic acid in this type of AG.[14] Figure 10 shows similarly obtained chromatograms for Japanese larch AG (*Larix leptolepis*).[21] Again, the uronic acid content is seen to exceed that of Western larch. The same general profile seen in Figure 10 (using low ion strength eluent) has been seen before in SEC analysis of Japanese larch AG, but misinterpreted. Thus, Teratani, *et al.*[22] identified the fast running peaks as consisting of all of the AG-A, and the A0 peak (plus its low-MW shoulder) as consisting entirely of AG-B. These workers also assigned MW values based on SEC retention times, and made no mention of uronic acid units or their possible effect on the SEC results. This failure to recognize the ion-exclusion effect is also seen in the literature for Western larch AG,[6,23] and it has resulted in faulty estimates of the relative amounts and of the MW's of both AG-A and AG-B. Eremeeva and Bykova[24] described the consequences of this effect in their SEC study of Siberian larch AG (*Larix sibirica*), and they identified uronic acid residues as the cause, but they speculated that it was due to intramolecular electrostatic effects rather than to ion exclusion.

CONCLUSION

A survey of the literature shows that the "trace" of uronic acid residues which had been earlier reported to occur in Western larch AG has been largely ignored. This is

especially true in the recent literature for Western larch AG, perhaps because of a consensus that the earlier reports might be unreliable (*e.g.*, due to an impurity). The present work not only shows that a small amount of acidic units are indeed a real component of the structure of AG, but also that these units can have profound effects on the interaction of AG species with other charged substances. This may have implications for some medical uses of AG, since a charge(s) on an AG structure might influence some biological activities, *e.g.*, cell wall interactions.

EXPERIMENTAL

Materials and General Methods. All AG samples were supplied by Larex, Inc (St. Paul, MN). The purified AG-A, marketed as "Larex UF", was produced by ultrafiltration of Stractan 2 (Champion Corp., Tacoma, WA) with 100 and 10 kDa membranes (the latter removes AG-B). The Stractan 2 was initially purified by treatment with MgO.[25] The crude samples of AG from Western and Eastern larch (Figures 7-9) were obtained from "pressate" solutions generated by mechanical compression of wood chips.[3] These chips were generally from the whole tree, but in the case of Figure 8, chips from Western larch center poles were used. Pressate samples were produced by Larex, then centrifuged in our laboratory, and diluted and filtered (0.45 μm) prior to SEC analysis. AG from Japanese larch (Figure 10) was obtained from Mitsubishi Rayon. It is a cream-colored powder, probably produced by water extraction of chips or sawdust, followed by purification using an ultrafiltration protocol.[26]

Techniques used for compositional, methylation and NMR analyses are described in Part II of this series. Ultrafiltrations were performed with a 50 mL ultrafiltration cell (Amicon, Inc., Beverly, MA) and Diaflo ultrafilters XM50 and YM30. Calcium glucarate (Sigma) was converted to the more soluble sodium form by treatment with Amberlite IR-120 (Na^+) ion exchange resin. All other reagents (*e.g.*, sodium glucuronate, *etc.*) were obtained from common commercial sources and used as received. Amberlite IRA-900 (Cl^-) (Sigma) was washed in a column (3 x 8 cm) with 2 M NaOH (100 mL), then with water until the effluent was neutral. AG-A (50 mg in 10 mL water) was eluted through the column (3 drops/s), then the effluent was concentrated and analyzed by SEC to produce Figure 4.

Chromatography. Except for Figure 1, all chromatograms were produced by a system consisting of a Waters 501 HPLC pump, Waters R401 differential refractometer, Waters 486 Tunable Absorbance Detector, Shodex KB-804 HPLC column (cross-linked

polyhydroxymethylmethacrylate gel, 8 x 300 mm), Shodex KB-800P pre-column, and Waters Baseline 810 Chromatography Workstation 3.3 software operating on a PC through a Waters System Interface Module. The column was used at ambient temperature with a flow rate of 1 mL/min, and eluents were degassed by stirring under vacuum prior to use. Injections consisted of 40 μL of eluent solution containing 1% (w/w) of analyte. The sampling interval was 1 s, and Baseline data files were processed with Axum 4.0 for Windows.

To condition the HPLC column for the methylene blue experiment, aqueous methylene blue (2 mM) + Na_2HPO_4 (10 mM) was pumped through the column for 75 min, followed by 0.2 mM $NaNO_3$ for two days. Then removal of some of the dye, by eluting 0.2 mM H_2SO_4 for about 30 min through the column, was necessary to achieve a relatively stable system. After another day of eluting 0.2 mM $NaNO_3$ through the column, the chromatograms of Figure 5 were obtained using AG-A further purified by ultrafiltration (50k membrane). Elution of 0.2 mM H_2SO_4 through the system for several days was necessary to remove all traces of the dye.

Figure 1 was produced by eluting 25 mg of AG-A through a column of Sephadex G-100 (2.6 x 90 cm), using deionized water eluent at 0.7 mL/min maintained by a Waters 501 pump. The sample was loaded using a 1.1 mL loop on a Rheodyne 7125 injection valve. A Waters R401 differential refractometer was used for detection, and the signal was monitored at 2 min intervals and accumulated using a PC fitted with an A/D converter.

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8. a) We observe an inverse relation between the response of the RI detector and the concentration of the eluent, such that the detector response is about 20% greater with 0.2 mM than with 50 mM $NaNO_3$.
b) Pullulan calibration of the HPLC column indicates that the A0 peak corresponds to MW ca.17 kDa. The discrepency between this and AG's larger actual weight is due in part to the fact that AG is highly branched, whereas pullulan is linear.
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